NTNU – Trondheim
Norwegian University of
Science and Technology

Molecular Study of Dimethylsulfoniopropionate (DMSP) Metabolism in the Coccolithophore *Emiliania huxleyi*

Meriam Kamlow

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Supervisor: Atle M. Bones, IBI
Co-supervisor: Tore Brembu, IBI

Norwegian University of Science and Technology
Department of Biology

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Abstract

The compatible solute dimethylsulfoniopropionate (DMSP) is made in prodigious amounts by many single-celled marine phytoplankton. *Emiliania huxleyi* is the most prominent coccolithophore, distributed across the world's oceans and forming regular blooms that can cover over hundred thousand square kilometers. The blooms act as an important source of dimethyl sulfide (DMS). The enzymatic cleavage of DMSP to DMS and either acrylate or a proton 3-hydroxypropionate (3-HP) appears via the action of the enzymes known generically as DMSP lyases. The emitted DMS can be transformed by DMS-consuming bacteria or released into the atmosphere and can be oxidized further to sulfate aerosols that form cloud condensation nuclei (CCN), which may influence global climate by increasing albedo.

The metabolism of DMSP was investigated and compared in two *E. huxleyi* strains (NCMA1516 and 373) previously reported to exhibit low and high DMSP lyase activity, respectively. The *EhDddD* gene, encoding a putative DMSP lyase, was sequenced in both strains, and an expression analysis of *EhDddD* was performed. Furthermore, DMSP lyase activity was studied by determining extracellular DMS and DMSP concentrations and *in vitro* measurements of DMSP lyase activity (DLA).

The amino acid sequence of EhDddD in 1516 contains an insertion of one amino acid at the N-terminus compared with 373, which may be aspartate or serine. The amino acid (aa) sequence contains eleven amino acid substitutions between 1516 and 373, of which six are non-conserved. *EhDddD* encodes an additional C-terminal protein domain of 200 amino acids in length that is missing in bacteria. The additional domain may be involved in the function of the protein or in the regulation of the protein.

Expression of the *EhDddD* gene was 8.414 times higher in 373 than 1516, whereas *in vitro* DMSP lyase activity was observed to be 60.6 times higher in 373 than 1516. In determination of extracellular DMS and DMSP concentrations, extracts of both strains produced DMS from DMSP, but the DMS production was 0.8 times lower in 373 than 1516. The DMSP concentration in 373 was observed to be 1.2 time higher than 1516. These results did not correlate with *in vitro* DMSP lyase activity.

These results suggest that the observed differences in DMSP lyase activity between *E. huxleyi* strains are due to structural differences and adaptation to different environmental conditions.

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Abbreviations

2-ME	2-Mecaptoethanol
3-HP	3-hydroxypropionate
A	Adenine
aa	Amino acid
att	Attachment sites
bp	Base pair
C	Cytosine
$CaCl_2$	Calcium chloride
Car-PDMS	Carboxen and polydimethylsiloxane
CCN	Cloud condensation nuclei
CDD	Conserved domain database
cDNA	Complimentary DNA
$C_6H_8O_7 \cdot H_2O$	Citric acid (monohydrate)
CO_2	Carbon dioxide
CoA	Coenzyme A
Ct	Crossing threshold
ddNTP	Dideoxynucleotide
DLA	DMSP lyase activity
DMS	Dimethyl sulfide
DMSHB	4-dimethylsulfonio-2-hydroxybutyrate
DMSO	Dimethyl sulfoxide
DMSP	Dimethylsulfoniopropionate
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E1	ATP-dependent ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E. coli	*Escherichia coli*
EhDddD	*Emiliania huxleyi* DMSP lyase gene
ESTs	expression sequence tags
FA	Formaldehyde
g	The Earth's gravitational acceleration
G	Guanine
gDNA	Genomic DNA
GC-MS	Gas chromatography-mass spectrometry
Hz	Hertz
In vitro	Process acting in a reaction tube
In vivo	Process acting in the cell
kb	Kilo base pair
kDa	Kilodalton
LB	Luria-Bertani
MACAW	Multiple alignment construction and analysis workbench
MeSH	Methanethiol
MMPA	methylmercaptopropionate
mRNA	Messenger RNA
MS	Mass spectrometer
MTA-CoA	Methylthioacryloyl-CoA
MQ	Milli-Q (Ultra-pure water)

m/z	Mass-to-charge ratio
NaCl	Sodium chloride
Na_2HPO_4	Dibasic sodium phosphate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NCMA	National Center for Marine Algae and Microbiota
NRT	Negative reverse transcription
NTC	No template control
Oligo-dT	Deoxy-thymine nucleotides
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
pg	Picogram
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse transcription PCR
REST	Relative expression software tool
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNase A	Ribonuclease A
RT	Reverse transcription
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SIM	Selected ion monitoring
SPME	Solid-phase microextraction
ssDNA	Single-stranded DNA
T	Thymine
TAE	Tris-acetate-EDTA
THF	Tetrahydrofolate
T_m	Melting point
UV	Ultraviolet
X-gal	5-brom-4-chloro-3-indolyl-β-D-galactopyranoside

1. Introduction

1.1 Description of *Emiliania huxleyi*

Coccolithophores (also called coccolithophorids) are unicellular marine phytoplankton (Curson et al., 2011) of the Class Prymnesiophyceae, which include all Haptophyte algae whose cells are yellow-brown and covered by several layers of calcium carbonate plates called coccoliths (30 coccoliths per cell) (Green and Leadbeater, 1994; Townsend et al., 1994). The coccolith has a diameter of 2.5 μm and a weight of 18 picogram (pg) (Thierstein and Young, 2004). *Emiliania huxleyi* is the most dominant and widespread coccolithophore, with the exception of polar waters (Holligan et al., 1993), and regularly forms extensive blooms at temperate and tropical latitudes (Mackinder et al., 2011).

E. huxleyi cells are small and spherical with a diameter of circa 5 μm. The cells lack a third flagellum-like multi-functional organelle called haptonema. Haptonema can attain a length over 100 μm. It is responsible to capture prey, and act as an obstacle-sensing device. Coccolithophores including *E. huxleyi* are characterized by possessing two flagella and two golden brown chloroplasts with chlorophylls a and c (Thierstein and Young, 2004).

1.2 Life-cycle of *Emiliania huxleyi*

The *E. huxleyi* life cycle involves several cell types. These cell types include the non-motile coccolith-bearing cell (C-cell), the non-motile naked cell (N-cell) and the motile scale-bearing cell with flagella (S-cell) (Klaveness, 1972). C-cells are the most familiar form, and produce blooms in both oceanic and coastal waters (Green et al., 1996). Each cell type is capable of independent asexual reproduction by simple binary fission. The first stage of cell division is multiplication from two chloroplasts to four. In the second stage, the cell become oblong and a constriction is formed. Coccoliths in the C-cells will also constrict. Two daughter cells connected by a very thin plasmatic cord are formed in the final stage (Klaveness, 1972). All three cell types have also been observed to give rise to amoeboid cells in the stationary phase (Green et al., 1996). Amoeboid cells are sausage-shaped and are thought to be protoplasts that have shed their covers (Klaveness, 1972).

A sexual life cycle may also exist in *E. huxleyi* by alternation between C-cell and S-cell types (Klaveness, 1972). Since the DNA content of S-cells is half that of the C-cells and N-cells, S-cells appear to be haploid, and C-cells and N-cells diploid. A sexual fusion with the S-cells

acting as gametes in culture has not yet been observed; neither has the meiosis leading to gamete formation in the C- and N-cells been recorded (Green et al., 1996).

S-cells (Figure 1.1 B) differ from N- and C-cells (Figure 1.1 A), first and foremost by having two flagella and a single external layer of organic scales produced by the Golgi apparatus. The sizes and shapes of the organic scales are variable. Before cell division, the flagella are moved inwards so that they no longer protrude, but when cell division is complete the flagella reappear (Klaveness, 1972). The length of flagella varies from 2.5 μm to 5 μm (Green et al., 1996). Structures of mitochondria, chloroplasts and intracellular membrane systems in S-cells do not differ from those of N- and C-cells, except the lacking of coccolith-forming apparatus (Klaveness, 1972). N-cells, which are void of body scales, are considered to be mutant diploid stages that have lost the ability of coccolith production (Thierstein and Young, 2004).

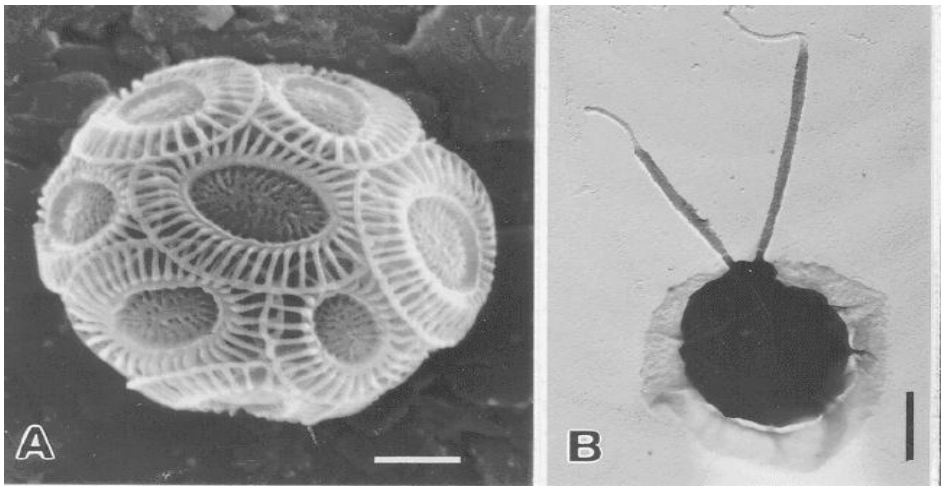


Figure 1.1 Comparison of C-cell and S-cell of *E. huxleyi*. A) A non-motile coccolith-forming C-cell showing overlapping calcium carbonate plates (coccoliths) (scale bar: 1.0 micron). B) The motile scale-bearing S-cell (B) (scale bar: 1.0 micron) displays two flagella and a single external layer of organic scales glued to the cell body. The length of flagella varies from 2.5 μm to 5 μm, and the sizes and shapes of organic scales are also variable. Before cell division, the flagella move inward, but they reappear when cell division is completed.
Pictures taken from http://www.noc.soton.ac.uk/soes/staff/tt/eh/lifecycle.html.

Little is still known about the environmental conditions that favor the transition from one cell type to another. According to Wilbur and Watabe (1963), lowering the amount of nitrate in the medium may induce the transition from S-cells to C-cells. Transition from C-cells to S- and N-cells may be triggered by senescence of the cultures. Figure 1.2 shows the possible life cycle and transitions between the three cell types as observed in culture. C-cells give rise to N-cells and both C- and N-cells give rise to S-cells. The cells reorganize several of their cytoplasmic components during the transition from C- or N-cells to S-cells. This

reorganization includes disappearance of coccolith-forming apparatus, forming of flagella apparatus and the ability of the cell to make uncalcified organic scales. Both the N- and C-cells lack the existence of uncalcified organic scales (Klaveness, 1972).

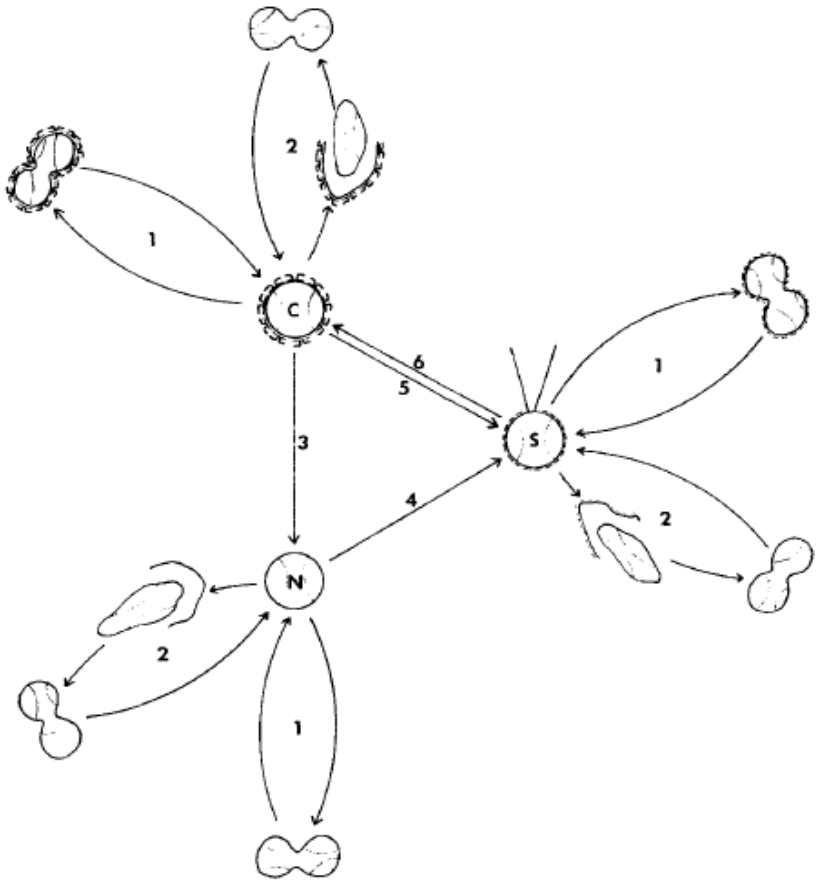


Figure 1.2 Possible modes of life cycle and transitions between cell types of *Emiliania huxleyi*. Some factors appear to favor the transition from C- cells to N- and S-cells and the transition of S-cells to C-cells. Senescence of the cultures is one of the factors that seem to trigger the transition from C-cells to N- and S-cells and the appearance of S-cells in cultures of N-cells. Insufficient amount of nitrate is another factor that may induce the transition from S-cells to C-cells. During the transition from C- or N-cells to S-cells some changes will appear. These changes include the disappearance of coccolith-forming apparatus, formation of flagellar apparatus and the ability of the cell to produce uncalcified organic scales. 1. Normal asexual reproduction. 2. Before cell division, the cell leaves the cover as an amoeboid. Amoeboid (sausage-shaped) cells are found in stationary cultures of C-, N- and S-cells at the bottom of the culture flasks. These cells appear to be protoplasts that have shed their covers. 3. N-cells appear when the C-cells loss its ability to produce calcified coccoliths. 4. S-cells appear in pure cultures of N-cells. 5. S-cells appear in pure cultures of C-cells. 6. C-cells appear in pure cultures of S-cells. From Klaveness (1972).

1.3 Morphological and geographical variation in strains of *Emiliania huxleyi*

The cosmopolitan *E. huxleyi* exhibits variations in morphology of coccoliths that can be related to the environmental conditions. Molecular phylogenetic studies exposed that *E. huxleyi* contain at least two mitochondrial sequence groups with various temperature tolerances. The cold-water group occur in subarctic North Atlantic and the warm-water group occur in the subtropical Atlantic and in the Mediterranean Sea.

Hagino et al. (2011) revealed that *E. huxleyi* consist of two major clades, I and II, showing different biogeographic distributions. Clade I include strains 1516 and 373 (Type A), whereas clade II include strains 370, 379, 920/8 and 920/9. Strains of clade I were isolated from tropical to temperate waters while strains of clade II were collected from boreal subarctic waters. Studies of different environmental parameters showed that clade I and II strains are statistically different from each other with regard to annual and monthly mean temperature, phosphate concentration and in annual mean nitrate concentration, but equal when it comes to annual and monthly salinity and in monthly mean nitrate concentration. In conclusion, clade I strains (1516 and 373) originate from warm tropical or temperate water, while clade II strains (370 and 379) originate from colder subarctic water (Hagino et al., 2011).

1.4 Dimethylsulfoniopropionate (DMSP), DMSP lyases and the production of dimethyl sulfide (DMS)

Dimethylsulfoniopropionate ($(CH_3)_2S^+CH_2CH_2COO^-$; DMSP) (Thierstein and Young, 2004) is an anti-stress and a zwitterionic compound synthesized by unicellular marine phytoplankton (coccolithophores, diatoms and dinoflagellates) under unfavorable conditions (Curson et al., 2011). DMSP is a multifunctional compound, it acts as compatible solute in cell metabolism and may have other important physiological roles as part of an overflow mechanism and as the basis of antioxidant cascade (Franklin et al., 2010). Stefels et al. (2007) hypothesized that the production of DMSP might be served as an overflow mechanism for excess reduced sulfur when carbon and nitrogen are limited. The antioxidant cascade is another hypothesis which suggests that the DMSP and its breakdown products (dimethyl sulfide, acrylate and dimethyl sulfoxide) create an efficient antioxidant system that scavenges hydroxyl radicals and other reactive oxygen species. The antioxidant protection is increased by the enzymatic cleavage of DMSP because DMS and acrylate are 60 and 20 times more effective in scavenging hydroxyl radicals than DMSP. The DMSP and its breakdown products have another metabolic function.

This includes the defence against grazing due to the toxicity of acrylate to zooplankton (Sunda et al., 2007).

DMSP is also the key precursor of dimethyl sulfide ($(CH_3)_2S$; DMS) production in the atmosphere (Thierstein and Young, 2004). DMS is a partially volatile organic sulfur compound which accounts for 50-60% of the total amount of natural reduced sulfur supplied to the atmosphere, including emissions from volcanoes and vegetation (Stefels et al., 2007). DMS production appears to occur mainly through the activity of an enzyme known as DMSP lyase (Thierstein and Young, 2004). DMSP lyase activity (DLA) has been reported in coccolithophores (*E. huxleyi*) and dinoflagellates, but the molecular description of these enzymes is still unknown (Curson et al., 2011; Franklin et al., 2010). DLA was reported to be highly variable between strains of *E. huxleyi*, suggesting that variation in DLA behavior could contribute to strain-specific differences within this species (Franklin et al., 2010). Steinke et al. (1998) reported that it has been found evidence for both membrane-bound and soluble DMSP lyase activity within *E. huxleyi*. The production of DMS *in vivo* occurs when the cell is exposed to grazing by microzooplankton, infection or nutrient limitation (Thierstein and Young, 2004). DMSP is a major source of carbon and sulfur for marine bacteria, which catabolize it to the volatile DMS and acrylate or a proton 3-hydroxypropionate (3-HP). The production of DMS by bacterial lyase enzymes is 300 million tonnes each year in the oceans (Curson et al., 2011).

The synthesis of DMSP for a range of marine algae including *E. huxleyi* is initiated by transamination of methionine. Further, the pathway continues via reduction and methylation reactions to the novel intermediate 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB). DMSHB is then converted to DMSP via oxidative decarboxylation. Depletion of cellular amino acids favors the transamination step, which promote the DMSP synthesis under nitrogen limitation (Gage et al., 1997). The transamination reaction produces a NH_3^+ group for general cell metabolism which appear to accommodate the suggestions that DMSP is synthesized when nitrogen is limiting (Thierstein and Young, 2004). The release of DMSP (Figure 1.3) from phytoplankton is induced by grazing, senescence or viral lysis (Curson et al., 2011). DMSP in the seawater can either be demethylated to methylmercaptopropionate (MMPA) by a demethylation pathway or cleaved to produce DMS and either acrylate or 3-HP by a cleavage pathway. The demethylation pathway differs from the cleavage pathway by not resulting in release of DMS. Produced DMS can either be released into the air or transformed by bacterial DMS consumers to dimethyl sulfoxide (DMSO), methanethiol (MeSH) and

tetrathionate (Curson et al., 2011). In the air, DMS is rapidly oxidized during the day via hydroxyl radicals and at night via nitrate radicals (Thierstein and Young, 2004). The oxidation reaction of DMS leads to the formation of DMSO or sulfate aerosols which can act as cloud condensation nuclei (CCN) and further increase albedo (sunlight reflected back into space). The global sulfur cycle return DMS back to land through precipitation (Curson et al., 2011).

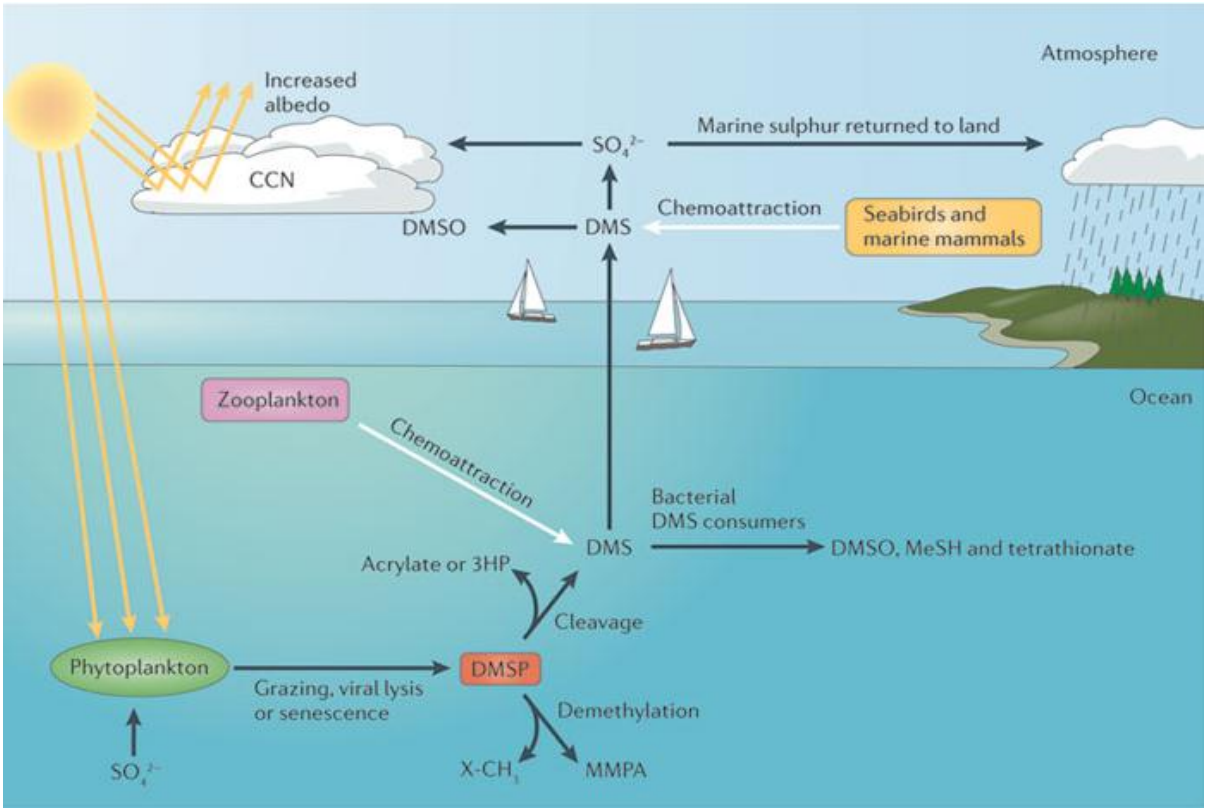


Figure 1.3 The bacterial degradation of dimethylsulfoniopropionate and fate of dimethyl sulfide. The release of dimethylsulfoniopropionate (DMSP) from phytoplankton is subjected to bacterial degradation by demethylation and cleavage pathway. The demethylation pathway produces methylmercaptopropionate (MMPA), whereas the cleavage pathway leads to the production of dimethyl sulfide (DMS) and either acrylate or 3-hydroxypropionate (3HP). The emitted DMS from the cleavage pathway can either be released into the air or transformed by bacterial DMS consumers to dimethyl sulfoxide (DMSO), methanethiol (MeSH) and tetrathionate. DMS in the atmosphere can be converted to DMSO or sulfate aerosols, which can act as cloud condensation nuclei (CCN) and further leads to an increase in the amount of sunlight reflected back into space. The global sulfur cycle return the marine sulfur to land via rain or snow. DMS is essential to zooplankton, seabirds and marine mammals as a chemoattractant. From Curson et al. (2011).

1.4.1 Bacterial enzymes involved in cleavage and demethylation of DMSP

Enzymes that act on DMSP are known as DMSP lyases. Five different classes of DMSP lyases proteins that split DMSP into acrylate and DMS have been identified in bacteria: DddL, DddP, DddQ, DddW and DddY. DddD is the only known enzyme with DMSP lyase activity that generates 3-HP as byproduct instead of acrylate. 3-HP may not be the initial product in the catabolic fate of DMSP. The enzyme DddD may produce a CoA derivative of DMSP before the rapid conversion to 3-HP, but a CoA intermediate has not yet been detected. DddP belongs to the M24 protease family, but does not contain metal cofactors and cleaves the S-C bond of DMSP instead of cleaving an amino bond like other members. In addition to the generation of DMS and acrylate, the enzymes DddL, DddQ and DddW have more common features. They contain metal binding carboxy-terminal domains called cupins, and the polypeptides are small (16-26 kDa). The DMSP lyase DddY is found in the betaproteobacterium *Alcaligenes faecalis* str. M3A. Unlike other cytoplasmically located lyases, DddY is associated with the cell surface (Curson et al., 2011).

Todd el al. (2007) reported that the enzymatic mechanism for DMS releasing involves modification of DMSP by the addition of acyl coenzyme A (CoA) rather than the immediate liberate of DMS by a DMSP lyase, the suggested mechanism. The predicted function of DddD protein is thought to be a type III acyl CoA transferase. The DddD cleavage is predicted to involve the addition of CoA to DMSP and subsequent cleavage and release of DMS and 3-HP instead of acrylate in DMSP lyase cleavage. In this thesis, the investigated protein DddD in *E. huxleyi* strains 1516 and 373 will be hereafter defined as DMSP lyase to compare to other studies.

The DMSP demethylase DmdA catalyzes the first step of the demethylation pathway by transferring a methyl group from DMSP to tetrahydrofolate (THF), forming MMPA. THF is used as the methyl acceptor. MMPA is further catabolized via MMPA-CoA and methylthioacryloyl-CoA (MTA-CoA) to acetaldehyde. The catabolism from MMPA to acetaldehyde involves DmdB, DmdC and DmdD. Figure 1.4 shows an overview of biochemical pathways for DMSP cleavage and demethylation (Curson et al., 2011).

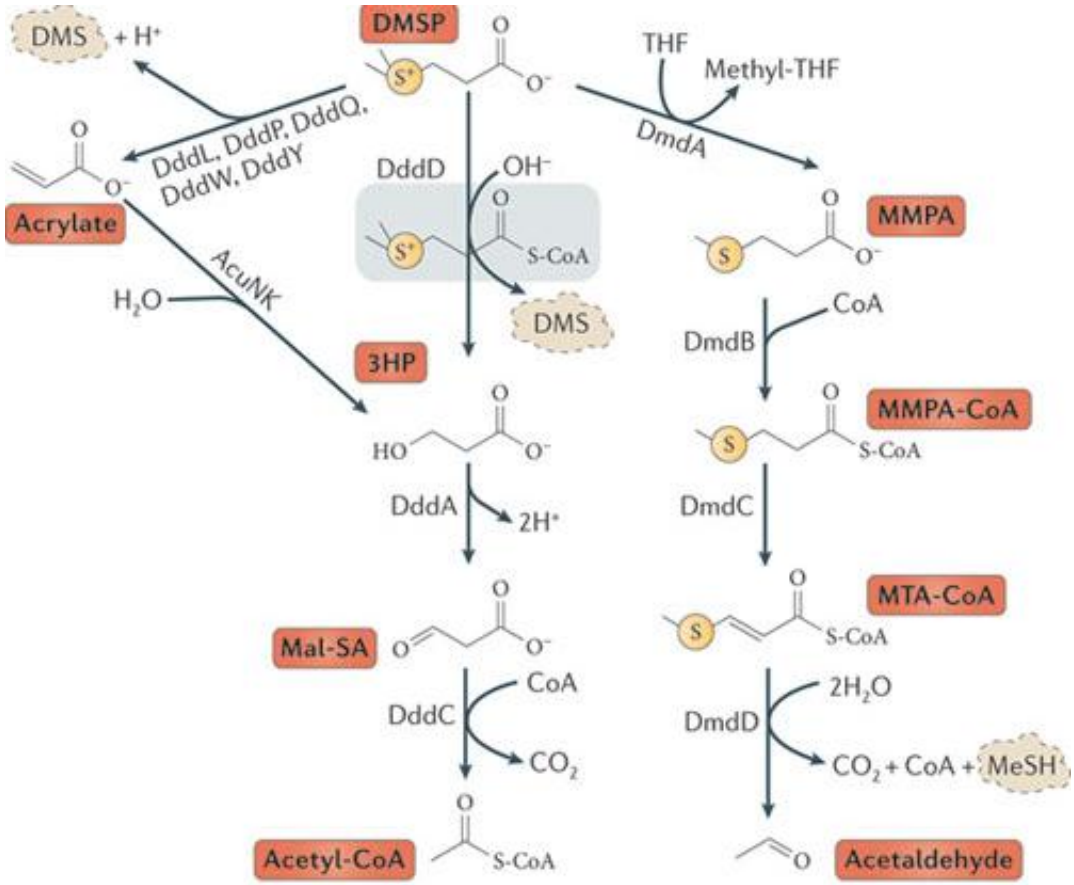


Figure 1.4 Pathways for degradation of dimethylsulfoniopropionate. The DMSP lyases DddL, DddP, DddQ, DddW and DddY break down DMSP to acrylate with the release of DMS. The acrylate is further converted to 3-HP by AcuNK. DddD converts DMSP to 3HP with the production of the intermediate DMSP-CoA (grey box). 3HP is further converted to malonate semi-aldehyde (Mal-SA) and acetyl-CoA by DddA and DddC. In the demethylation pathway, the DMSP demethylase DmdA transfer a methyl group from DMSP to tetrahydrofolate (THF) as the methyl acceptor to produce methylmercaptopropionate (MMPA). MMPA is further catabolized, via MMPA-CoA and methylthioacryloyl-CoA (MTA-CoA) to acetaldehyde with the release of another volatile, methanethiol (MeSH) by DmdB, DmdC and DmdD. From Curson et al. (2011).

1.5 DMSP lyase isozymes in several strains of *Emiliania huxleyi*

Steinke et al. (1998) partially characterized and compared DMSP lyase activity in crude extracts of six axenic *E. huxleyi* strains (CCMP370, 373, 374, 379, 1516 and strain L) with respect to pH and sodium chloride (NaCl). All strains except 1516 showed high intracellular concentrations of DMSP (from 157 to 242 mM). Strain 1516 had a DMSP concentration of 50 mM. Extracts of all strains produced DMS from DMSP *in vitro*, but the enzyme activity varied greatly among strains and did not correlate with the intracellular DMSP concentration.

Strains 373 and 379 showed very high DMSP lyase activity compared to 1516, 370, L and 374 (Figure 1.5). DMS production rate in 374 was very low, whereas 370, 1516 and L were observed to have a higher DMS production of 0.01 to 0.03 fmol DMS $cell^{-1}$ min^{-1}. The DMSP lyase activity in 373 was more than 100-fold higher than 1516.

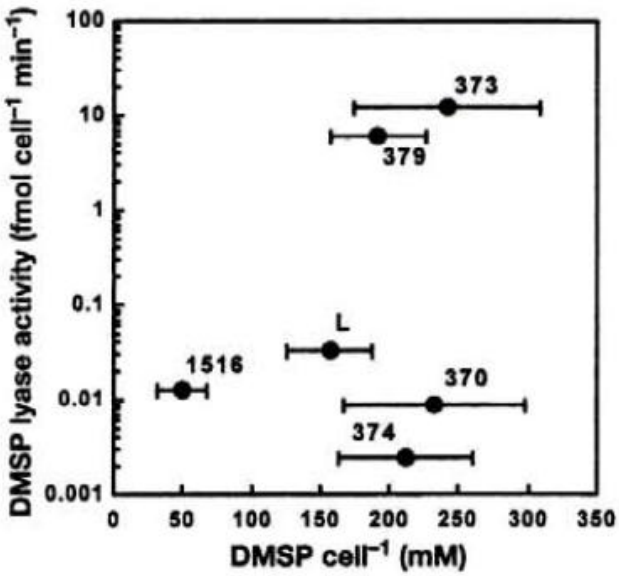


Figure 1.5 Comparison between *in vitro* DMSP lyase activity and intracellular DMSP concentration in six strains of *Emiliania huxleyi*. Intracellular DMSP concentrations were very similar in 370, 373, 374 and 379, but the DMSP lyase activities were higher in 373 and 379 compared to 370 and 374. The DMS production rate in 374 was very low, but 370, 1516 and strain L showed a higher DMS production of 0.01 to 0.03 fmol DMS $cell^{-1}$ min^{-1}. Compared to these production rates, the DMSP lyase activities in 373 and 379 were more than 100-fold higher (6.1 to 12.5 fmol DMS $cell^{-1}$ min^{-1}). Most of the cultures showed high concentrations of intracellular DMSP ranged from 157 to 242 mM, except for 1516 which showed a concentration of 50 mM DMSP $cell^{-1}$. From Steinke et al. (1998).

These observations led to the conclusion that intracellular DMSP concentration did not correlate with DMSP lyase activity in the investigated strains. Intracellular DMSP concentrations were highly similar in 370, 373, 374 and 379, but the DMSP lyase activity in 373 and 379 were higher compared with 370 and 374.

The addition of NaCl to the cultures of *E. huxleyi* can have some effect on DMSP lyase activities. Increasing NaCl concentrations resulted in increased DMSP lyase activity in the 370, 1516 and L strains, and reduced DMSP lyase activity in the 373 and 374 strains.

Various pH levels in citric acid/phosphate buffer can also have different effects on DMSP lyase activities. Strains 373 and 379 with high lyase activity exhibited a sharp pH optimum around pH 6, with less than 40% of maximal activity at pH 4 and 7. A pH optimum around pH 5 was observed in the low activity strains (374, 1516 and L), except strain 370, which showed increasing activity with increasing pH. The alkaline pH reduced DMSP lyase activity in all investigated strains of *E. huxleyi* except for 370. This might explain the reason for low DMS production *in vivo*.

The difference in pH and NaCl requirements in the investigated strains of *E. huxleyi* indicates that DMSP lyase enzymes in different strains may be structurally different, which can contribute to activity variations. DMSP lyase enzymes in *E. huxleyi* differ also in cellular locations, they can be membrane-bound and soluble. This can be the second reason for the activity variation among the DMSP lyase isozymes. The cellular location of DMSP is still unknown and need further investigation (Steinke et al., 1998).

Sunda et al. (2007) investigated the effect of nitrogen-limitation on DMSP lyase activity in semi-continuous cultures of *E. huxleyi*. It has been observed an increase in DMS in nitrogen limited cultures. This can be concluded that nitrogen-limitation increase the activity of DMSP lyase activity.

1.6 Factors that influence the production of DMSP in Haptophyte

Low or high temperature, osmotic stress, nitrogen and light intensity are factors that may influence the intracellular DMSP concentration in Haptophytes such as *E. huxleyi*. DMSP increases in concentration when the growth temperature is reduced. Under nitrogen-limitation the compatible solute will be preferentially synthesized (Thierstein and Young, 2004). The marine phytoplankton might favor the production of intracellular DMSP when nitrogen availability is low. This hypothesis has been supported by some evidence. *E. huxleyi* grown in a medium with no nitrate supplement showed a higher intracellular DMSP concentration compared with a culture grown in a medium supplemented with nitrate (Green and Leadbeater, 1994). In contrast, Sunda et al. (2007) reported that under nitrogen-limitation, *E. huxleyi* strain (CCMP374) resulted in no measureable increase in DMSP production.

Sunda et al. (2002) demonstrated that in *E. huxleyi*, increased ultraviolet (UV) radiation, carbon dioxide (CO_2) limitation and iron deficiency resulted in increased cellular DMSP levels. However, another study registered no apparent increase in intracellular DMSP concentration when *E. huxleyi* was exposed to UV light (Van Rijssel and Buma, 2002). Studies on DMSP production versus light intensity have produced mixed results (Thierstein and Young, 2004).

1.7 Environmental effects of DMS emissions

The ocean is the major source of volatile organic compound DMS, which enters the atmosphere via air-sea exchange. DMS is ubiquitous in the surface seawater and is the dominant volatile sulfur compound in seawater (Green and Leadbeater, 1994). Atmospheric DMS photo-oxidizes rapidly to DMSO or sulfate aerosols. Sulfate aerosols can act as CCN, initiating cloud cover over the oceans, thereby influencing the climate by reflecting radiation from the sun back into space (albedo) (Thierstein and Young, 2004). Charlson et al. (1987) presented CLAW hypothesis as a possible role for DMSP in regulating climate. The doubling of atmospheric CO_2 can be balanced by an approximate doubling of CNN. DMS appears to be the major source of CNN over the oceans (Charlson et al., 1987). For humans, DMS contributes to the tangy smell of the seaside (Curson et al., 2011).

CLAW hypothesis described by Charlson et al. (1987) starts with sunlight increasing the growth rates of phytoplankton (coccolithophores) in the ocean, which further increase the synthesis of DMSP. The increased production of DMSP leads to an increase in the

concentration of DMS. In atmosphere DMS is oxidized to form sulfate aerosols. These aerosols act as CCN and increase cloud droplet number and cloud area. This leads to enhanced cloud albedo which reflects the sunlight back to space and cooling the earth. Figure 1.6 shows a schematic diagram of CLAW hypothesis.

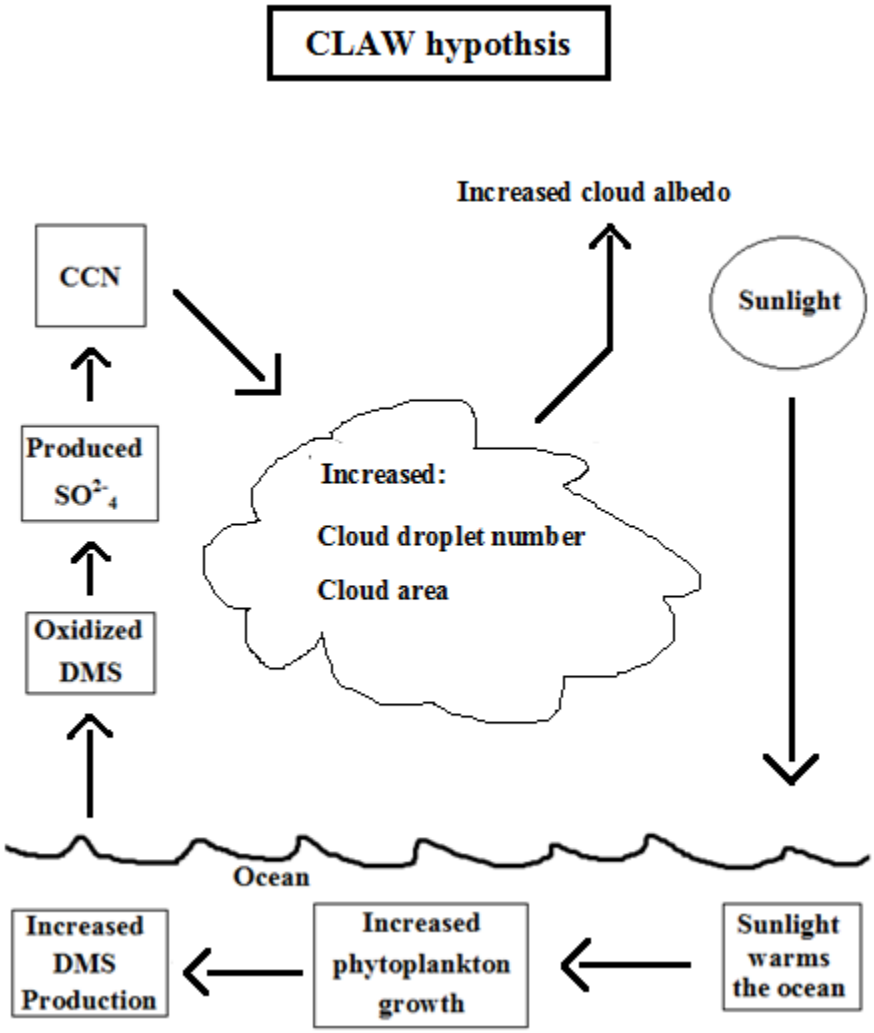


Figure 1.6 Schematic diagram of CLAW hypothesis. Enhanced energy from the sun leads to increase the growth rates of phytoplankton. The enhanced growth of phytoplankton increases the synthesis of DMSP which in turn increase the concentration of DMS. In the atmosphere DMS is oxidized to sulfate aerosols which act as cloud condensation nuclei (CCN) and further increase cloud droplet number. This results in increased cloud albedo which reflects solar radiance back into space and so cooling the earth. From Charlson et al. (1987).

1.8 Aim of study

The overall aim for this project was to study the metabolism of dimethylsulfoniopropionate (DMSP) in *Emiliania huxleyi*.

The aims of the *in vitro* system were as following:
(i) Sequencing the *EhDddD* gene in two strains of *E. huxleyi* 1516 and 373.
(ii) Studying the gene expression of *EhDddD* in two strains of *E. huxleyi* 1516 and 373.
(iii) Measuring the DMSP lyase activity in two strains of *E. huxleyi* 1516 and 373.
(iv) Determining extracellular DMS and DMSP concentrations in two strains of *E. huxleyi* 1516 and 373.

2 Materials and methods

2.1 Experimental work

Emiliania huxleyi (Lohmann) Hay et Mohler, strains NCMA1516 and NCMA373 were used for all experiments described in this thesis.

Non-axenic cultures of *E. huxleyi* were obtained from the National Center for Marine Algae and Microbiota (NCMA, USA). The origin information for the investigated strains of *E. huxleyi* is as follows:

Emiliania huxleyi strains	Origin
1516	South East Pacific
373	North East Atlantic

2.1.1 Growth conditions

The cultures were kept *in vitro* in a growth room at 18°C under cool white fluorescent light at a scalar irradiance (E_{PAR}) of 115 μmol m^{-2} s^{-1} under continuous white light conditions. The cultures were grown in 50 ml flasks containing 35 ml L1-Si (silisium) medium and 3.5 ml cells to a total volume of 38.5 ml. The L1-Si medium was made from filtered (pore size 0.2 μm) and autoclaved seawater enriched with nutrients. The recipe for L1-Si medium is presented in Appendix 1. Once a week the cultures were diluted 1:11.

The experiments were performed at 18°C under cool white fluorescent light at a scalar irradiance (E_{PAR}) of 210 μmol m^{-2} s^{-1} under continuous white light conditions.

2.1.2 Cell harvesting

The cells were dispersed into 50 ml tubes and centrifuged at 4495 g, 18°C for 10 minutes. Cell pellets were transferred into 1.5 ml microcentrifuge tube and centrifuged at 16.060 g, 4°C for two minutes. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C for further analysis.

2.1.3 Cell counting

Cell counting of *E. huxleyi* strains was performed using Bürker counting chamber and light microscope. The cells were treated with 1 drop of Lugol′s solution (potassium iodide) to kill and stain the cells. The cells were diluted 1:4 with L1-Si medium.

The cell density (cells/ml) was calculated by counting the cells observed in six squares (diagonal) and multiplying the mean number of the cells with 10^4.

2.1.4 Axenity test

0.5 ml of culture was mixed with 5 ml f/2 with peptone (1g/L) in a 15 ml tube. The tube was covered with aluminum foil and checked for bacterial growth after one week.

2.2 DNA isolation

E.Z.N.A.® SP Plant DNA Kit is a rapid method used for the extraction of high quality cellular DNA from plant species. HiBind matrix binds DNA and removes proteins, polysaccharides and other contaminants under optimal conditions (Omega-bio-tek, 2010).

Buffer SP1 contains a detergent that lyses cell walls to release the DNA. Buffer SP2 precipitates proteins and polysaccharides, while buffer SP3 contains a high concentration of chaotropic salt that helps DNA to bind the silica membrane. SPW Wash buffer contains alcohol (ethanol) and non-chaotropic salt. The Wash buffer is used to remove residual proteins, polysaccharides and pigments (Omega-bio-tek, 2010). The role of ethanol is to remove chaotropic salts and precipitate the DNA (Reece, 2004). Elution buffer contains low salt concentration to elute DNA from the column (Omega-bio-tek, 2010).

Procedure:

1. Frozen samples of *E. huxleyi* were homogenized in TissueLyser (QIAgen) for two minutes at an oscillation frequency of 25 Hz.
2. 400 µl of buffer SP1 was added to each sample followed by the addition of 5 µl of RNase A (10 µg/µl, Sigma). RNase A is an enzyme that breaks down RNA (Reece, 2004). The samples were incubated at 65°C for 10 minutes. During the incubation the samples were inverted several times.
3. 140 µl of buffer SP2 was added to each sample. The samples were incubated for five minutes on ice and then centrifuged at 10.000 g for 10 minutes.

4. The supernatant was transferred to an Omega Homogenizer Column placed into a 2 ml collection tube. The Omega Homogenizer Column removes the remaining precipitates and cell debris (Omega-bio-tek, 2010).
5. The tubes were immediately centrifuged at 10.000 g for two minutes.
6. Cleared lysates were transferred into 1.5 ml tubes without disturbing the pellet.
7. 600 µl of buffer SP3 was directly added to the cleared lysate and vortexed to obtain a homogenous mixture.
8. 650 µl of the supernatant was transferred to a HiBind DNA Mini Column placed into a 2 ml collection tube. The samples were centrifuged at 10.000 g for one minute to bind DNA.
9. Step 8 was repeated with the remaining sample.
10. The HiBind DNA Column was placed into a new 2 ml collection tube and 650 µl of SPW Wash buffer was added. The tubes were centrifuged at 10.000 g for 1 minute and the flow-through was discarded.
11. The wash step was repeated by adding another 650 µl SPW Wash buffer. The tubes were centrifuged at 10.000 g for 1 minute and the flow-through was discarded.
12. The empty column was centrifuged for two minutes at maximum speed (16.060 g) to dry.
13. The HiBind DNA Mini Column was transferred into a clean 1.5 ml tube. 50 µl of Elution buffer pre-warmed to 65°C was applied, and the tubes were incubated at room temperature for five minutes followed by centrifugation at 10.000 g for one minute to elute DNA.
14. To increase the DNA yield, the eluted DNA was returned back into the HiBind DNA Mini Column, incubated at 65°C for five minutes and then centrifuged at 10.000 g for one minute to elute DNA. The DNA was stored at -20°C (Omega-bio-tek, 2010).

2.2.1 NanoDrop Spectrophotometry

NanoDrop spectrophotometry was used to measure the concentration (ng/µl) and quality of the nucleic acids. NanoDrop is designed for small samples (0.5-2.0 µl). NanoDrop measures the absorbance of nucleic acids in samples to determine the level of contaminants. The absorbance ratio of 260/280 defines the purity of nucleic acids (NanoDrop®Technology, 2007) while the absorbance ratio of 260/230 defines the degree of co-purified contaminants (Thermo-Scientific, 2009). Pure DNA has a 260/280 ratio at 1.8 while pure RNA has a

260/280 ratio at 2.0. Ratios of DNA and RNA below 1.8 and 2.0, respectively, indicate presence of proteins, phenols or other contaminants (NanoDrop®Technology, 2007). The 260/230 ratio of 1.8-2.2 indicates pure nucleic acids (Thermo-Scientific, 2009).

Procedure:

1.5 µl of each sample was directly pipetted onto the pedestal to measure the concentration and purity of DNA to determine if it is pure enough for further analysis.

2.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique invented by Kary Mullis (Baumforth et al., 1999) that was used for amplifying specific DNA sequences of *E. huxleyi* DMSP lyase (*EhDddD*) gene *in vitro* to yield sufficient quantities to be cloned, sequenced and analyzed. The technique is extremely effective because it amplifies billions of identical copies of a certain DNA strand in a short time through repeating cycles (Baumforth et al., 1999).

The PCR commences with an initial denaturation step usually at 95°C to ensure the complete separation of the double-stranded DNA (dsDNA) and terminates with a final extension step at 70°C to ensure that all amplified copies of a certain DNA fragment is in double stranded conformation (Baumforth et al., 1999).

The LA *Taq* DNA polymerase is provided by TaKaRa La Taq^{TM} kit with GC Buffer for GC-rich PCR. TaKaRa LA *Taq* consists of a combination of *Taq* polymerase and a proofreading DNA polymerase with 3' to 5' exonuclease activity. Proofreading polymerase increases fidelity (6.5x) compared to *Taq* polymerase alone, which lacks 3' to 5' exonuclease activity (TaKaRa, 2012a). *Taq* polymerase has a terminal transferase activity that provides the PCR products with adenine (A) overhangs at their 3' ends. This property of *Taq* is useful in TOPO TA cloning, whereby PCR products can directly be cloned into cloning vectors with a thymine (T) 3' overhang (Invitrogen, 2006). The GC-optimized Buffer I is specifically designed for amplification of DNA templates (2-3 kb fragments) with high GC content (TaKaRa, 2012b). The *EhDddD* gene has a GC content of 74.70%.

Procedure:

To amplify a specific DNA sequence of *EhDddD* gene, the PCR reagents were mixed in PCR tubes as following:

Reagent	Volume
TaKaRa LA *Taq* (5 U/µl)	0.125 µl
dNTP mixture (10 mM)	2 µl
2 X GC Buffer I	12.5 µl
Template	1 µl
MQ water	7.5 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
Total	25.125 µl

The sequences for the forward and reverse primers are provided in Table A1, Appendix 4.

The DNA fragment of *EhDddD* gene was amplified in a thermal cycler containing the following conditions:

1	95°C, 1 min	
2	95°C, 30 sec 60-65°C, 30 sec 70°C, 2:30	35 cycles
3	70°C, 5:00 min	
4	4°C, Hold	

2.2.3 Gel electrophoresis

Gel electrophoresis is a technique for separation of DNA fragments based on size in an electric field. The DNA molecule is negatively charged because of the phosphates that form the sugar-phosphate backbone. The agarose gel is agarose (extracted from seaweed) dissolved in boiling Tris-acetate-EDTA (TAE) buffer. The concentration of agarose ranges between 1 and 3%. Pore size is inversely correlated with agarose concentration; therefore, agarose gels made with 1% agarose will give good separation of large DNA fragments, whereas 2% agarose gels give good resolution of small fragments (Reece, 2004).

When an electric current is applied, DNA fragments will migrate to the positive electrode (anode). A small DNA fragment will migrate faster than a large DNA fragment because a small molecule can move more easily through the pores of the gel. GelRed is a compound that binds the DNA molecule and fluoresces by ultraviolet (UV) exposure. GelRed is mixed with the agarose gel to visualize the DNA fragments. GeneRuler ladder is used to identify the size of the bands (Reece, 2004). Recipe for the 1% agarose gel is provided in Appendix 2.

Procedure:

1. 2.5 µl of 1:10 diluted Loading Dye was added to each PCR reaction.
2. 5 µl of GeneRuler^{TM} 1 kb Plus DNA ladder (Appendix 8) and 25 µl of PCR reaction were applied into wells in the gel placed in TAE buffer (Appendix 2).
3. The gel was connected to a power supply (rear) at 80 V for about one hour and 30 minutes.
4. PCR products were cut by scalpel under UV radiation and placed in a 1.5 ml microcentrifuge tube. PCR products were stored at -20°C for further analysis.

2.2.4 Purification of PCR products from agarose gel

Wizard® SV Gel and PCR Clean-Up System kit by Promega was used to extract and purify PCR products from agarose gel. PCR products are purified to eliminate excess of primers and nucleotides. This method is based on the binding of DNA to silica membrane in the presence of chaotropic salts. The bands of interest are excised and dissolved in the presence of guanidine isothiocyanate (Promega, 2010). Purified DNA was further used for TOPO TA cloning (described in chapter 2.2.5).

Procedure:

The purification of DNA fragments from agarose gel was performed according to the Wizard® SV Gel and PCR Clean-Up System protocol (Promega, 2010). DNA was eluted with 30 µl Nuclease-Free Water, instead of 50 µl Nuclease-Free Water.

2.2.5 TOPO TA cloning

TOPO® TA cloning is a molecular biology technique that provide an easy, fast and highly efficient method for cloning PCR products into a plasmid vector (Invitrogen, 2006). *EhDddD* gene was cloned into pCR™ II-TOPO® vector using TOPO TA cloning® Kit Dual Promoter (Invitrogen). A plasmid map of pCR™ II-TOPO® vector is provided in Figure A1, Appendix 3.

Procedure:

1. The TOPO® Cloning Reaction (2 µl purified PCR product, 1 µl salt solution, 2 µl water and 1 µl pCR™ II-TOPO® vector) was gently mixed and incubated for five minutes at room temperature. Placed the reaction on ice after incubation.
2. 3 µl of the TOPO® Cloning Reaction was transformed into DH5α RbCl competent E.coli cells, mixed gently and incubated on ice for 30 minutes.
3. 30 µl of X-gal (20 mg/ml) was plated onto kanamycin (50 µg/ml) Luria-Bertani (LB) plates and incubated at 37°C.
4. The cells were heat-shocked for 30 seconds at 42°C without shaking and then immediately incubated on ice for two minutes.
5. 1 ml of room temperature LB medium was added into the cells.
6. The cells were incubated in an incubator shaker series (200 rpm) at 37°C for one hour.
7. Plated 100 µl from each transformation on prewarmed LB plates and then incubated the plates overnight at 37°C.
8. White colonies from LB plates were picked and transferred into 13 ml tube containing 3 ml LB medium and 3 µl kanamycin (50 µg/ml). The tubes were incubated in an incubator shaker (200 rpm) at 37°C for 16 hours.

2.2.6 Plasmid isolation by miniprep

QIAprep Miniprep kit was used to isolate plasmid DNA under alkaline conditions. This system utilizes a silica membrane for adsorption of plasmid DNA and elimination of chromosomal DNA, proteins and high-molecular-weight RNA. The procedure consists of bacterial lysis under alkaline conditions, adsorption of DNA onto the QIAprep membrane in the presence of high salt concentration, washing, and elution of plasmid DNA in low-salt buffer (Qiagen, 2006).

Buffer P1 is a resuspension buffer provided with ribonuclease A (RNase A). Buffer P2 contains sodium hydroxide (NaOH) and sodium dodecyl sulfate (SDS). NaOH ensure alkaline conditions, while SDS denatures plasmid DNA, proteins, chromosomal DNA and cell membranes. Neutralization buffer (buffer N3) contains guanidine hydrochloride and acetic acid to give the lysate high salt concentrations. Chromosomal DNA, cellular debris and proteins will precipitate. Buffer PE is a wash buffer containing ethanol, and buffer EB (10 mM Tris·Cl, pH 8.5) is an elution buffer that contains low salt concentration to elute plasmid DNA (Qiagen, 2006).

Procedure:

The plasmid DNA isolation was performed according to the QlAprep Miniprep Handbook (Qiagen, 2006). To avoid degradation of DNA, the plasmids were stored at -20°C

The concentration and quality of isolated DNA was measured using NanoDrop (described in chapter 2.2.1).

2.2.7 Restriction cutting of plasmid

Restriction cutting of plasmid can be used as a control to confirm that a PCR product was correctly inserted in a plasmid. This is done by digesting the plasmid DNA with one or more restriction enzymes, followed by analysis of the resulting fragments by gel electrophoresis. Restriction enzymes are able to recognize and cleave DNA at specific sequences. The restriction enzyme *Eco*RI has the recognition site 5'-GAATTC- 3' (Reece, 2004).

Procedure:

The restriction reaction mixture was prepared as following and incubated at 37°C for two hours. After incubation the fragments were separated on 1% agarose gel by gel electrophoresis.

Digestion performed with *Eco*RI:

Reagent	Volume
*Eco*RI (10 U/ µl)	0.5 µl
10 x Buffer	1 µl
Plasmid DNA	3 µl
MQ water	5.5 µl
Total	10 µl

Digestion performed with HindIII and XhoI:

Reagent	Volume
Hind III (20 U/ µl)	0.5 µl
Xho I (20 U/ µl)	0.5 µl
10 x NEBuffer 2	1 µl
Plasmid DNA	3 µl
10 x BSA	1 µl
MQ water	4 µl
Total	10 µl

2.2.8 DNA sequencing

BigDye® Terminator v 3.1 sequencing protocol was used to amplify a DNA fragment of interest. The nucleotide sequence within a DNA fragment can be determined by automated sequencing. Automated DNA sequencing is achieved by using dideoxynucleotide (ddNTP) labelled with a different dye. The synthesis of growing DNA sequence will terminate when ddNTP is incorporated so that different lengths of DNA with different acceptor dyes will be produced. Polyacrylamide gel is used to separate DNA fragments of different lengths. The sequence of DNA molecule is determined when the fluorescence detector measures the wavelengths of the fluorescence emitted by the separated DNA molecules (Reece, 2004).

Procedure:

The mixture of sequencing reaction was prepared according to the BigDye v3.1 sequencing protocol (UNN, 2010). The samples were sent to the University of Tromsø for automated sequencing.

Reagent	Volume
Big-Dye v3.1	1 µl
Sequencing buffer	3 µl
Template	200 ng
Primer (3.2 pmol)	1 µl
MQ water	14 µl
Total	20 µl

The sequences of used forward and reverse primers are provided in Table A1, Appendix 4.

A thermal cycler was provided to amplify the DNA fragment of *EhDddD* gene by PCR as following:

1	96°C, 5 min	
2	96°C, 10 sec 50-53°C, 5 sec 60°C, 4 min	25 cycles
3	4°C, Hold	

2.2.9 DNA sequence analyzing

Chromas Lite version 2.01 software was used to analyze DNA sequences. The software also provides information about the quality of sequences and the presence of contamination in the PCR products (Technelysium, 2005).

Multiple Alignment Construction & Analysis Workbench (MACAW) is a program for analyzing, locating and comparing of sequences. The program was used to find similarities and differences between sequences of different strains by linking and aligning them together into a multiple alignment (Biology-Software-List, 1999-2012).

2.3 RNA isolation

Spectrum™ Plant Total RNA Kit was used to isolate and purify high quality total RNA from strains of *E. huxleyi* without using solvents such as phenol and chloroform. The Lysis Solution containing 2-Mercaptoethanol (2-ME) releases RNA and inactivates ribonucleases. Filtration column removes the residual debris from lysate, while Binding Solution captures the RNA onto the binding column and prevents genomic DNA and polysaccharides from clogging the column. Residual DNA is eliminated by wash solutions and purified RNA is

eluted by RNase-free water. DNase digestion removes most of the DNA, which is necessary for very sensitive applications (Sigma-Aldrich, 2010).

Procedure:

1. The cells of *E. huxleyi* were harvested, flash frozen in liquid nitrogen and stored at -80°C.
2. The frozen cells were grounded in the TissueLyser (QIAgen) for 2x2 minutes at 25 Hz.
3. 10 µl of 2-ME was added for every 1 ml of Lysis Solution. 500 µl of the mixture was added to each RNA preparation and placed in the TissueLyser (QIAgen) for 2x2 minutes at 25 Hz.
4. The samples were incubated at 56°C for five minutes and then centrifuged at maximum speed (16.060 g) for three minutes to pellet cellular debris.
5. The lysate supernatant was pipetted into a filtration column (blue retainer ring) placed in a 2 ml collection tube and centrifuged at 16.060 g for one minute to remove the residual debris.
6. 500 µl of Binding Solution was added to the lysate and mixed by pipetting several times.
7. 750 µl of the mixture was transferred into a binding column (red retainer ring) placed in a 2 ml collection tube. The samples were centrifuged at 16.060 g for one minute to bind RNA. The flow-through was decanted and residual liquid was removed by tapping the collection tube on an absorbent paper.
8. 300 µl of Wash Solution 1 was transferred into the binding column and centrifuged at 16.060 g for one minute. After centrifugation the flow-through was decanted.
9. 80 µl of DNase I in DNase digestion buffer from Qiagen (RDD buffer) was added onto the center of the filter inside the binding column. The sample was incubated at room temperature for 15 minutes.
10. 500 µl of Wash Solution 1 was added into the binding column and the tube was centrifuged at 16.060 g for one minute to remove the digested DNA. The flow-through was decanted.
11. 500 µl of Wash Solution 2 was added twice into the binding column and the tube was centrifuged at 16.060 g for 30 seconds. The flow-through was decanted and the column was centrifuged at 16.060 g for one minute to dry. The column was transferred into a new 2 ml collection tube.

12. 50 µl of Elution Solution was added onto the center of the binding matrix and incubated at room temperature for one minute. Centrifuged the column at 16.060 g for one minute to elute the purified RNA.
13. 1.2 µl of RNasin (1 µl RNasin per 40 µg RNA) from Promega was added to each sample to inhibit ribonuclease activity (Sigma-Aldrich, 2010). The concentration and purity of RNA was measured by NanoDrop and Qubit (described in chapter 2.3.1) for further analysis, and the samples were stored at -80°C.

2.3.1 Qubit

Quant-iT™ assay kits uses dyes selective for dsDNA, RNA and proteins. The presence of contaminants, nucleic acids and proteins in the samples will not affect the quantitation of RNA (Invitrogen, 2007). Qubit® fluorometer was used to measure the exact quantitation of RNA in the samples.

The samples were prepared by using Quant-iT^{TM} RNA Assay Kit, according to the Quant-iT^{TM} Assays Abbreviated Protocol (Invitrogen, 2009).

2.3.2 Formaldehyde gel electrophoresis

Formaldehyde (FA) gel electrophoresis is used to denaturate and separate RNA molecules on an agarose gel to determine the quality of RNA for further analysis. The recipe for 1.2% FA agarose gel and FA running buffer are provided in Appendix 2.

Procedure:

1. The samples were centrifuged for 30 seconds, incubated at 65°C for five minutes and applied into wells in the gel placed in FA running buffer.
2. The samples were electrophoresed at 80 V for one hour.

2.4 Complimentary DNA synthesis for qRT-PCR

RNA molecules are unstable and therefore need to be converted to complimentary DNA (cDNA) by the process of reverse transcription (RT). The synthesis was conducted by using QuantiTect Reverse Transcription kit produced by Qiagen. cDNA is a more stable molecule and contain no introns, whereas genomic DNA (gDNA) contain introns. A negative reverse transcription (NRT) reaction was prepared to control the level of the DNA contamination in RNA samples (Qiagen, 2009).

gDNA Wipeout buffer eliminate effectively gDNA contamination from RNA samples. RT-primer mix contains an optimized mixture of deoxy-thymine nucleotides (Oligo-dT) and random primers that enables cDNA synthesis from all regions of RNA transcripts, even from 5' regions. The enzyme reverse transcriptase, RT-primer mix and RT-buffer transcribes RNA to cDNA in RT reaction (Qiagen, 2009).

Procedure:

1. The RNA samples were placed on ice and the gDNA Wipeout buffer, Quantiscript reverse transcriptase, RT-buffer, RT-primer mix and RNase-free water were thawed at room temperature.
2. The gDNA elimination reaction was prepared on ice by mixing 2 µl of gDNA Wipeout buffer and RNase-free water to each 1 µg of template RNA to a total volume of 14 µl.
3. The gDNA elimination reaction was incubated at 42°C for two minutes. After incubation the reaction mix was placed on ice. NRT reactions was prepared in same manner but with half of the volume.
4. The master mix of reverse transcription reaction was prepared by mixing 1 µl of Quantiscript reverse transcriptase, 4 µl of RT-buffer and 1 µl of RT-primer mix per reaction.
5. 6 µl of master mix was added to each sample (14 µl) to a total volume of 20 µl and incubated at 42°C for 15 minutes.
6. The reactions were incubated 95°C for three minutes to inactivate Quantiscript reverse transcriptase. The samples were stored at -20°C.
7. The master mix of NRT reaction was prepared with half of volume and RNase-free water was used instead of Quantiscript reverse transcriptase.

2.5 Quantitative reverse transcription PCR (qRT-PCR)

Light cycler 480 SYBR® Green I Master kit was used for amplification and detection of any DNA or cDNA using LightCycler® 480 Instrument. This kit is suited for hot-start PCR applications, minimizes primer-dimers and is capable for very sensitive detection of defined DNA sequences. The fluorescence reporter SYBR® Green emits light when it binds dsDNA in the minor groove (Reece, 2004). The emitted light can be detected in each cycle throughout the reaction to obtain an amplification plot (Roche-Applied-Science, 2011). The fluorescent signal increases proportionally to the amount of PCR product in a reaction. The advantages of

SYBR Green are that it is sensitive and inexpensive, whereas the disadvantage is that it will bind to any dsDNA. As a result, SYBR Green also binds to non-specific reaction products or primer-dimers (Reece, 2004).

PCR amplification using the Light Cycler® 480 (Roche) instrument consist of the following steps: pre-incubation, amplification, melting curve and cooling. The pre-incubation step is for denaturation of DNA and activation of FastStart Taq DNA polymerase. The amplification step consists of PCR amplification of target DNA. The melting curve step identifies the PCR product. The cooling step cools down the PCR plate (Roche-Applied-Science, 2011).

To improve the specificity of targets rich in GC content in PCR amplification, a variety of PCR enhancing agents can be included. Betaine and dimethyl sulfoxide (DMSO) are the most commonly used enhancing agents, but DMSO was chosen instead of Betaine because it was more effective in increasing the specificity of the reaction. DMSO facilitates strand separation by disrupting base pairing and reduces the melting temperature of DNA and primers (Frackman et al., 1998; Jensen et al., 2010).

Procedure:

1. cDNA samples and NRT were diluted 1:10 to dilute possible PCR inhibitors that can interfere with the amplification of the target sequence. The primers were also diluted 1:10 to avoid generation of primer-dimers and non-specific annealing. The primers used are listed in Table A2, Appendix 4.
2. A master mix for qRT-PCR with the Light cycler® 480 SYBR Green I Master kit was prepared by mixing 2 µl of PCR grade water, 2 µl of diluted PCR primer, 1 µl of DMSO (final concentration 5% v/v) and 10 µl of SYBR® Green per reaction to a total volume of 15 µl.
3. 5 µl of template cDNA and 15 µl of master mix were added to each well on a 96-multiwell PCR plate. In the no template control (NTC) reaction wells, the template was substituted by PCR grade water. NTC is used to reveal if there are there are any contaminations from other sources than the cDNA (Roche-Applied-Science, 2011).

The PCR conditions in the Roche Light Cycler® were set up with the following pre-incubation (1), amplification (2), melting curve (3) and cooling (4) programs:

1	95°C, 5 min	
2	95°C, 10 sec 65°C, 10 sec 72°C, 10 sec	45 cycles
3	95°C, 5 sec 65°C, 1 min 99°C, -	
4	40°C, 10 sec	

2.6 Quantitative RT-PCR data analysis

Quantitative RT-PCR (qRT-PCR) generates data to quantify the level of gene expression of one or several genes in a sample. The fit point method was used to determine the crossing threshold (Ct) value by drawing a line parallel to the x-axis in the log-linear region of the fluorescence intensity curve (Luu-The et al., 2005). The Ct-value is defined as the number of cycles it takes each reaction to reach the fluorescence threshold (VanGuilder et al., 2008). The generated Ct-values were used to analyze and compare the levels of relative expression of a target gene (*EhDddD*) versus a reference gene in two samples. Actin was used as a reference gene for normalization.

2.6.1 PCR primer efficiency calculation

Samples in which the gene of interest has a high expression level will have a higher starting concentration of template and will reach the threshold faster, resulting in a low Ct-value. Conversely, high Ct-values indicate that the sample needs more cycles to reach the defined threshold and therefore have a low starting concentration of template (Ruijter et al., 2009)

The mean PCR efficiency per amplicon was calculated using the LinRegPCR software. LinRegPCR is a program for the analysis of qRT-PCR data, and functions by performing a baseline correction on each sample followed by a linear regression analysis to fit a straight line through the PCR data set. The PCR efficiency of each sample is calculated by the slope of the line (Ruijter et al., 2009).

2.6.2 Melting curve analysis

The melting temperature (T_m) is defined as the temperature when 50% of the DNA in a sample appears as dsDNA and 50% as single-stranded DNA (ssDNA). The resulting melting curves differ between primer-dimers and specific PCR products. The primer-dimers melt at lower temperature (around 70°C) than specific products (over 80°C) (Roche-Applied-Science, 2011).

2.6.3 REST analysis

The Relative Expression Software Tool (REST) 2009 is used in gene expression studies, where it compares two groups; treated and untreated. The calculated mean PCR efficiency per amplicon and the Ct-values of reference gene and of the target gene (*EhDddD*) were used to compare the expression level between two strains of *E. huxleyi*.

2.7 cDNA synthesis for full-length gene amplification

Messenger RNA (mRNA) was reverse transcribed to cDNA by an enzyme known as reverse transcriptase. Oligo-dT primer was used to obtain a full length of cDNA. Since RNA has a poly-A 3' tail, the Oligo-dT primer binds poly-A tail and provide a free 3'-OH end that can be used by reverse transcriptase to create cDNA (Reece, 2004).

cDNA synthesis was performed using the PrimeScript™ 1st strand cDNA Synthesis Kit by TaKaRA, according to the standard protocol (TaKaRa, 2012c).

2.7.1 Gradient PCR

The Gradient function of gradient PCR was used to vary the temperature distributed across the block to determine the optimum annealing temperature.

Procedure:

To amplify the *EhDddD* gene, the reaction mixture of Gradient PCR was performed by mixing the reagents together in PCR tubes to a total volume of 25 µl as following. The samples were then amplified in 6 different annealing temperatures (55°C, 55.6°C, 57.8°C, 59.1°C, 61.8°C and 64.2°C) to determine the optimum annealing temperature.

Reagent	Volume
TaKaRa LA *Taq* (5 U/ µl)	0.125 µl
dNTP mixture (10 mM)	2 µl
2 X GC Buffer I	12.5 µl
Template	1 µl
DMSO (4%)	1 µl
att Forward primer (10 pmol/µl)	1 µl
att Reverse primer (10 pmol/µl)	1 µl
MQ water	6.4 µl
Total	25.025 µl

The sequence of *att* forward and reverse primers are provided in Table A1, Appendix 4.

A thermal cycler was used to amplify *EhDddD* gene by Gradient PCR as following:

1	95°C, 1 min	
2	94°C, 30 sec 55-64.2°C, 30 sec 70°C, 3 min	35 cycles
3	70°C, 5 min	
4	Hold, 4°C	

2.8 Determining of dimethyl sulfide (DMS) and dimethylsulfoniopropionate (DMSP) by solid-phase microextraction and gas chromatography-mass spectrometry

Determination of dimethyl sulfide (DMS) and dimethylsulfoniopropionate (DMSP) in the samples was achieved using solid-phase microextraction (SPME) followed by gas chromatography-mass spectrometry (GC-MS), as described in Niki et al. (2004). The target compound is extracted on the fiber and introduced directly to the GC column through the GC injection port. The coating of the SPME fiber that was utilized in this experiment was a combination of carboxen and polydimethylsiloxane (PDMS), and is abbreviated to Car-PDMS. To determine the abundance of DMS and DMSP in the samples, 6 of 12 samples were treated with sodium hydroxide (NaOH) to hydrolyze DMSP to DMS. The amount of DMSP was calculated as DMSP = DMS+DMSP (treated samples) - DMS (untreated samples) (Niki et al., 2004). For quantification of DMS, the molecular ions at mass-to-charge ratios (*m/z*) 62.15 and 47 were used.

Procedure:

1. Treated samples were prepared by adding 0.2 ml of 5 M NaOH into 4 ml glass vials containing 2 ml of the cells to hydrolyze DMSP to DMS. Untreated samples were prepared by adding 0.2 ml L1-Si medium into 4 ml glass vials containing 2 ml of the cells.
2. The treated samples were equilibrated in a thermostatic bath shaker for 30 minutes at 25°C, while untreated samples were equilibrated for 10 minutes at 25°C.
3. Before the SPME procedure, the Car-PDMS fiber was pretreated in the injection port of the GC for one minute at 250°C.
4. The samples were vortexed for 30 seconds and the Car-PDMS fiber was exposed in the headspace of the vial for 10 minutes at 22°C.
5. The DMS on Car-PDMS fiber was immediately desorbed in the injection port (250°C) of the GC in splitless mode. An overview of instrument control parameters is presented in Table 2.1.
6. DMS was detected by a mass spectrometer (MS) running in selected ion monitoring (SIM) mode (monitored *m/z* were 62.15 and 47.0) (Niki et al., 2004).

Table 2.1 Instrument control parameters in GC-MS

Control information	**Oven**	**Front intlet**
Sample intlet: GC	Initial temperature: 50°C	Mode: Splitless
Injection source: Manual	Final temperature: 150°C	Initial temperature: 250°C
Injection location: Front	Rate: 15°C/min	Pressure: 7.58 psi
Use MS: Yes	Run time: 6.67 min	Gas type: Helium
General Information	**Tune parameter for signal-to-noise ratio (S/N)**	**MS acquistion parameters**
Uploaded method: DMSP-7 minute	Ionization voltage 70eV	Acquistion mode: SIM
Fiber: Car-PDMS		Plot 1 ion: 62.15
		Plot 2 ion: 47.0
Transfer line temperature		MS Quadrupole: 150°C
200°C		MS Source: 230°C

2.8.1 DMS standard curve

Three replicas of nine different concentrations of DMS (1 µM, 0.1 µM (100 nM), 0.01 µM (10 nM), 0.001 µM (1 nM), 0.0001 µM (100 pM), 0.00001 µM (10 pM), 0.000001 µM (1 pM), 0.0000001 µM (100 fM) and 0.00000001 µM (10 fM)) were used to make a standard curve to determine the concentrations of DMS and DMSP in the extracts of *E. huxleyi* strains 1516 and 373. The method was performed in 4 ml glass vials.

Procedure:

1. To obtain a concentration of 1 µM DMS, 219 µl of DMS was mixed with 2781 µl MQ water in a 15 ml tube to make a start concentration of 1 mM DMS. The tube was vortexed for a few seconds and 300 µl of the mixture was transferred into the next tube, which contained 2700 µl MQ water to obtain a DMS concentration of 100 µM. The tube of 100 µM DMS was vortexed and 300 µl of the mixture was transferred into the next tube which contained 2700 µl MQ water to obtain a DMS concentration of 10 µM. The tube was vortexed for few seconds and 300 µl of the mixture was transferred into a new 15 ml tube containing 2700 µl MQ water for a final concentration of 1 µM DMS. Further dilutions of DMS (100 nM, 10 nM, 1 nM, 100 pM, 10 pM, 1 pM, 100 fM and 10 fM) were generated by repeated transfer of 300 µl to a new tube containing

2700 µl MQ water. The tubes were vortexed for a few seconds and 2.2 ml was transferred into a 4 ml glass vial for GC-MS.

2. Solid-phase microextraction and gas chromatography-mass spectrometry was performed as described in chapter 2.8, steps 3-6.

2.9 DMSP lyase activity measurements by solid-phase microextraction and gas chromatography-mass spectrometry

DMSP lyase activity varies in *E. huxleyi* strains, and intracellular DMSP concentration is not consistent with DMSP lyase activity (Steinke et al., 1998). To measure the DMSP lyase activity in two strains (1516 and 373) of *E. huxleyi*, the cells were harvested according to the method described by Steinke et al. (1998).

Procedure:

1. Test buffer was made by mixing 32.1 ml of dibasic sodium phosphate (Na_2HPO_4) (1.2 M) and 17.9 ml of citric acid ($C_6H_8O_7$) · H_2O (0.1 M) to obtain a pH of 6. The mixture was diluted with MQ water to a total volume of 100 ml. 500 mM sodium chloride (NaCl), 20 mM calcium chloride ($CaCl_2$), 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (1 tablet, Roche) were added to the mixture.
2. 600 ml cell culture of *E. huxleyi* strains 1516 and 373 were distributed to three 250 ml centrifuge cups and centrifuged at 3500 g for 20 minutes and 15°C. The supernatant was removed. Each cell pellet was resuspended in 10 ml medium and all the content of the three centrifuge cups was collected into one 40 ml centrifuge cup.
3. The cells were centrifuged once more at 20.000 g, 4°C for 10 minutes.
4. The resulting pellet was resuspended in 3 ml test buffer, and the cells were homogenized on ice using ultrasound (2x5 seconds).
5. The cells were distributed by adding 500 µl in 6 centrifuge tubes (1.5 ml) and stored at -20°C (Steinke et al., 1998).
6. 245 µl of test buffer was mixed with 50 µl of resuspended pellet and 5 µl DMSP (1.2 M).
7. Before the SPME procedure, the Car-PDMS fiber was pretreated in the injection port of the GC for one minute at 250°C.
8. The samples were vortexed for 30 seconds and the Car-PDMS fiber was exposed in the headspace of the vial for 10 minutes at 22°C.

9. The Car-PDMS fiber was desorbed in the injection port (250°C) of the GC in splitless mode.
10. DMS was detected by a MS running in SIM mode (monitored *m/z* were 62.15 and 47.0). The condition of instrument control parameters in GC-MS that was utilized in this part of the experiment is shown in Table 2.1.

2.9.1 DMS standard curve for DMSP lyase activity measurements

Three replicas of seven different concentrations of DMS (100 μM, 10 μM, 1 μM, 0.1 μM (100 nM), 0.01 μM (10 nM), 0.001 μM (1 nM) and 0.0001 μM (100 pM)) were used to make a standard curve to determine the concentration of DMS in the extracts of *E. huxleyi* strains 373 and 1516 of DMSP lyase activity measurements (described in chapter 2.9). The method was performed in 1.5 ml glass vials.

Procedure:

1. 7 μl of DMS was mixed with 993 μl of test buffer (described in chapter 2.9) in a centrifuge tube (1.5 ml) to obtain a concentration of 100 μM DMS. Further dilutions of DMS (10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM,) were generated by repeated transfer of 100 μl to a new tube containing 900 μl of test buffer. The centrifuge tubes were vortexed in a few seconds and 300 μl of the mixtures were transferred into 1.5 ml glass vials for GC-MS.
2. Solid-phase microextraction and gas chromatography-mass spectrometry was performed as described in Chapter 2.8, steps 3-6.

2.10 Statistical tests

The significance of differences in DMS and DMSP concentrations between the 1516 and 373 strains was calculated using student's t-test in Microsoft Excel 2010. P-values less or equal to 0.05 were defined as significant.

3 Results

In this thesis, the *Emiliania huxleyi* DMSP lyase (*EhDddD*) gene has been examined in two strains, NCMA1516 and NCMA373 (hereafter named 1516 and 373, respectively), by several methods to study the cleavage of dimethylsulfoniopropionate (DMSP) to dimethyl sulfide (DMS) by enzymes known as DMSP lyases.

The differences of DMSP lyase enzymes between the 1516 and 373 strains were identified by sequencing the *EhDddD* gene, studying the level of gene expression of *EhDddD* gene, measuring the DMSP lyase activity (DLA), and determining extracellular DMS and DMSP concentrations.

3.1 Cloning of the *EhDddD* gene in 1516 and 373

3.1.1 Gradient PCR

The *EhDddD* gene in 1516 and 373 was amplified with *att* primers using gradient PCR to determine the optimum annealing temperature. The best annealing temperature of the ones tested for amplifying *EhDddD* gene in strain 1516 and 373 appeared to be 64.2°C, as shown in Figure 3.1. The expected size (3450 bp) of the PCR product of 1516 and 373 is marked with a red square.

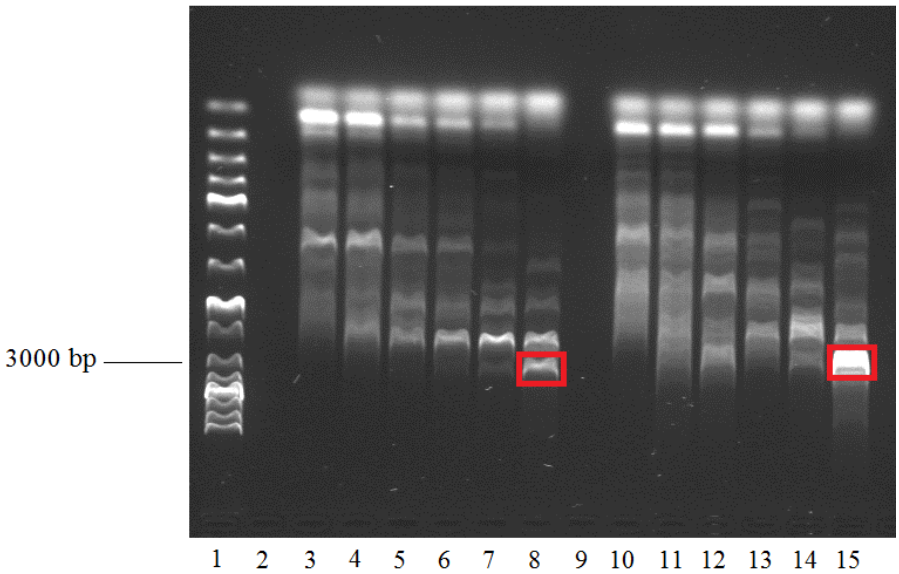


Figure 3.1 PCR products of the *EhDddD* gene in 1516 and 373 strains with different annealing temperatures separated by gel electrophoresis. Lane 1: GeneRuler™ 1kb Plus DNA ladder (Fermentas), lane 2: space, lane 3: 1516 55°C, lane 4: 1516 56.6°C, lane 5: 1516 57.8°C, lane 6: 1516 59.1°C, lane 7: 1516 61.8°C, lane 8: 1516 64.2°C, lane 9: space, lane 10: 373 55°C, lane 11: 373 56.6°C, lane 12: 373 57.8°C, lane 13: 373 59.1°C, lane 14: 373 61.8°C, lane 15: 373 64.2°C. The red squares indicate the expected size of PCR product (3450 bp) obtained at annealing temperature of 64.2°C.

The amplified fragment was excised from the agarose gel and purified from agarose gel using Wizard® SV Gel and PCR Clean-Up System kit (described in chapter 2.2.4) and DNA quality and quantity were checked using NanoDrop spectrometry (described in chapter 2.2.1). The PCR products were cloned into the pCR™ II-TOPO® vectors using TOPO TA cloning® Kit Dual Promoter (described in chapter 2.2.5). Five colonies from each agar plate were picked and cultured, and plasmid DNA was isolated from these clones using QIAprep Miniprep kit (described in chapter 2.2.6) and quantified using NanoDrop.

3.1.2 Verification of *EhDddD* by restriction enzymes

Restriction cutting of plasmid was used as a control to confirm that a PCR product was correctly inserted in the pCRII-TOPO vector. Digestion with *Eco*RI produced two fragments. The size of PCR product was about 3.5 kilo-bases (kb), while the size of the vector was 3.9 kb. Since both fragments had almost the same size, the plasmids were digested with HindIII and XhoI. Based on the predicted *EhDddD* gene sequence, this digestion was expected to produce six fragments (1617, 787, 707, 273, 36 and 33 bp). Only the four largest restriction fragments are shown on the agarose gel.

Figure 3.2 A shows *Eco*RI digestion and Figure 3.2 B shows HindIII and XhoI digestion of plasmid DNA from the pCRII-TOPO-*EhDddD* clones.

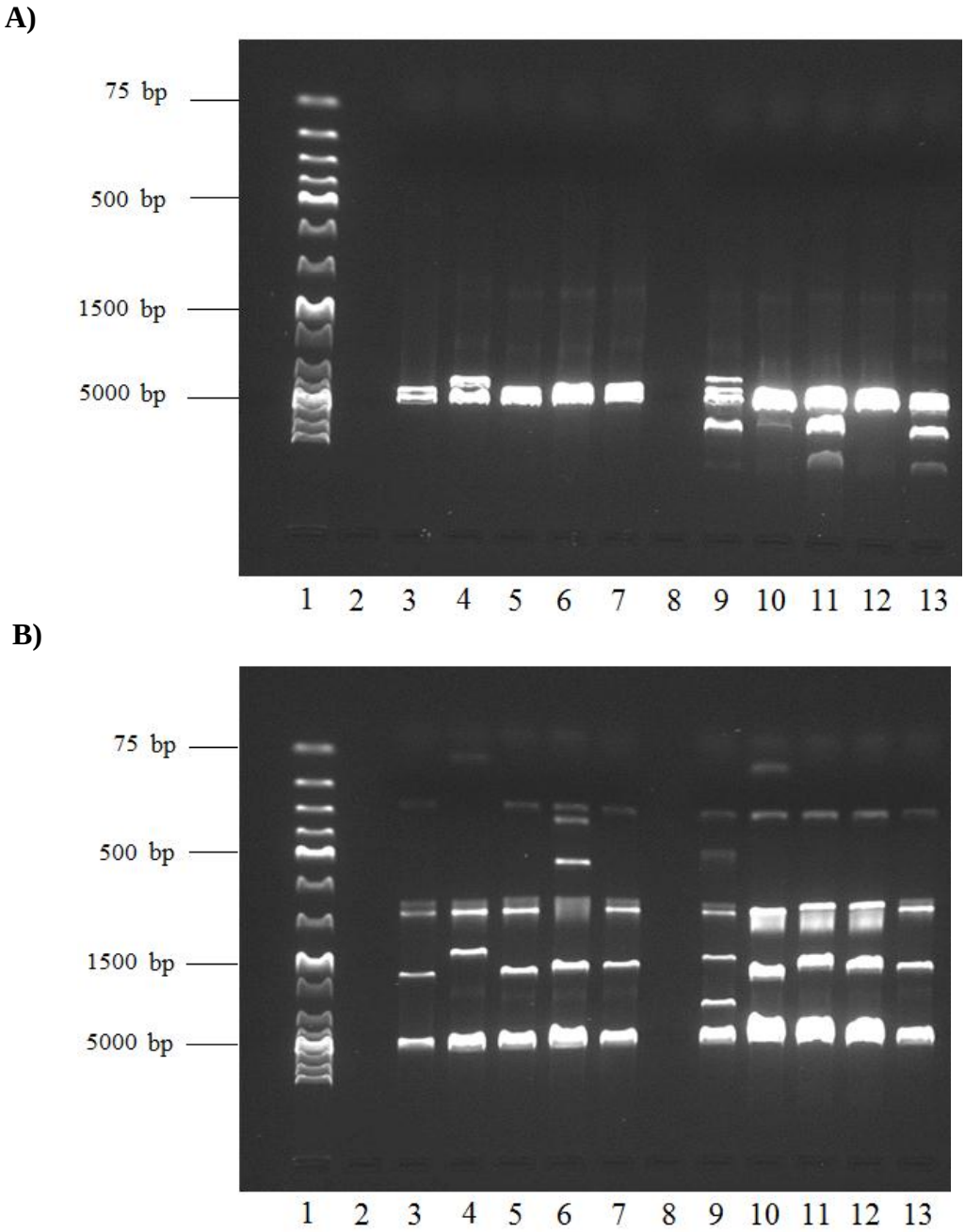


Figure 3.2 Verification of insertion of the *EhDddD* gene into pCRII-TOPO by HindIII and XhoI. Agarose gel electrophoresis of pCR™ II-TOPO® vector cloned with *EhDddD* gene of 1516 and 373, that has been digested with A) *Eco*RI and B) HindIII and XhoI restriction enzymes. Lane 1: GeneRuler™ 1kb Plus DNA ladder (Fermentas), lane 2: space, lane 3-7: 1516 clone 1-5, lane 8: space, lane 9-13: 373 clone 1-5.

Figure 3.2 A shows that 1516 clone 3-5 and 373 clone 2 and 4 seemed to contain the plasmid with *EhDddD* gene. The digestion with HindIII and XhoI revealed that 1516 clone 1, 3 and 5 and 373 clone 2-5 contained the plasmid with *EhDddD* gene. Three of five clones of 1516 and four of five clones of 373 were sequenced.

3.1.3 DNA sequences of *EhDddD*

For further verification of the *EhDddD* gene in 1516 and 373, and for studying the differences of the DMSP lyase activity between these two strains, the full-length *EhDddD* gene was sequenced. The selected pCRII-TOPO-*EhDddD* clones were subjected to amplification using the BigDye® Terminator v3.1 sequencing protocol (described in chapter 2.2.8) and sent to the University of Tromsø for automated sequencing.

The cDNA sequence of *EhDddD* gene was translated into a polypeptide chain to compare the difference in amino acid (aa) sequence in 1516 and 373. Strain 1516 contained 1136 aa, whereas strain 373 contained 1135 aa as shown in Figure 3.3. The order of aa in both strains differ in eleven aa, where five of eleven differences were conserved (having similar chemical properties). In addition to this, 1516 had an insertion of one aa close to the N-terminal (position 13), which may be serine or aspartate. These observed differences may be the reason for the variation in DLA.

```
                 *        20         *        40         *        60
373 DddD  : MASTFSSSSSDGG-RTAPLDGLHVVEIGPFLACPLTARHLLDLGASVTAVVRPESARGQR :   59
1516 DddD : MASTFSSSSSDGDSRTAPLDGLRVVEIGSFLACPLTARHLLDLGASVTAVVRPESARGQR :   60

                 *        80         *       100         *       120
373 DddD  : AEQAWRPETTRALRSGKDVVTLDLKSPAGQEALDELLVAADAVVVGFAPAVCRRLRLTAE :  119
1516 DddD : AEQAWRPETTRALRSGKDVVTLDLKSPAGQEALDELLVAADAVVIGFAPAVCRRLRLTAE :  120

                 *       140         *       160         *       180
373 DddD  : RVHEVNPRAVLAHLPGFATGDAERSKIEAWEASILAEAGVFRDMGISRQLAGKLASYSPL :  179
1516 DddD : RVHEVNPRAVLAHLPGFATGDAERSKIEAWEASILAEAGVFRDMGINRQLAGKLASYSPL :  180

                 *       200         *       220         *       240
373 DddD  : PLASSYASIFAALGVVSAERKRTTLPPGAAPRLSLEVPLASALCDALVHNSLQHEVPEEY :  239
1516 DddD : PLASSYASIFAALGVVSAIRKRTTLPPGAAPRLSLEVPLASALCDALVHNSLQHEVPEEY :  240

                 *       260         *       280         *       300
373 DddD  : RSRRQRALDKQRAGEPLDYFETLELTDPFFSHYTTADARPFYLVAPCHLRHQRRAIAVLG :  299
1516 DddD : RSRRQRALDKQRAGEPLDYFETLELTDPFFSHYTTADARPFYLVAPCHLRHQRRAIAVLG :  300

                 *       320         *       340         *       360
373 DddD  : IEEQVAALGVPLAATYAASAVGANAPRHGFGAGQVGDDWAPKLRKLMRRAFLTRTAYEWE :  359
1516 DddD : IEKQVAALGVPLAATYAASAVGANAPRHGFGAGQIGDDWAPKLRKLMRRAFLTRTAYEWE :  360

                 *       380         *       400         *       420
373 DddD  : AAFGAAGVPGSAHRTTAEWLTCPHARAAGLVREDESGAVSPAAMTWVVQQELPPPLPSPS :  419
1516 DddD : AAFGAAGVPGSAHRTTAEWLTCPHARAAGLVREDESGAVSPAAMTWVVQQELPPPLPSPS :  420

                 *       440         *       460         *       480
373 DddD  : LPSPCRSPEGRPSSADAAPHFGGGAGGAGRPAAVRPAAGWATRAEAAPAAPPPPPSPAAA :  479
1516 DddD : LPSPCRSPEGRPSSADAAPHFGGGAGGAGRPAAVRPAAGWATRAEAAPAAPPPPPSPAAA :  480

                 *       500         *       520         *       540
373 DddD  : REGGRSGGGWMEGVEVLDLCNVIAGPTIGTMLARFGAKVTKVDSPRPTYSPEITVLYGLA :  539
1516 DddD : WEGGRSGGGWMEGVEVLDLCNVIAGPTIGTMLARFGAKVTKVDSPRPTYSPEITVLYGLA :  540

                 *       560         *       580         *       600
373 DddD  : ANAGKRSVLLDVSPAEAAGRAAFEALVARVDVVVYSGTSDALERLGITPAELHRMNPNVV :  599
1516 DddD : ANAGKRSVLLDVSPAEAAGRAAFEALVARVDVVVYNGTSDALERLGITPAELHRMNPNVV :  600

                 *       620         *       640         *       660
373 DddD  : LSRFDAYGGPNEGKGERADHISYDDNLQAALGIMERFGGGLGRVEEHAHVGTIDVAAGVA :  659
1516 DddD : LSRFDAYGGPNEGKGERADHISYDDNLQAALGIMERFGGGLGRVEEHAHVGTIDVAAGVA :  660
```

```
                  *       680          *       700          *       720
373 DddD  : GALATAATLLLRERRATGELPPAPPRSLLIARASLASVGQMVQFPFCCGPPAALAAEGDR :  719
1516 DddD : GALATAATLLLRERRATGELPPAPPRSLLIARASLASVGQMVQFPFCCGPPAALAAEGDR :  720

                  *       740          *       760          *       780
373 DddD  : SVDTPLNRGPECRGEHSLLHCYSTADGSWLLLVASLLPPLRMGEAELKTVLRHLSLADGR :  779
1516 DddD : SVDTPLNRGPECRGEHSLLHCYSTADGSWLLLVASLLPPLRMGEDELKTVLRHLSLADGR :  780
                  *       800          *       820          *       840
373 DddD  : LHAALRPALERGVGGVSDAALEAAVGGALRADPSASWWAERLGAVGVSAVPLASFDVLRE :  839
1516 DddD : LHAALRPALERGVGGVSDAALEAAVGGALRAGPSASWWAERLGAVGVSAVPLASFDVLRE :  840

                  *       860          *       880          *       900
373 DddD  : SNILAAEDCTVDLGGSTFQFLRHGSHPLGSPLVMFAPCSVRTPGGCGLAVPLEDAPRYGE :  899
1516 DddD : SNILAAEDCTVDLGGSTFQFLRHGSHPLGSPLVMFAPCSVRTPGGRGLAVPLEDAPRYGE :  900

                  *       920          *       940          *       960
373 DddD  : HTLEVLGELGVDPTLLLSRHAAATGWCDDYLPGKASQTLPDIPRPALKTCPVCLDPIKRH :  959
1516 DddD : HTLEVLGELGVDPTLLLSRHAAATGWCDDYLPGKASQTLPDIPRPVLKTCPVCLDPIKRH :  960

                  *       980          *      1000          *      1020
373 DddD  : VGLACSHWLCHDCAVRCSNAGLASCPVCRHPQLLDPVRLARRSVEWRAAYGSWRQGGVRG : 1019
1516 DddD : VGLACSHWLCHDCAVRCSNAGLASCPVCRHPQLLDPVRLARRSVEWRAAYGSWRQGGVRG : 1020

                  *      1040          *      1060          *      1080
373 DddD  : SKGEASSISSAAQAPARSLVTSAAGDLAKGSFRKWSGASLAHSSPIRAMKSCAAGLSLAE : 1079
1516 DddD : SKGEASSISSAAQAPARSLVTSAAGDLAKGSFRKWSGASLAHSSPIRAMKSCAAGLSLAE : 1080

                  *      1100          *      1120          *
373 DddD  : VREQELLRRRDSDASSARSSARLSAGSGRSPGAPRPAHELRDSLGSEVPTRPASRS : 1135
1516 DddD : VREQELLRRRDSDASSARSSARLSAGSGRSPGAPRPAHELRDSLGSEVPTRPASRS : 1136
```

Figure 3.3 Amino acid sequence alignment of the EhDddD protein of strains 1516 and 373. Every 10th aa along the sequence alignment is indicated with an asterisk. Black highlighted area shows the similarity of aa sequence, while not shaded area shows the differences in aa in both strains.

Searches with the EhDddD protein sequences for conserved domains (CDD search, NCBI) revealed that EhDddD contains two N-terminal domains with strongest similarity to bacterial acyl-CoA transferases of the CaiB type, and a C-terminal RING domain (Figure 3.4). The RING domain in EhDddD seems to be missing in bacteria (Figure A6, Appendix 9). In bacteria, the CaiB domains are close to each other, whereas they in EhDddD are separated by about 40 aa. A BlastP search against the NCBI protein database was performed, and showed that L-carnitine dehydratase/bile acid-inducible protein F in *Hoeflea phototrophica* DFL-43 (ZP_02165273.1) was most homolog to EhDddD protein, with 34% identical and 47% conserved aa compared with EhDddD protein of 1516 and 373. DddD from *Marinomonas* sp. MWYL1 (Todd et al., 2007) showed 31% identity and 45% conservation to the EhDddD protein.

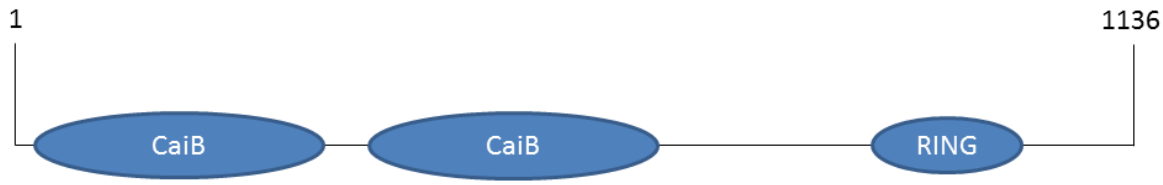


Figure 3.4 Domain organization of the EhDddD protein. EhDddD contains two domains that are similar to CaiB domains in bacteria, and a RING domain.

3.2 Expression analysis of the *EhDddD* gene in 1516 and 373 by qPCR

Four biological replicas of *E. huxleyi* cultures (200 ml) of 1516 and 373 were kept in a Vötsch growth cabinet at 18°C under cool white fluorescent light at a scalar irradiance (E_{PAR}) of 210 µmol m^{-2} s^{-1} under continuous white light conditions. The cells were harvested after obtaining a cell density of 3.5 x 10^6 cells ml^{-1}. Total RNA was isolated from harvested cells using Spectrum™ Plant Total RNA Kit (described in chapter 2.3)

Figure 3.5 shows two different total RNA profiles for 1516 and 373 by agarose gel electrophoresis. The RNA in strain 373 seemed to be somewhat degraded (lane 3-6) compared to 1516 (lane 8-11). The RNA profile for 1516 appeared to have a higher intensity of fluorescence from RNA than 373 in the 28S ribosomal RNA (rRNA) band. In 373, the fragments of degraded RNA are smaller than intact RNA and thus the fragments will migrate faster and locate on the top of the gel. The intensity of fluorescence from degraded RNA fragments in 373 seemed to be higher than 1516.

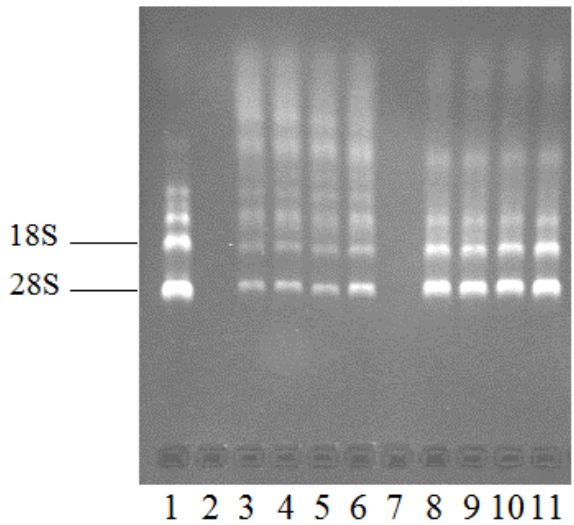


Figure 3.5 Comparison of total RNA profiles for strain 1516 and 373. Lane 1: A*rabidopsis thaliana* total RNA (control), lane 2: space, lane 3-7: 373, replicates 1-4, lane 7: space, lane 8: 1516, replicates 1-4.

Figure 3.5 may also indicate that strain 373 have less RNA compared to 1516. In addition to the NanoDrop analysis, total RNA was also quantified using a fluorometric method (Qubit). Table 3.1 shows that NanoDrop has only overestimated 1516 replica 4. Both NanoDrop and Qubit measurements showed higher values of RNA quantity in 373 compared to 1516.

Table 3.1 RNA quantity in 1516 and 373 measured by NanoDrop and Qubit.

Strains	**Replica**	**NanoDrop (ng RNA/µl)**	**Qubit (ng RNA/µl)**
1516	1	420	522
1516	2	530	579
1516	3	517	536
1516	4	429	372
373	1	639	683
373	2	591	669
373	3	538	619
373	4	627	713

qRT-PCR was performed on cDNA (described in chapter 2.4) to investigate potential differences in *EhDddD* gene expression level between the two strains (1516 and 373). Actin was used as a reference gene to normalize the expression data between 1516 and 373. The expression of the *EhDddD* gene in 373 was 8.414 times higher than in 1516, as shown in Figure 3.6.

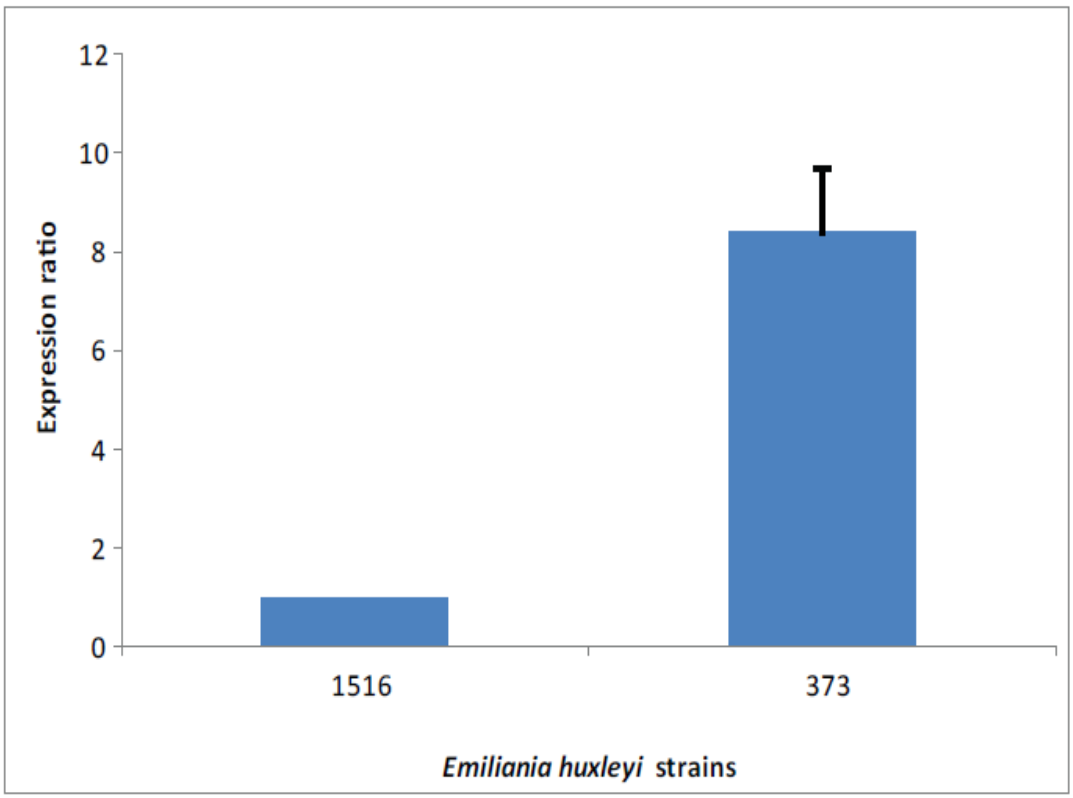


Figure 3.6 Relative gene expression of the *EhDddD* gene in 373 compared to 1516. Expression of *EhDddD* in 1516 is defined as 1. The actin gene was used as a reference gene for normalizing. n = 4.

The obtained Ct-values (Table A3) of 1516 and 373 by qPCR, REST results (Table A4 and Figure A2) and calculation of standard deviation (Table A5) are presented in Appendix 5.

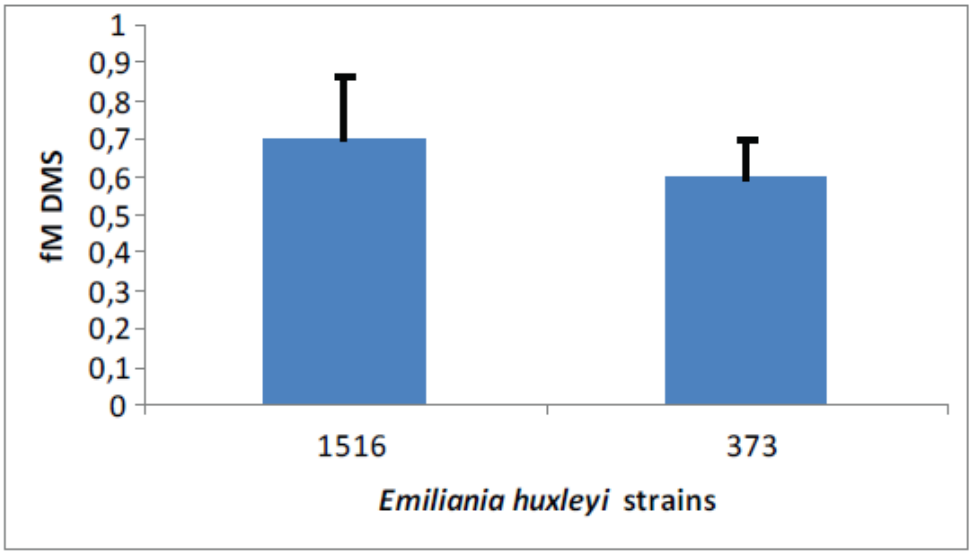
3.3 Measurement of extracellular DMS and DMSP concentrations in 1516 and 373 by gas chromatography-mass spectrometry

Three biological replicas of *E. huxleyi* cultures (100 ml) of 1516 and 373 were kept in a Vötsch growth cabinet at 18°C under cool white fluorescent light at a scalar irradiance (E_{PAR}) of 210 μmol m^{-2} s^{-1} under continuous white light conditions. The cell density of 373 was 4.1 x 10^6 cells ml^{-1}, while 1516 had a cell density of 3.0 x 10^6 cells ml^{-1}. Six of 12 samples were treated with sodium hydroxide (NaOH) to hydrolyze the DMSP to DMS (Niki et al., 2004). Measurements of DMS and DMSP amount were performed by gas chromatography-mass spectrometry (GC-MS). A DMS standard curve was made to determine the concentrations of DMS and DMSP in the extract of 1516 and 373. The obtained results were adjusted for the difference in the cell density in both strains.

Figure 3.7 A and B shows the concentrations of DMS and DMSP in the extracts of 1516 and 373. In 1516, DMS concentration was 0.7 femtomolar (fM), while DMSP concentration was 152.3 fM. DMS concentration in 373 was observed to be 0.6 fM, while the concentration of DMSP was 182.8 fM. In the extracts of both strains, the DMS concentration was 0.8 times lower in 373 than 1516, whereas the DMSP concentration was 1.2 time higher in 373 than 1516.

The ratio of DMS between 1516 and 373 was not significant (Student's t test; $p = 0.324$), while the ratio of DMSP between 1516 and 373 was significant ($p = 0.012$). After adjustment for cell density, this difference was no longer significant. The ratio of DMS showed a non-significant value of $p = 0.231$, whereas the ratio of DMSP showed a non-significant value of $p = 0.063$.

A)



B)

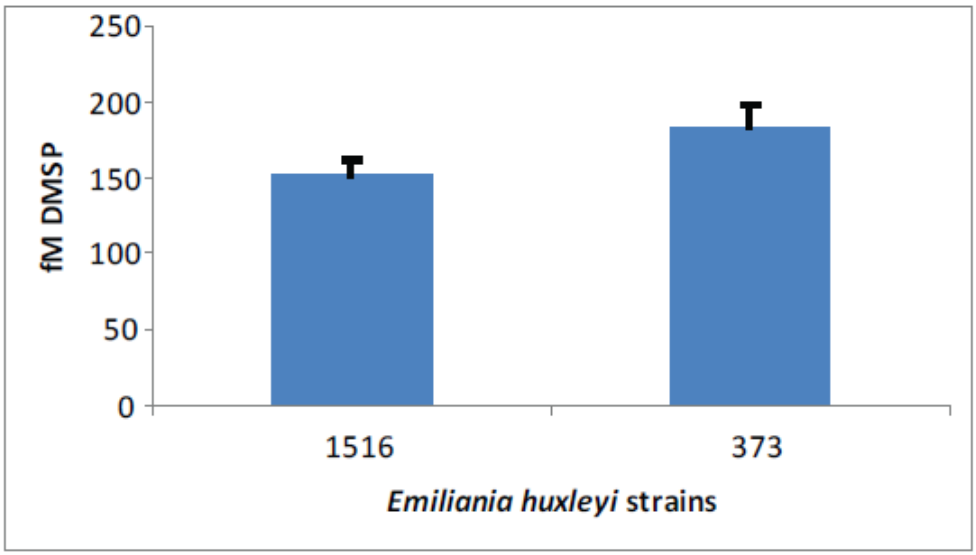


Figure 3.7. DMS (A) and DMSP (B) concentration in extracts of 1516 and 373. The concentration of DMS and DMSP is presented in fM. n = 3.

The raw data (Table A7) and calculated means (Table A6) of the experiment, standard curve (Table A8 and Figure A3) measured by GC-MS and calculation (Table A9, A10, A11 and A12) are presented in Appendix 6.

3.4 Measurement of DMSP lyase activities by GC-MS

The activity of DMSP lyase enzymes in extract of 1516 and 373 were measured using GC-MS. Three cultures of each strain (600 ml) were grown *in vitro* in a Vötsch growth cabinet at 18°C under continuous white light conditions and harvested in the exponential growth phase with a cell density of 3 x 10^6 cells ml^{-1}.

The DMS production rate in 1516 was very low and showed a DMS production of 1.04 nmol $cell^{-1}$ min^{-1} (Figure 3.8). 373 showed a higher DMS production of 63.2 nmol $cell^{-1}$ min^{-1}, indicating that the DMSP lyase activity was 60.6 times higher in 373 than 1516. This correlates with the results seen from Steinke et al (1998), which showed high DMSP lyase activity in 373 and low DMSP lyase activity in 1516.

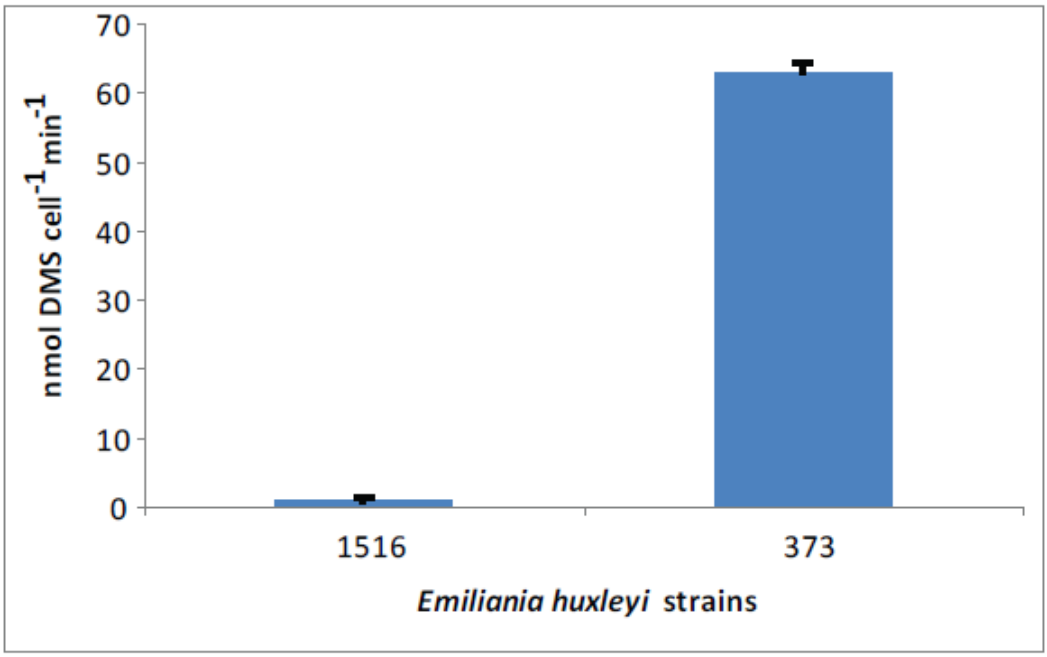


Figure 3.8 *In vitro* comparison of DMSP lyase activity in *Emiliania huxleyi* strains 1516 and 373. Strain 373 shows 60.6 times higher DMSP lyase activity than strain 1516. The DMSP lyase activity is presented in nmol $cell^{-1}min^{-1}$. n = 3.

The raw data (Table A14) and calculated means (Table A13) of the experiment, standard curve (Table A15, A16 and Figure A4) measured by GC-MS and calculation (Table A17 and A18) are presented in Appendix 7.

4 Discussion

The investigated protein DddD in *Emiliania huxleyi* (EhDddD) was defined as DMSP lyase for comparing to other studies since most other studies have investigated DMSP lyase. Several experiments were conducted on two *Emiliania huxleyi* strains (NCMA1516 and 373) to study the metabolism of dimethylsulfoniopropionate (DMSP). The cultures of *E. huxleyi* were treated equally to investigate the difference of *E. huxleyi* DMSP lyase (*EhDddD*) gene in 1516 and 373.

4.1 Variation in the amino acid sequence of the EhDddD protein of strains 1516 and 373

As presented in Figure 3.3, the order of amino acids (aa) in the sequence of 1516 and 373 differs to some extent by showing different positions of individual aa throughout the sequence. The order of aa in both strains differ in eleven aa, where five of eleven differences are conserved, having the same chemical properties. The first difference is aa number 30 in the sequence, which involves proline in 373 and serine in 1516. Proline is nonpolar and hydrophobic, while serine is polar and uncharged. Proline has an aliphatic side chain with a cyclic structure. Serine is more hydrophilic than proline, due to the functional group that forms hydrogen bond with water. The second difference was aa number 304 in the sequence, which involved glutamate in 373 and lysine in 1516. Glutamate is negatively charged because of a second carboxyl group. In contrast to glutamate, lysine is positively charged due to a second amino group. The third difference was aa number 482 in the sequence which involved arginine in 373 and tryptophan in 1516. Arginine is positively charged, while tryptophan is aromatic. The fourth difference was the aa number 766 in the sequence, which involved alanine in 373 and aspartate in 1516. Alanine is nonpolar and hydrophobic and aspartate is negatively charged. The side chain of alanine together with valine, leucine and isoleucine tend to cluster together within the protein and stabilize the structure via the hydrophobic interactions. The fifth difference was aa number 813 in the sequence, which involved aspartate in 373 and glycine in 1516. Aspartate is negatively charged, while glycine is nonpolar and hydrophobic. Glycine is characterized by its very small side chain which leads to no real contribution to hydrophobic interactions. The sixth and last difference appeared to be in aa number 887 in the sequence, which involves cysteine in 373 and arginine in 1516. Cysteine is polar and uncharged while arginine is positively charged. Cysteine can be oxidized to form a covalently linked dimeric aa called a cysteine bridge, which has a special role in the structure of a protein by forming covalent link between parts of a polypeptide chain

molecule (Nelson et al., 2008). The differences observed in the aa sequence is caused by a change of codon, likely due to a point mutation in the DNA.

In the first aa difference, proline in 373 and serine in 1516 were not conserved to the conserved motive of glutamine found in bacteria. In the second difference, glutamate in 373 and lysine in 1516 were not conserved to the conserved motive of aspartate in bacteria. In the third difference, arginine in 373 and tryptophan in 1516 could not be compared to bacteria, since the bacteria had a gap in that position. This may indicate that arginine and tryptophan are not essential for the activity of the protein and may have occurred by genetic drift. In the fourth difference, alanine in 373 was only conserved to two bacteria (*Agrobacterium* sp. H13-3 and *Hoeflea phototrophica* DFL-43), while aspartate in 1516 was not conserved to any of the bacteria. In the fifth difference, aspartate in 373 and glycine in 1516 were not able to be compared due the gap found in the bacteria, which indicate that aspartate and glycine are also not essential for the activity of the protein. The sixth and last difference, cysteine in 373 was not conserved to any of the bacteria, while arginine in 1516 was conserved to three bacteria (*Halomonas* sp. HTNK1, *Hoeflea phototrophica* DFL-43 and *Ruegeria pomeroyi* DSS-3) (Figure A6, Appendix 9).

In addition to these observed differences, strain 1516 has an insertion of one aa, which may be serine or aspartate, in position number 13, which is N-terminal to the first CaiB domain. The CaiB domains form the catalytic part of the EhDddD protein. The insertion of one amino acid at N-terminus may have no effect on the function of the protein since it is not a part of CaiB domain.

Three clones of each strain were compared and checked for possible sequence errors. It was found that the observed differences were due polymorphisms since all the three clones contained the same difference. The observed variation in aa sequence in both strains and the insertion of one aa in 1516 may be the reason for the higher gene expression of *EhDddD* gene (Figure 3.6) and higher DLA in 373 than 1516 (Figure 3.8). These results suggest that the structures of DMSP lyase enzymes within strains of *E. huxleyi* are different which leads to variation in DLA (Steinke et al., 1998).

Nearly all known DMSP catabolizing bacteria are in the phylum Proteobacteria (for example *Halomonas*, *Agrobacterium*, *Marinomonas* MWYL1 and *Psychrobacter*). A C-terminal protein domain of 200 aa found in 1516 and 373 was observed to be missing in these bacteria (Figure A6, Appendix 9). The additional domain in EhDddD appears to contain a RING

domain, which is probably involved in mediating protein-protein interactions. The RING domain is a type of zinc-finger domain that comprises 40-60 residues and characterized by a specific motif, which coordinates two zinc ions in a cross brace arrangement.

Ubiquitin is a small regulatory protein which may regulate EhDddD protein by tagging the protein for degradation by proteasomes. Ubiquitin tags may also direct the proteins to other locations in the cell. It is the number of ubiquitin molecules that determine how the cell interprets the ubiquitin message. A polyubiquitin chain targets a protein for degradation, while monoubiquitin addition regulates the transport of membrane proteins (Alberts et al., 2008). It has been reported that DMSP and DMSP lyase are located in different compartments within the cell, and DMS is produced when the cell is exposed to stress or cell damage (Van Rijssel and Gieskes, 2002). This may explain the production of DMS by ubiquitin tagging which transports EhDddD protein into the same compartment as DMSP. The covalent modification of proteins by the addition of ubiquitin depends on a cascade of three enzymes. These enzymes include an ATP-dependent ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). The cascade is initiated by the activation of ubiquitin via E1 protein. The activated ubiquitin molecule is further transferred to the catalytic cysteine in E2 enzyme forming E2-ubiquitin conjugate, which engages E3 ligase. E3 promotes the transfer of ubiquitin molecule from E2 to a lysine in the target protein. E3 ligase contains a RING domain, which plays an essential role in promoting ubiquitin transfer. The ability of the RING domain to promote the transfer of ubiquitin depends on its capacity to bind E2-ubiquitin conjugate (Budhidarmo et al., 2012). No other functions have been described to the RING domain beyond a role in dimerization of several proteins (Joazeiro and Weissman, 2000).

EhDddD of 1516 and 373 has a higher similarity to translated expression sequence tags (ESTs) in another haptophyte (*Prymnesium parvum*) than to bacteria sequences (Brembu, unpublished results). This is expected since both species are haptophytes.

In addition to two possible pathways for DMSP catabolism via the action of DMSP lyase and DMSP demethylase by different bacteria (Curson et al., 2011), Todd et al. (2007) reported a third pathway by DddD cleavage. The DddD is predicted to modify DMSP by the addition of coenzyme A (CoA) to DMSP and the release of DMS and 3-hydroxypropionate (3-HP) instead of acrylate. Nearly all known DMSP-catabolizing bacteria are in the phylum Proteobacteria (Curson et al., 2011). The proteobacterium *Marinomonas* MWYL1 was the first DMSP-cleaving species to be studied. The species grow well on DMSP as the sole

carbon source, and growth was accompanied by the release of DMS. This phenotype is termed Ddd^+ (DMSP-dependent DMS). The production of DMS is induced by DMSP, not by acrylate or 3HP. This is due increased expression of both *dddD* and the *dddTBCR* operon. The *dddR* encodes a LysR-type (transcriptional regulator), which responds to DMSP. The function of the DddC and DddB proteins may be involved in modifying the DMSP either before or after the addition of acyl CoA. The DddT protein functions as the DMSP transporter. In *Halomonas* the Ddd^+ phenotype is induced by DMSP, 3-HP and acrylate. It seems that the transcriptional regulator in *Halomonas* responds to DMSP, 3-HP and acrylate (Todd et al., 2007). Curson et al. (2011) reported that *E. huxleyi* contained a gene that encodes a predicted polypeptide with 30% identity to bacterial DddD proteins. Protein Blast searches showed that the EhDddD polypeptide have class III CoA transferase domains that are similar to CaiB domains found in bacteria. CaiB is a homo-dimer that adds acyl CoA to carnitine. This might suggest that the mechanism behind DMSP lyase activity in EhDddD is the same as the CoA transferase activity found in DddD by Todd et al. (2007). Carnitine is a betaine with structure similar to DMSP, and DddD is predicted to add acyl CoA to DMSP (Todd et al., 2007). *Hoeflea phototrophica* DFL-43 have a protein with 34% identical and 47% conserved aa compared with EhDddD. The published DddD protein from Marinomonas MWYL1 (Todd et al., 2007) has 31% identical and 45% conserved residues compared with EhDddD protein. The DddD protein is most likely the protein mentioned by Curson et al (2011).

4.2 The variation in *EhDddD* gene expression of strains 1516 and 373

The gene expression of *EhDddD* was 8.414 times higher in 373 than 1516 (Figure 3.6) when the expression was normalized to the reference gene, actin. The actin gene was chosen as a reference gene because it is assumed to be expressed equally in both strains. Mackinder et al. (2011) reported that actin and elongation factor 1 (*EFG1*) were the most stable reference genes for studying strains of *E. huxleyi*. *EFG1* was not used for normalization in this thesis because it resulted in too high Ct-values (Ct>35). Histone H4 (*EhH4*) is another example of a reference gene that was not used due the same reason above.

The actin transcripts in 1516 were ca. 4 times higher than in 373. Strain 1516 showed a mean Ct-value of 20.24, while 373 showed a mean Ct-value of 22.63. Degraded RNA (Figure 3.5) may be the reason for detection of fewer actin transcripts in 373. To avoid degradation affects, the expression of *EhDddD* gene was adjusted with actin which is assumed to be equally expressed in both strains. With or without normalization to actin, the *EhDddD* gene was more

up-regulated in 373. Without normalization the *EhDddD* gene was 1.88 times higher expressed in 373 than 1516.

Figure 3.5 showed some RNA degradation in 373. The RNA profile for 1516 seemed to have higher fluorescence intensity from RNA in ribosomal RNA (rRNA) compared to 373. The band of 28S rRNA was more clearly (higher intensity) than 18S rRNA in 373, which indicates less degradation. Degraded RNA fragments are smaller than intact RNA and thus will migrate faster and gathered on the top of the gel as it shown in Figure 3.5. Degradation of RNA may have occurred during cell harvesting or RNA isolation.

The differences between the intensity of fluorescence from RNA in 28S and 18S of 1516 and 373 may indicate less amount of RNA in 373. In addition to NanoDrop, Qubit was run to control if NanoDrop over- or underestimated RNA amount (ng/µl) in the samples. Qubit measurements are more exact and measure only RNA in the samples in contrast to NanoDrop, which detects RNA, DNA and proteins. Both NanoDrop and Qubit measured equal amounts of RNA in the samples, except for 1516 replica 4, which was overestimated by NanoDrop (Table 3.1). This indicates that the amounts of RNA in both strains are the same. Equal amounts of RNA but two different RNA profiles (Figure 3.5) may be because 373 is degraded or the ratio of rRNA and mRNA in 1516 and 373 can be different.

4.3 The correlation between extracellular DMS and DMSP concentrations in the extracts of strains 1516 and 373

As presented in Figure 3.7 A and B, the DMS concentration in 1516 was 0.7 femtomolar (fM), while the DMS concentration in 373 was 0.6 fM. On the other hand, the DMSP concentration in 373 was 182.8 fM, while 1516 showed a DMSP concentration of 152.3 fM. The obtained DMS concentration did not correlate with the result presented in DMSP lyase activity measurements (Figure 3.8). It was expected to measure higher DMS concentration in 373 than 1516, since the DMSP lyase activity was observed to be 60.6 times higher in 373 and qPCR results showed 8.414 times higher gene expression of *EhDddD* in 373. The DMS production should be higher in 373 than 1516, since the concentration of DMSP was observed to be higher in 373.The DMSP concentration was 1.2 times higher in 373 than 1516, but it was expected to observe even higher concentration of DMSP in 373.

Despite higher *in vitro* enzyme activity (Figure 3.8) and higher DMSP concentration (Figure 3.7 B) in 373, DMS production was 0.8 time lower in 373 than 1516. This may imply that the cleavage reaction in 373 was strongly repressed at that time. The DMSP and DMSP lyase are

segregated within the cells, and DMS production is only activated when the cells are under conditions that result in cell stresses (chemical or physical) or cell damage (Wolfe and Steinke, 1996). One explanation for the small difference in DMS production could be that the cells during the experiment were not stressed, which resulted in low DMS production. Cell harvesting may stress the cells and lead to the cleavage reaction of DMSP to DMS. In the determination of DMS and DMSP concentration in the extracts of both strains, the cells were not harvested. Since DMSP lyase and DMSP are located in different compartments in *E. huxleyi*, this may explain the low DMS production (van Rijssel and Gieskes, 2002).

4.4 The variation of DMSP lyase activity within *Emiliania huxleyi* strains 1516 and 373

Both strains of *E. huxleyi* had detectable DMSP lyase activity under similar culture conditions, measured as production of DMS using GC-MS. The investigated strains exhibited variability in DMSP lyase activity. 1516 showed a DMS production of 1.04 nmol $cell^{-1}$ min^{-1}, whereas 373 showed a DMS production of 63.2 nmol $cell^{-1}$ min^{-1} (Figure 3.8). These results correlate with the results presented by Steinke et al. (1998), who compared DMSP lyase enzymes in extracts of six axenic cultures of *E. huxleyi*. The investigated strains appeared to fall into two groups; high enzyme activities and low enzyme activities. Strains 373 and 379 showed higher enzyme activities than 370, 374, 1516 and L, circa 100-fold per cell higher. Strain 373 showed a DMS production of 12.5 fmol $cell^{-1}$ min^{-1} while 1516 showed a DMS production of 0.02 fmol $cell^{-1}$ min^{-1}.

DMSP lyase enzymes within *E. huxleyi* strains may have various requirements of pH levels. The cells of both strains were sonicated in citric acid/phosphate of pH 6. Steinke et al. (1998) reported that strains 373 and 379 had a narrow pH optimum around pH 6, whereas strains 374, 1516 and L had a pH optimum of pH 5. An exception was strain 370, which showed increasing enzyme activity with increasing pH. This may explain some of the difference in DMSP lyase activity between 1516 and 373. If 1516 was exposed to citric acid/phosphate of pH 5, the activity of DMSP lyase may be somewhat higher than observed in this work.

High gene expression of the *EhDddD* gene correlates with high DMSP lyase activity. In 373, the *EhDddD* gene was 8.414 times higher expressed than 1516 (Figure 3.6). This may explain the higher enzyme activity observed in 373. Light intensity might be another reason for the variability of enzyme activity within these two strains. From own experience of working with 1516 and 373, both strains showed various requirements for light intensity. The cells of strain 1516 grew much better at a light intensity of 210 μmol than 87 μmol, whereas the cells of 373

showed similar growth rates under low (87 µmol) and high (210 µmol) light intensities. Van Rijssel and Gieskes (2002) reported that high light intensity and low temperature influenced growth rate and cell size in batch cultures of *E. huxleyi*. 1516 may be adapted for even higher light intensities than 210 µmol. DMSP lyase activity may increase with increasing light intensity. Less differences of enzyme activity between these two strains may be observed under higher light intensities than 210 µmol.

The lower enzyme activity in 1516 can also be explained with differences in enzyme stability in the extracts of the 1516 and 373 strain, but since the extracts were frozen at -20, the DMSP lyase activity will remain constant over several months (Steinke et al., 1998).

1516 and 373 were isolated from different geographic regions. 1516 was isolated from South East Pacific, whereas 373 was isolated from North East Atlantic. Different ecotypes/strains may be adapted to specific environmental conditions, resulting in phenotypic differences such as in morphology (form and structure features of organisms) or physiology (function in living systems) (Steinke et al., 1998).

1516 obtained from National Center for Marine Algae and Microbiota (NCMA) was non-axenic, while 373 obtained from NCMA was axenic in the starting point. An axenity test was performed using f/2 medium with peptone. The axenity test showed that both strains were non-axenic. Contamination from bacterial DMSP lyases may be involved in DMS production in 1516. 1516 showed much lower enzyme activity than 373, suggesting that bacterial DMSP lyase was not present.

The higher enzyme activity in 373 can be explained with higher gene expression of *EhDddD* gene or higher catalytic activity of the DMSP lyase.

5 Conclusion

The research in this thesis was done to investigate the putative DddD protein in *Emiliania huxleyi* (EhDddD), here defined as DMSP lyase, to study the metabolism of dimethylsulfoniopropionate (DMSP) in two strains 1516 and 373.

The *EhDddD* gene in *E. huxleyi* strains 1516 and 373 was cloned into the pCR™ II-TOPO® vector and sequenced. The amino acid sequence of 1516 contains an insertion of one amino acid compared with 373, which may be aspartate or serine. Furthermore, the EhDddD amino acid sequence of 373 was observed to be different from 1516 in 11 positions, where five of eleven substitutions were conserved. EhDddD of strain 1516 had more amino acids in common with bacteria than 373 in those positions where there are differences between 1516 and 373, which may suggest that EhDddD protein in 373 is a further development of EhDddD protein in 1516.

By comparing the amino acid sequence of EhDddD and DddD homologs in bacteria, an additional protein domain of 200 amino acids in length was found to be missing in bacteria. It is likely that the additional domain which include a RING domain in *E. huxleyi* is needed for the protein to function more effectively or for better regulation of the protein.

The gene expression of *EhDddD* gene was found to be 8.414 times higher in 373 than in 1516, and the enzyme activity was shown to be 60.6 times higher in 373 than 1516. The extracellular DMSP concentration was observed to be 1.2 higher in 373 than 1516, while the DMS concentration was found to be 0.8 time lower in 373 than 1516. These results did not correlate with gene expression analysis and *in vitro* DMSP lyase activity measurement. The higher enzyme activity found in 373 can be explained by higher expression levels of the *EhDddD* gene or differences in enzyme processivity.

In conclusion, these results suggest that structural and transcriptional differences in DMSP lyase between *E. huxleyi* strains may lead to variation in activity, perhaps due to adaptation to different environmental conditions.

6 Further work related to the *EhDddD* gene in 1516 and 373

The diatom *Thalassiosira pseudonana* is also observed to produce DMSP but at lower concentrations than *E. huxleyi*. *T. pseudonana* does not generate DMS. A possible further experiment is to transform *EhDddD* gene from 1516 and 373 in *T. pseudonana* and analyze for DMS production. The enzyme activity can also be compared between *T. pseudonana* transformed with *EhDddD* from 1516 and *T. pseudonana* transformed with *EhDddD* from 373 to observe if the enzyme activity shows same result as presented in this work.

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Appendixes

Appendix 1: Media

LB medium (1L)	
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

The reagents were dissolved in 1 L MQ water. The medium was autoclaved at 121°C for 20 minutes. The antibiotic (1 ml) was added when the medium was cooled to 50°C. The medium was stored at room temperature.

LB agar plates (1L)	
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g

The reagents were dissolved in 1 L MQ water. The solution was autoclaved at 121°C for 20 minutes. The antibiotic (1 ml) was added when the solution was cooled to 50°C. The solution was poured into petri dishes and stored at -20°C.

L1-Si medium (1L)	
Sodium nitrate ($NaNO_3$)	1 ml
Sodium phosphate (NaH_2PO_4)	1 ml
Vitamins	0.5 ml
L1 trace metals	1 ml

The seawater provided from the department of biotechnology was first filtered (pore size 0.2 µm) and then autoclaved at 121°C for 20 minutes. The reagents were added and the medium was stored at room temperature.

f/2 medium (1L)	
Sodium nitrate ($NaNO_3$)	1 ml
Sodium phosphate (NaH_2PO_4)	1 ml
Sodium silicate (Na_2SiO_3)	1 ml
Vitamins	0.5 ml
f/2 trace metals	1 ml

The seawater provided from the department of biotechnology was first filtered (pore size 0.2 µm) and then autoclaved at 121°C for 20 minutes. The reagents were added and the medium was stored at room temperature.

f/2 with peptone (1L)	
Peptone	1 g

The f/2 medium was mixed peptone (1g/L) and stored at room temperature.

Appendix 2: Gels and running buffers for electrophoresis

1% agarose gel for standard electrophoresis	
Agarose	1 g
TAE buffer	100 ml
Gel Red	5 µl

1 g of agarose for routine was solved in 100 ml TAE buffer and heated in a microwave oven until the solution was totally dissolved. 5 µl of Gel Red™ produced by Biotiom was added before gel casting to stain the nucleic acids.

TAE running buffer (1L)	
Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA	100 ml
MQ water	To final volume

The solution was mixed and autoclaved at 121°C for 20 minutes and stored at room temperature.

1.2% FA agarose gel for denaturing electrophoresis	
Agarose	0.6 g
10 x FA gel buffer	5 ml
MQ water	To final volume
37% Formaldehyde	900 µl
Ethidium bromide	0.5 µl

0.6 g of agarose for routine was solved with 5 ml 10 x FA gel buffer and a total volume of 50 ml with MQ water was heated in a microwave oven until the solution was totally dissolved. After the solution was cooled to 65°C, 900 µl of 37% formaldehyde and 0.5 µl of ethidium bromide was added before gel casting. The gel was retained in FA running buffer for 30 minutes before applying the samples.

FA running buffer (0.5L)	
10 x FA gel buffer	50 ml
37% Formaldehyde	10 ml
MQ water	To final volume

The FA gel buffer and formaldehyde were dissolved in autoclaved MQ water to a total volume of 500 ml.

Appendix 3: Vector maps

The vector used for cloning experiments in this thesis is described in Figure A1.

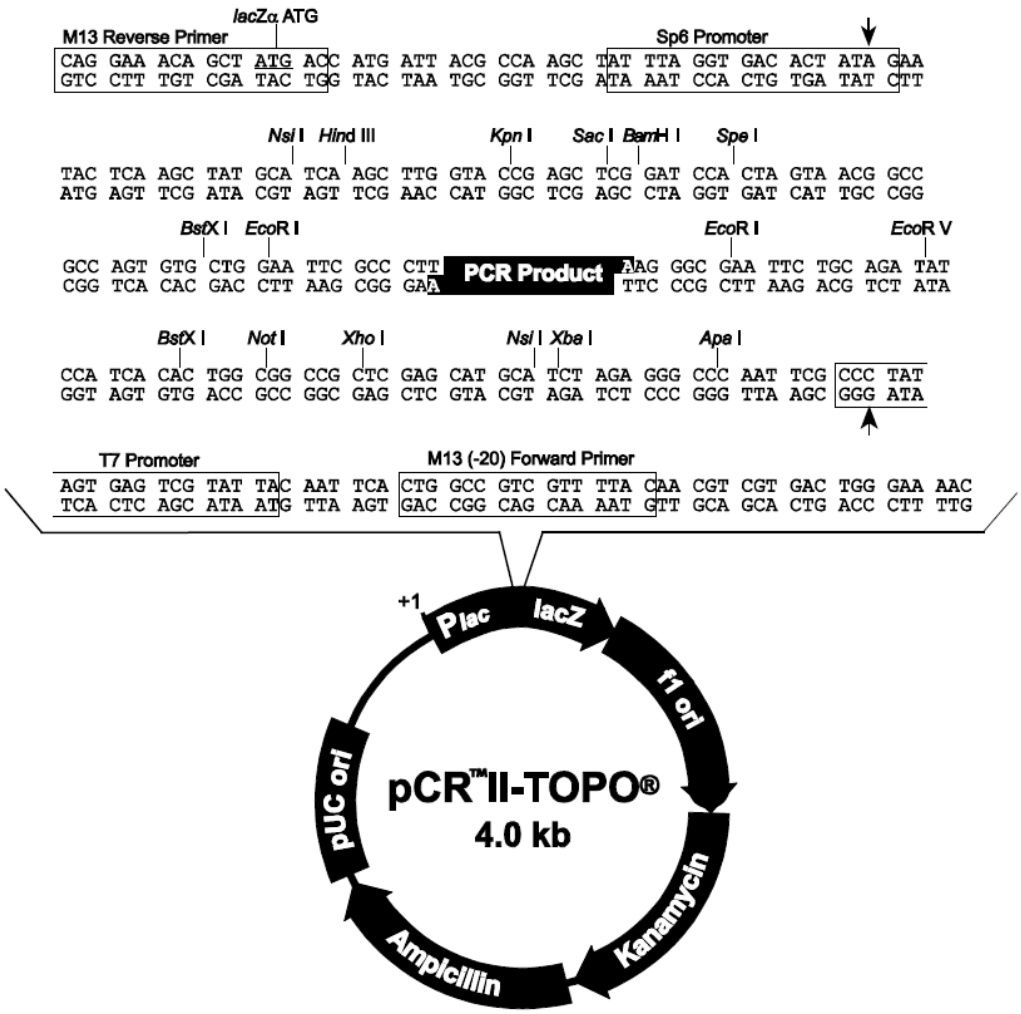


Figure A1 pCR™ II-TOPO® plasmid map present major features of the vector. Labelled restriction sites indicate the cleavage site. Start of transcription for Sp6 and T7 are indicated by arrows. The vector consist of lacZα, M13 Reverse priming site, Sp6 promoter, Multiple Cloning Site, T7 promoter, M13 (-20) Forward priming site, f1 origin, kanamycin resistance ORF, ampicillin resistance ORF and pUC origin (Invitrogen, 2006).

Appendix 4: List of primer sequences for *EhDddD* gene

The primers used for amplification of DNA in PCR and qPCR in this thesis are presented in Table A1 and A2.

Table A1 Primers used in PCR.

Primers	Sequence	Orientation and specification
qDddDF1/F2	5' GCGCTCCCTCGTCACCTCG 3'	Forward
qDddDR1	5' CACCTCCGCGAGACTCAGC 3'	Reverse
qDddDF4	5' GACCATCGGCACGATGCT 3'	Forward
qDddDR4	5' AGCATCGTGCCGATGGTT 3'	Reverse
qDddDF3	5' TGCTCGACCTCTGCAACGTG 3'	Forward
qDddDR2B	5' GGCGCACGACTTCATCGC 3'	Reverse
EhDddDattF	5' GGGGACAAGTTTGTACAAAAA AGCAGGCTCGATGGCCTCGACCTT CAG 3'	Forward
EhDddDattR	5' GGGGACCACTTTGTACAAGAA AGCTGGGTCTAGGAGCGCGACGCC GG 3'	Reverse
M13UNI	5' CGCCAGGGTTTTCCCAGTCACGAC 3'	Forward sequencing primer for pDONR and pTOPO vectors
M13REV	5' AGCGGATAACAATTTCACACAGGA 3'	Reverse sequecing primer for pDONR and pTOPO vectors
EhDddDF1	5' CCGAGACATGGGCATCAACC 3'	Forward
EhDddDR2	5' CCGACGTGCGCGTGCTCCT 3'	Reverse

Table A2 primers used in qPCR.

Primers	Sequence	Orientation
qDddDF2	5' TCCTTCCGCAAGTGGAGC 3'	Forward
qDddDR2B	5' GGCGCACGACTTCATCGC 3'	Reverse
EhActF	5' GACCGACTGGATGGTCAAG 3'	Forward
EhActR	5' GCCAGCTTCTCCTTGATGTC 3'	Reverse

Appendix 5: LinReg and REST output from analysis of qPCR

All data obtained by qPCR was analyzed in LinReg and REST. Table A3 shows the Ct-values for strains 1516 and 373. Table A4 presents the data obtained in REST and Figure A2 shows the result obtained in REST. The calculation of standard deviation (SD) is shown in Table A5.

Table A3 Ct-values from four replicas of 1516 and 373. The qF2R2B represent *EhDddD* and actin (EhAct) was used as a reference gene. The Ct-values were obtained by fit point method. The LinReg was used to define reaction efficiency of qPCR through linear regression. Reaction efficiency for qF2R2B was 1.841 and for EhAct was 1.873.

Strain	qF2R2B	EhAct
1516	29.90	20.52
	30.41	21.00
	29.39	19.85
	28.54	19.58
Mean	29.56	19.98
373	29.47	23.52
	28.76	22.78
	28.49	22.73
	27.38	21.47
Mean	28.53	22.63

Table A4 Relative expression results obtained by REST. REST software was used to calculate relative expression and statistical significance between amplicons in sample group (treated) and control group (untreated). 1516 was considered as untreated and 373 was considered as treated. The relative expression was normalized with a reference gene, actin. DMSP lyase gene is 8.414 times higher expressed in 373 than 1516. Standard (Std) error was used to calculate standard deviation (SD) in 373. P(H1) shows a value of 0.001, which means that the probability for correct result is 99.9%. Confidence interval (C. I.) shows a value range of 6.563-10.

Gene	Type	Reaction efficiency	Expression	Std. Error	95% C. I.	P(H1)	Result
qF2R2B	TRG	0.841	8.414	6.998-9.503	6.563-10	0.001	UP
EhAct	REF	0.873	1				

Figure A2 shows the gene expression of *EhDddD* in 373 is 8.414 times higher than 1516.

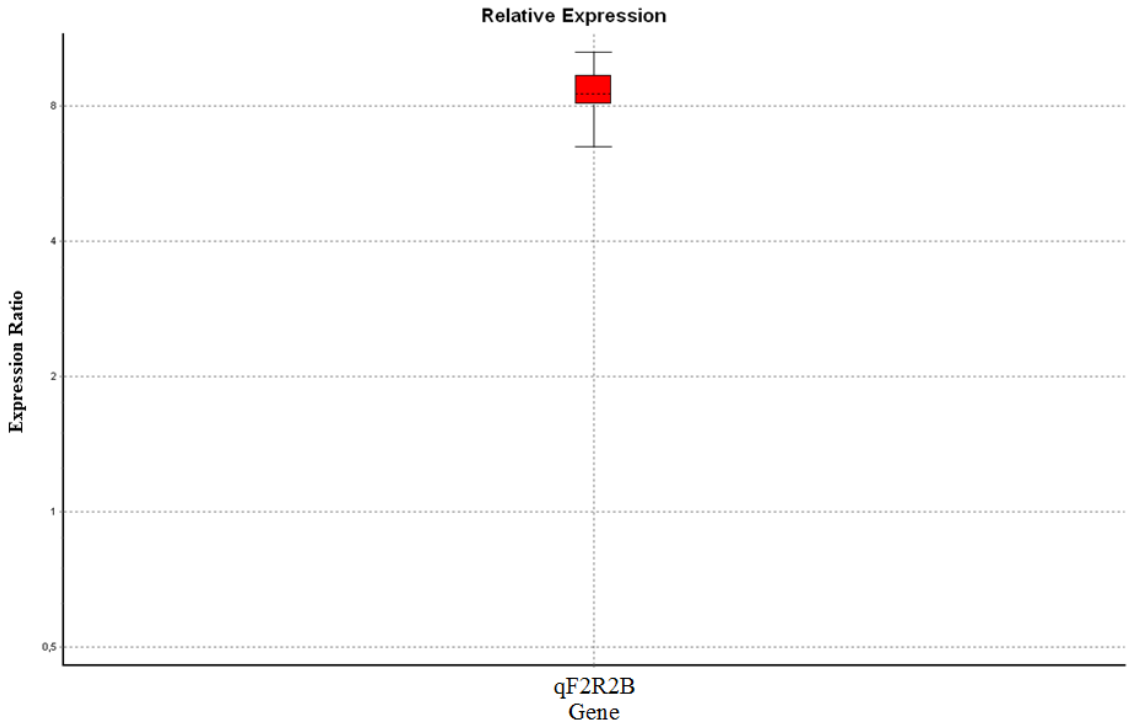


Figure A2 Relative gene expression of DMSP lyase gene (qF2R2B) in 373 compared to 1516. DMSP lyase gene in 373 is 8.414 times higher expressed than 1516. The expression was normalized to the reference gene, actin.

Table A5 Calculation of standard deviation. The value of standard (Std) error obtained by REST was used to calculate the standard deviation (SD).

Std. Error	6.998-9.503	
	A	**B**
	6.998	9.503
Mean	(6.998+9.503)/2 = 8.251	
SD	6.998-8.251 = 1.253	

Appendix 6: The raw data of determining extracellular DMS and DMSP concentrations and standard curve obtained by GC-MS

Summarized data of the experiment are shown in Table A6. Table A7-A8 shows detector responses for samples and standard curve obtained by GC-MS which were used for determining the concentrations of DMS and DMSP. Table A9-A12 shows the calculation of DMS and DMSP concentrations. Figure A3 shows the DMS standard curve.

Table A6 An overview of DMS and DMSP concentrations and standard deviation (SD) in strains 1516 and 373.

Strains	DMS (fM)	DMSP (fM)	SD (DMS)	SD (DMSP)
1516	0.7	152.3	0.1627	10
373	0.6	182.8	0.097	15

Table A7 Raw data of amount DMS and DMS + DMSP in extract of *E. huxleyi* strains 1516 and 373 measured by GC-MS. Detector response which constitutes of mass fragments 62.15 and 47 is measured in three replicas of each strain. Detector response shows the total amount of mass fragments. Retention time (RT) (not shown) had a range of 1.7-2.7 minutes. The DMS was quantified in this range.

Strain	Replica	Detector response (area peak)
DMS 1516	1	1086995
DMS 1516	2	889214
DMS 1516	3	696915
DMS 373	1	1045115
DMS 373	2	1149263
DMS 373	3	836418
DMS + DMSP 1516	1	186846389
DMS + DMSP 1516	2	194984318
DMS + DMSP 1516	3	171933656
DMS + DMSP 373	1	328098747
DMS + DMSP 373	2	294619642
DMS + DMSP 373	3	278332440

Table A8 Raw data of standard curve obtained by GC-MS. Detector response which constitutes of mass fragments 62.15 and 47 is measured in three replicas of nine different known concentrations of DMS. Detector response shows total amount of mass fragments. RT (not shown) had a range of 1.7 to 2.7 minutes. The DMS was quantified in this range.

DMS concentration (µM)	Replica	Detector response (area peak)
0.00000001	1	654 530
0.0000001	1	669 669
0.000001	1	8 410 544
0.00001	1	11 445 659
0.0001	1	13 431 161
0.001	1	19 075 181
0.01	1	25 275 891
0.1	1	60 025 136
1	1	473 790 485
0.00000001	2	533 493
0.0000001	2	696 129
0.000001	2	3 384 789
0.00001	2	2 112 805
0.0001	2	2 862 657
0.001	2	3 518 982
0.01	2	7 479 251
0.1	2	42 262 751
1	2	343 271 446
0.00000001	3	609 844
0.0000001	3	380 905
0.000001	3	620 007
0.00001	3	1 202 559
0.0001	3	912 420
0.001	3	1 885 972
0.01	3	4 789 580
0.1	3	46 731 480
1	3	370 267 252

Figure A3 shows a DMS standard curve with a linear function. The linear formula y = 396 868 866.21x was used to calculate the concentration of DMS and DMSP in the extract of *E. huxleyi* strains 1516 and 373. The regression (R^2) value is 0.97.

X-axis: DMS concentrations (0.00000001, 0.0000001, 0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1 and 1 µM)

Y-axis: Detector response

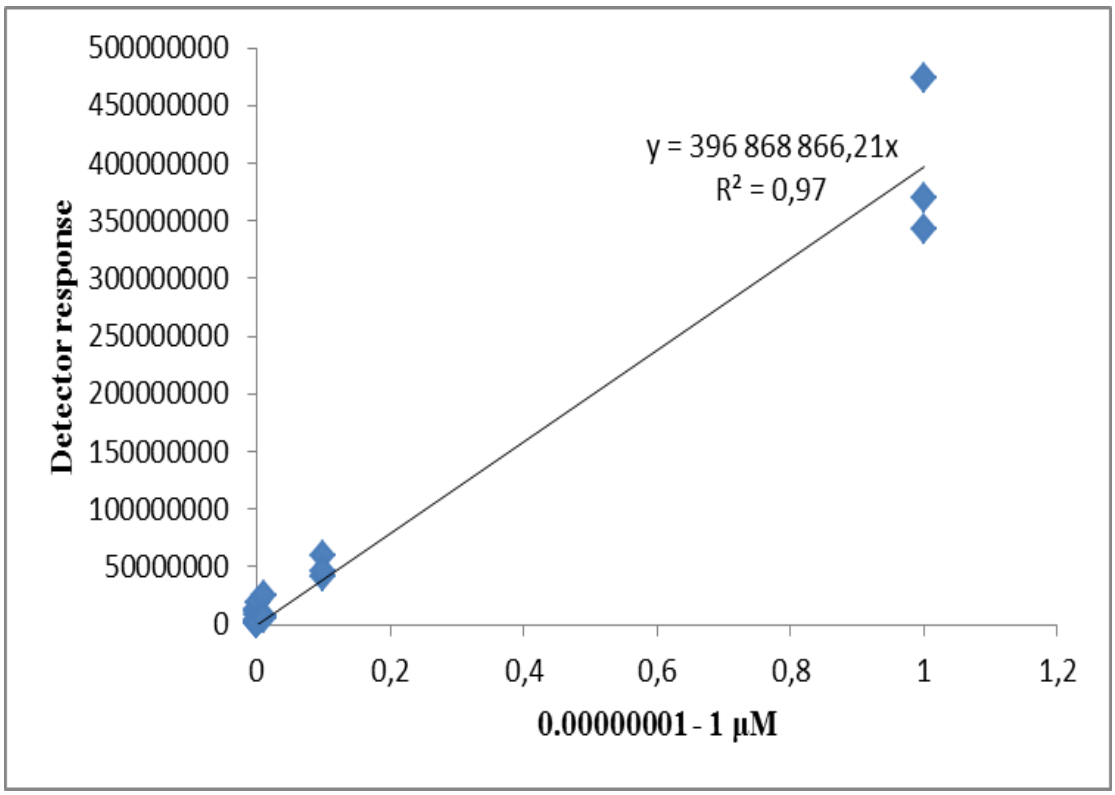


Figure A3 DMS standard curve for determination of DMS and DMSP concentrations in extract of *Emiliania huxleyi* strains. Nine different concentrations of DMS resulted in a regression (R^2) value of 1. The linear formula y = 396 868 866,21x was used to calculate the concentration of DMS and DMSP in the samples of *Emiliania huxleyi* strains given in µM.

Table A9 The calculation of DMS and DMS + DMSP concentrations in extract of *Emiliania huxleyi* strains 1516 and 373 using the linear formula (y = 396868866.21x). Detector response presents the total amount of mass fragments (*m/z* = 62.15 and 47) in three replicas. X stands for the concentrations of DMS and DMS + DMSP in μM.

Strain	**Detector response (replica 1)**	**Detector response (replica 2)**	**Detector response (replica 3)**
DMS 1516	1086995	889214	696915
DMS 373	1045115	1149263	836418
DMS + DMSP 1516	186846389	194984318	171933656
DMS + DMSP 373	328098747	294619642	278332440
Formula: y = 396868866.21x			
DMS 1516-1		DMS + DMSP 1516-1	
x = 1086995/ 396868866.21	= 0.0027	x = 186846389/ 396868866.21	= 0.4708
DMS 1516-2		DMS + DMSP 1516-2	
x = 889214/ 396868866.21	= 0.0022	x = 194984318/ 396868866.21	= 0.4913
DMS 1516-3		DMS + DMSP 1516-3	
x = 696915/ 396868866.21	= 0.0018	x = 171933656/ 396868866.21	= 0.4332
Mean: (0.0027+0.0022+0.0018)/3	= 0.0022 μM	Mean: (0.4708+0.4913+0.4332)/3	= 0.4651 μM
DMS 373-1		DMS + DMSP 373-1	
x = 1045115/ 396868866.21	= 0.0026	x = 328098747/ 396868866.21	= 0.8267
DMS 373-2		DMS + DMSP 373-2	
x = 1149263/ 396868866.21	= 0.0029	x = 294 619 642/ 396868866.21	= 0.7424
DMS 373-3		DMS + DMSP 373-3	
x = 836418/ 396868866.21	= 0.0021	x = 278332440/ 396868866.21	= 0.7013
Mean: (0.0026+0.0029+0.0021)/3	= 0.0025 μM	Mean: (0.8267+0.7424+0.7013)/3	= 0.7568 μM

Table A10 The calculation of DMSP concentration in the extracts of *Emiliania huxleyi* strains 1516 and 373 based on Niki et al. (2004) method.

Strain	Replica	DMS + DMSP concentration (µM)	DMS concentration (µM)	DMSP concentration (µM)
1516	1	0.4708	0.0027	0.4708–0.0027 = 0.4681
1516	2	0.4913	0.0022	0.4913–0.0022 = 0.4891
1516	3	0.4332	0.0018	0.4332–0.0018 = 0.4315
373	1	0.8267	0.0026	0.8267–0.0026 = 0.8241
373	2	0.7424	0.0029	0.7424–0.0029 = 0.7395
373	3	0.7013	0.0021	0.7013–0.0021 = 0.6992

Table A11 The calculation of DMS and DMSP concentrations per cell.

Strain	Cell density	Mean DMS	Mean DMSP	DMS per cell	DMSP per cell
1516	3040000	0.0022	0.4629	0.0022/3040000 = $7.38545*10^{-10}$ µM	0.4629/3040000 = $1.52259*10^{-07}$ µM
373	4126666	0.0025	0.7543	0.0025/4126666 = $6.16864*10^{-10}$ µM	0.7543/4126666 = $1.82776*10^{-07}$ µM

Table A12 The calculation of DMS and DMSP concentrations from µM into femtomolar (fM).

Strain	DMS (µM)	DMSP (µM)	DMS (fM)	DMSP (fM)
1516	$7.38545*10^{-10}$	$1.52259*10^{-07}$	$7.38545*10^{-10}*10^{9}$ = 0.7	$1.52259*10^{-07}*10^{9}$ = 152.3
373	$6.16864*10^{-10}$	$1.82776*10^{-07}$	$6.16864*10^{-10}*10^{9}$ = 0.6	$1.82776*10^{-07}*10^{9}$ = 182.8

Appendix 7: The raw data of enzyme activity measurement and standard curve obtained by GC-MS

Summarized data of the experiment are shown in Table A13. Table A14-A16 shows detector responses for samples and standard curve obtained by GC-MS which were used for determining the concentration of DMS. Table A17-A18 shows the calculation of DMSP lyase activity for strain 1516 and 373. Figure A4 shows the DMS standard curve for DMSP lyase activity.

Table A13 An overview of DMSP lyase activity and standard deviation (SD) in strains 1516 and 373.

Strains	DMS (nmol $cell^{-1}$ min^{-1})	Standard deviation (SD)
1516	1.04	0.5259
373	63.2	1.4170

Table A14 Raw data of amount DMS in extract of *E. huxleyi* strains 1516 and 373 measured by GC-MS. Detector response which constitutes of mass fragments 62.15 and 47 is measured in three replicas of each strain. Detector response shows the total amount of mass fragments. Retention time (RT) (not shown) had a range of 1.7-2.7 minutes. The DMS was quantified in this range.

Strain	Replica	Detector response (area peak)
1516	1	7458832
1516	2	12506058
1516	3	10923915
373	1	323551697
373	2	312555022
373	3	310674486

Table A15 Raw data of standard curve obtained by GC-MS. Detector response which constitutes of mass fragments 62.15 and 47 is measured in three replicas of seven different known concentrations of DMS. Detector response shows total amount of mass fragments. RT (not shown) had a range of 1.7 to 2.7 minutes. The DMS was quantified in this range.

DMS concentration (µM)	Replica	Detector response (area peak)
0.0001	1	4496674
0.001	1	5240030
0.01	1	9826786
0.1	1	56736844
1	1	525101346
10	1	1526763275
100	1	2810986716
0.0001	2	4228038
0.001	2	5578269
0.01	2	9849986
0.1	2	54931155
1	2	505614932
10	2	1122265952
100	2	1705554762
0.0001	3	3833477
0.001	3	5273180
0.01	3	8269441
0.1	3	58096023
1	3	456740168
10	3	987516451
100	3	1299935232

Figure A4 shows a DMS standard curve with a linear function. Only five of seven concentrations (Table A16) were used to obtain a linear standard curve with a regression (R^2) value of 1. The linear formula y = 490870295.04x + 5173226.30 was used to calculate the concentration of DMS in the extract of *E. huxleyi* strains 1516 and 373.

X-axis: DMS concentrations (0.0001, 0.001, 0.01, 0.1 and 1 µM)

Y-axis: Detector response

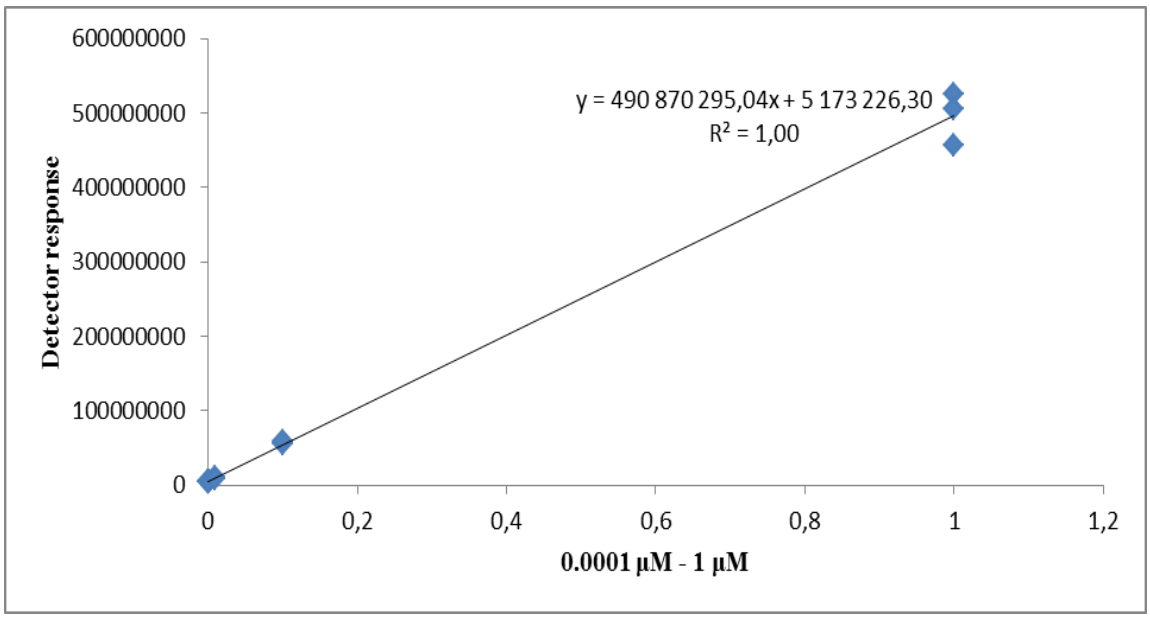


Figure A4 DMS standard curve for DMSP lyase activity. Five of seven DMS concentrations resulted in a regression (R^2) value of 1. The linear formula was used to calculate the concentration of DMS in the samples of *Emiliania huxleyi* strains given in µM.

Table A16 The concentrations of DMS used in standard curve. The green marked area shows the five of seven concentrations of DMS in three replicas used in the standard curve (Figure A4) and the total amount of detector response of each replica.

Replica	DMS concentrations (µM)	Detector response (area peak)
1	0.0001	4496674
2	0.0001	4228038
3	0.0001	3833477
1	0.001	5240030
2	0.001	5578269
3	0.001	5273180
1	0.01	9826786
2	0.01	9849986
3	0.01	8269441
1	0.1	56736844
2	0.1	54931155
3	0.1	58096023
1	1	525101346
2	1	505614932
3	1	456740168
1	10	1526763275
2	10	1122265952
3	10	987516451
1	100	2810986716
2	100	1705554762
3	100	1299935232

Table A17 The calculation of DMS concentrations in extract of *Emiliania huxleyi* strains 1516 and 373 using the linear formula (y = 490870295.04x + 5173226.30). Detector response presents the total amount of mass fragments (*m/z* = 62.15 and 47) in three replicas. X stands for the concentrations of DMS in μM.

Strain	**Detector response (replica 1)**	**Detector response (replica 2)**	**Detector response (replica 3)**
1516	7458832	12506058	10923915
373	323551697	312555022	310674486
Formula: y = 490870295.04x + 5173226.30			
1516-1		**373-1**	
7458832-5173226.30 = 2285605.7		323551697-5173226.30 = 318378471	
x = 2285605.7/490870295.04	= 0.0047	x = 318378471/490870295.04	= 0.6486
1516-2		**373-2**	
12506058-5173226.30 = 7332831.7		312555022-5173226.30 = 307381796	
x = 7332831.7/490870295.04	= 0.0149	x = 307381796/490870295.04	= 0.6262
1516-3		**373-3**	
10923915-5173226.30 = 5750688.7		310674486-5173226.30 = 305501260	
x = 5750688.7/490870295.04	= 0.0117	x = 305501260/490870295.04	= 0.6224
Mean: (0.0047+0.0149+0.0117)/3	= <u>0.0104</u> μM	**Mean**: (0.6486+0.6262+0.6224)/3	= <u>0.6324</u> μM

Table A18 The calculation of DMS amount from µM into nmol cell^{-1} min^{-1}.

Strain (replica)	Replica	DMS concentration (µM)
1516	1	0.0047
1516	2	0.0149
1516	3	0.0117
373	1	0.6486
373	2	0.6262
373	3	0.6224
		DMS (nmol cell^{-1} min^{-1})
1516	1	0.0047/10*1000 = 0.47
1516	2	0.0149/10*1000 = 1.49
1516	3	0.0117/10*1000 = 1.17
		Mean: 0.50+1.50+1.20/3 = <u>1.04</u>
373	1	0.6486/10*1000 = 64.9
373	2	0.6262/10*1000 = 62.6
373	3	0.6224/10*1000= 62.2
		Mean: 64.9+62.6+62.2/3 = <u>63.2</u>

Appendix 8: DNA standard

GeneRuler^{TM} 1kb DNA Ladder (Figure A5) from Fermentas was used as a standard, when the DNA fragments were separated on agarose gel.

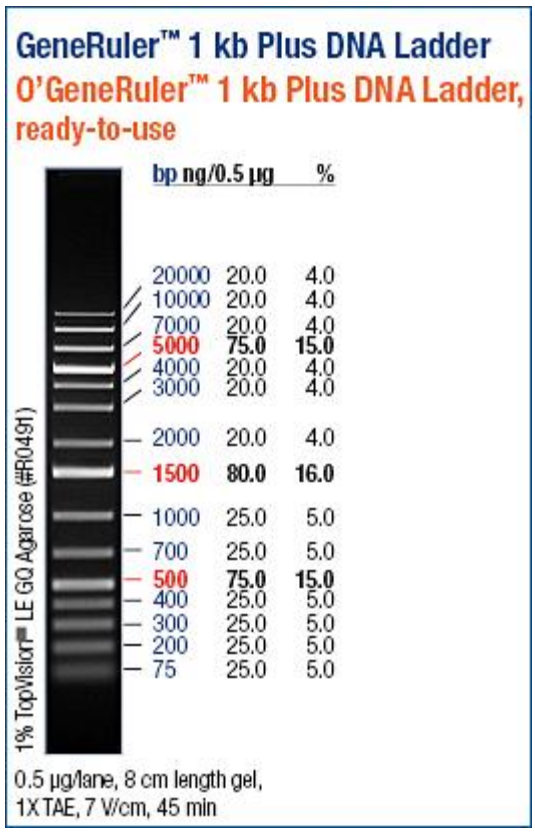
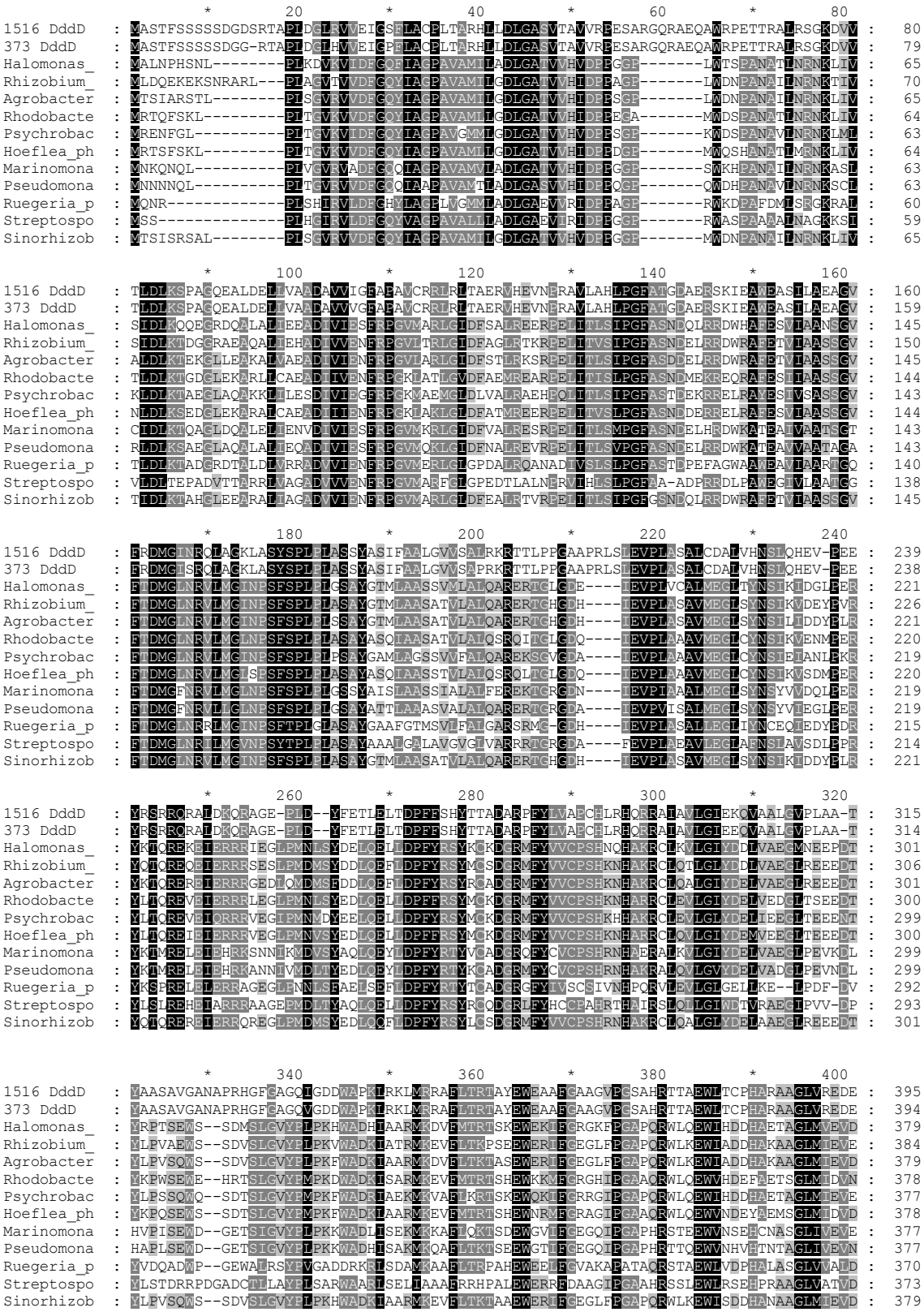
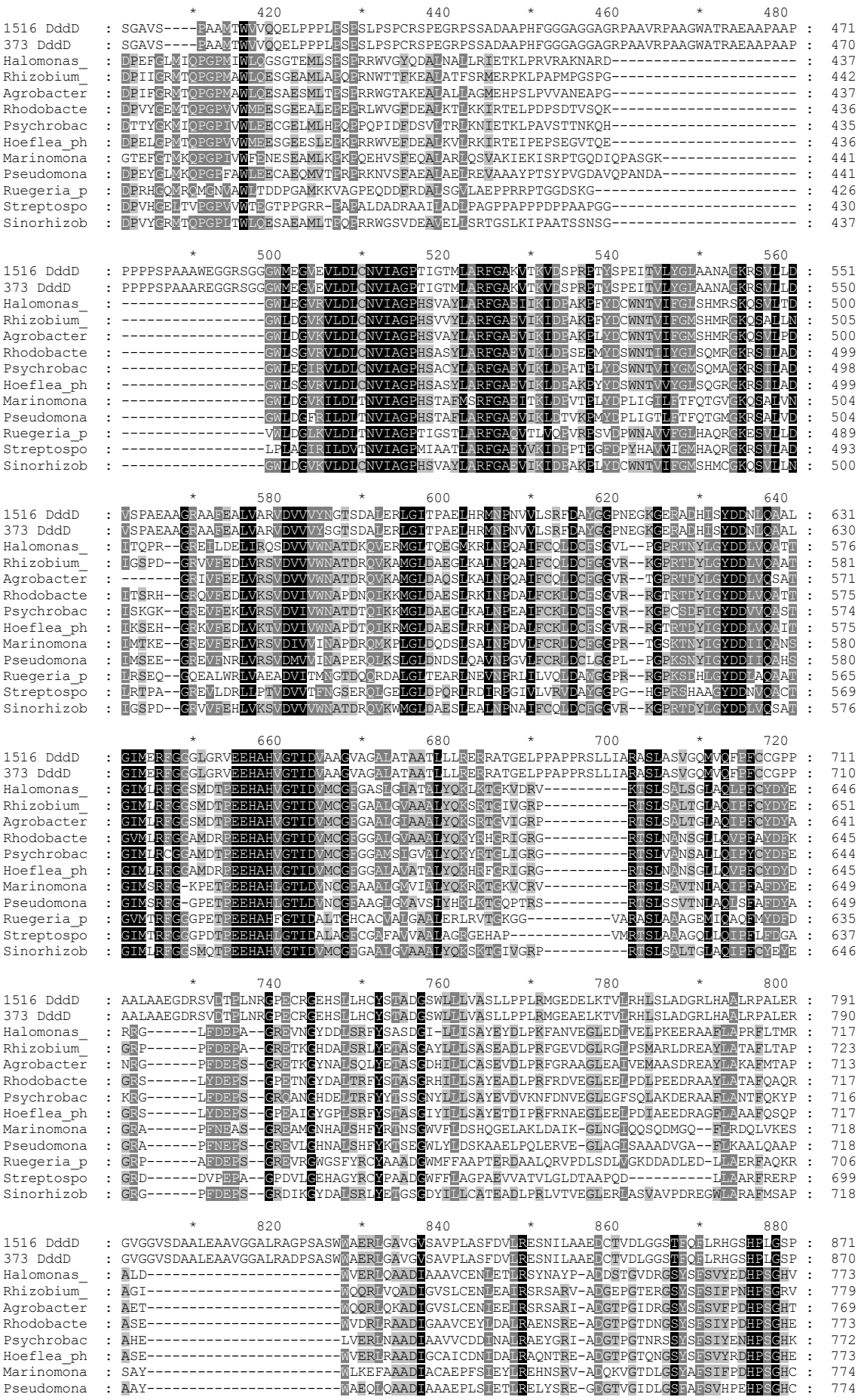


Figure A5 GeneRuler^{TM} 1kb Plus DNA ladder from Fermentas.

Appendix 9: Amino acid sequence alignment of DddD protein in bacteria and *Emiliania huxleyi* strains 1516 and 373.

The amino acid sequence alignment show the homolog of DddD protein in several bacteria (Brembu, unpublished results) compared *Emiliania huxleyi* strains 1516 and 373.





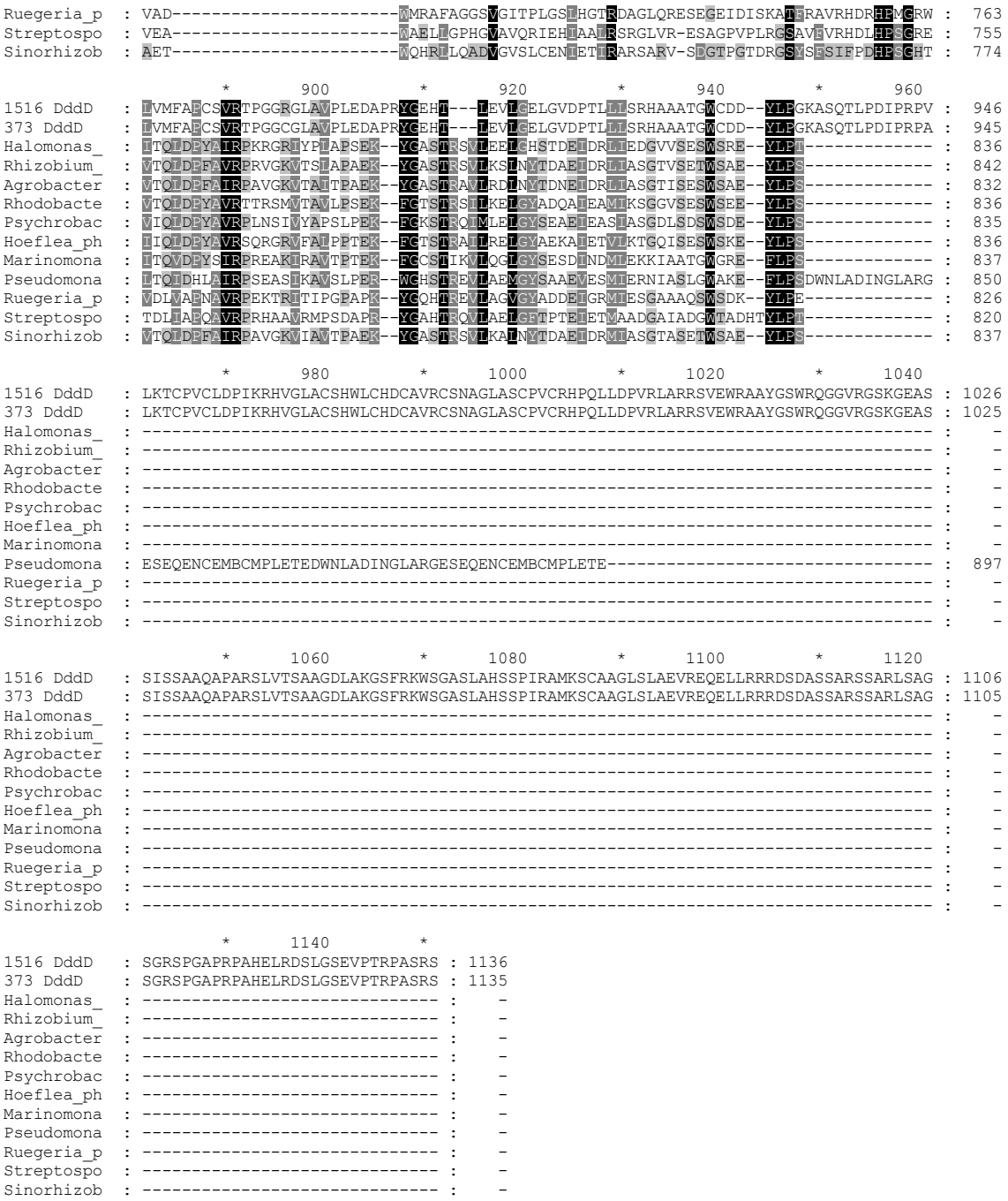


Figure A6 Amino acid sequence alignment of DddD protein in several bacteria compared to *Emiliania huxleyi* strains 1516 and 373.