

Partial characterisation of dimethylsulfonio-propionate (DMSP) lyase isozymes in 6 strains of *Emiliana huxleyi*

Michael Steinke^{1,*}, Gordon V. Wolfe², Gunter O. Kirst¹

¹Marine Botany, University of Bremen FB2, D-28334 Bremen, Germany

²College of Oceanic and Atmospheric Sciences, Oregon State University, Corvallis, Oregon 97331-5503, USA

ABSTRACT: We characterised and compared dimethylsulfoniopropionate (DMSP) lyase isozymes in crude extracts of 6 axenic *Emiliana huxleyi* cultures (CCMP 370, 373, 374, 379, 1516, and strain L). This enzyme cleaves DMSP to form dimethyl sulfide (DMS), acrylate and a proton, but the function of this reaction in algae is still poorly understood. Most of the cultures produced high concentrations of intracellular DMSP, which was constant over the growth cycle and ranged from 157 to 242 mM, except for 1516 which had 50 mM DMSP cell⁻¹. Extracts of all strains produced DMS from exogenous DMSP *in vitro*. DMSP lyases appeared constitutive, but enzyme activity and behaviour varied greatly among strains, and did not correlate with intracellular DMSP concentration. Strains 373 and 379 showed high DMSP lyase activities (12.5 and 6.1 fmol DMS cell⁻¹ min⁻¹, respectively), whereas DMS production was more than 100-fold lower in 370, 374, 1516 and L. This difference was intrinsic and the general pattern of high- and low-activity strains remained true over more than a 1 yr cultivation period. The cleavage reaction was optimal at pH 6 in the strains with high lyase activity and pH 5 was optimal for 374, 1516 and L. Strain 370 showed increasing activity with increasing pH. Experiments with additions of 0.125 to 2 M NaCl indicated halotolerant DMSP lyases in 373, 379 and 374. However, the halophilic DMSP lyases in 370 and L required 1 M NaCl addition for optimal DMSP cleavage, and 1516 showed optimal activity at 2 M NaCl. These results suggest that there are several structurally different DMSP lyase isozymes within *E. huxleyi*. However, it cannot be ruled out that varying concentrations of DMSP lyase per cell may have contributed to the differences in enzyme activity per cell. Comparison with other algal taxa indicates several families of DMSP lyases, hinting at possibly different cellular locations and functions, and varying DMS production under natural conditions.

KEY WORDS: Dimethylsulfoniopropionate (DMSP) lyase · Isozymes · Dimethyl sulfide (DMS) · Marine sulfur cycle · Biogeochemistry · *Emiliana huxleyi*

INTRODUCTION

Some marine micro- and macroalgae synthesise the sulfur compound dimethylsulfoniopropionate (DMSP), which is accumulated to high intracellular concentrations (mM to M) in some algal taxa (Ulvophyceae, Haptophyceae, Dinophyceae) (Reed 1983, Keller et al. 1989, Blunden et al. 1992). DMSP is probably the most important precursor for volatile dimethyl sulfide

(DMS), the major source of biogenic sulfur in the marine atmosphere (Andreae & Jaeschke 1992, Liss et al. 1997). DMS is important for atmospheric chemistry (Andreae et al. 1985, Plane 1989), and may affect the formation of cloud-condensation nuclei, hence influencing global climate (Bates et al. 1987, Charlson et al. 1987, Malin et al. 1992). Therefore, the production of DMSP and the microbial pathways resulting in DMS have received much attention recently (see Kiene et al. 1996, review by Malin & Kirst 1997).

Abiotic decomposition of DMSP to DMS, acrylate and a proton is slow in natural seawater (Dacey & Blough 1987), therefore cleavage via the enzyme DMSP lyase

*Present address: School of Environmental Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom.
E-mail: m.steinke@uea.ac.uk

is the major source of DMS. DMSP lyases have been found in bacteria (de Souza & Yoch 1995a, b, van der Maarel et al. 1996, Yoch et al. 1997), which utilise the cleavage product acrylate as a carbon source. They have also been identified in a heterotrophic dinoflagellate (Kadota & Ishida 1968), the marine fungus *Fusarium lateritium* (Bacic & Yoch 1998), and various marine algae (Cantoni & Anderson 1956, Stefels & van Boekel 1993, Nishiguchi & Goff 1995, de Souza et al. 1996, Steinke & Kirst 1996). However, enzymatic DMS production rates are highly species-specific (Steinke et al. 1996) and there is also evidence for strain-specific enzymatic cleavage of DMSP (Wolfe & Steinke 1996).

Among pelagic unicellular algae, the haptophytes *Phaeocystis* and *Emiliana* are particularly important DMSP producers, especially since both form large and almost monospecific blooms during spring and summer (Riegman et al. 1992, Malin et al. 1993, Matrai & Keller 1993). Additionally, DMSP lyase activity has been characterised in crude extracts of *Phaeocystis* sp. (Stefels & Dijkhuizen 1996) and in 2 *E. huxleyi* strains (Wolfe & Steinke 1996). However, the conversion of DMSP to DMS in *E. huxleyi* or *Phaeocystis* sp. is not yet fully understood.

The release of DMSP from algal cells after autolysis (Brussard et al. 1995) has not been demonstrated yet. However, viral lysis (Bratbak et al. 1995, Malin et al. 1998) or grazing by copepods (Dacey & Wakeham 1986, Levasseur et al. 1996) may be important for the subsequent bacterial production of DMS from dissolved DMSP (Ledyard & Dacey 1994). In *Emiliana huxleyi* grazing by herbivorous protists results in direct conversion of DMSP to DMS, excluding bacterial metabolic pathways (Wolfe & Steinke 1996). This finding may be coherent with a grazing-activated chemical defence mechanism in *E. huxleyi* (Wolfe et al. 1997), because the by-product of DMS production is acrylate, a compound with antimicrobial properties at elevated concentrations (Sieburth 1960).

Different sub-populations of marine algal species vary with respect to their physiology and qualitative protein content (Gallagher 1982, Beam & Himes 1987, Stable et al. 1990). Therefore, the strain-specificity of DMSP cleavage (Wolfe & Steinke 1996) could reflect a further level of diversity. Van Bleijswijk et al. (1991) were able to distinguish 2 morphotypes of *Emiliana huxleyi* using an immunofluorescence method. Different growth rates (Brand 1982, van Bleijswijk et al. 1994), long-chain alkenone composition (Conte et al. 1995), gene sequence data (Barker et al. 1994, Medlin et al. 1996) and taxonomical investigations (Young & Westbroek 1991) support the general pattern of diversity in different sub-populations of *E. huxleyi*.

In this study, we consider DMSP lyase activity in 6 *Emiliana huxleyi* strains, focusing on the characteristics of *in vitro* activity with respect to pH and NaCl requirements. Our findings are compared with published data on other algal DMSP lyases.

MATERIAL AND METHODS

Culture conditions. Axenic cultures of *Emiliana huxleyi* (Lohmann) Hay et Mohler were obtained from the Provasoli-Guillard Center for the Cultivation of Marine Phytoplankton (CCMP, West Boothbay Harbor, Maine, USA: strains 370, 373, 374 and 379) or from Brian Palenik (Scripps Institution of Oceanography, San Diego, USA: CCMP 1516 and strain L). The origin and isolation information for these strains are given in Table 1.

Stock cultures were kept in 250 ml Erlenmeyer flasks containing 150 ml seawater medium. This was made from filtered (pore size 0.2 µm) and autoclaved seawater (central North Sea) enriched with nutrients (modified 1/2-si after Guillard [1975], with iron concentration reduced by 50% or 5.8 µM Fe to lessen precipitation). Before autoclaving, the medium was adjusted to a

Table 1. *Emiliana huxleyi*. Origin and isolation information for strains investigated

Parameter	<i>Emiliana huxleyi</i> strain					
	370 ^a	373 ^a	374 ^a	379 ^a	1516 ^a	L ^b
Origin	North Sea	Sargasso Sea	Gulf of Maine	English Channel	North Pacific	Oslo Fjord
Year of isolation	1959	1960	1989	1950	1991	ca 1980
Collector	Paasche	Guillard	Holligan	Parke	Polans	Andersen
Synonyms	451 B F451	BT 6 CSIRO-CS-57	89 E	P-92 A UTEX 1016 CCAP 920/1A		

^aSources from the homepages of the Provasoli-Guillard Center for the Cultivation of Marine Phytoplankton (CCMP; <http://ccmp.bigelow.org/>) or The Culture Collection of Algae at the University of Texas (UTEX; <http://bluebonnet.pai.utexas.edu/infores/utex/>)

^bE. Paasche pers. comm. 1998

salinity of 30 practical salinity units (psu) with distilled water. Light was supplied by fluorescent tubes at $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ under a light-dark rhythm of 18:6 h. Culture and experimental temperatures were 15°C .

Growth experiments. Strains were transferred from stock cultures into 1 l square polycarbonate bottles (Nalgene) to reach initial cell densities of $3000 \text{ cells ml}^{-1}$. At a light intensity of $80 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a light-dark rhythm of 18:6 h, cell number, particulate DMSP and chlorophyll *a* were monitored daily at noon over a growth period of 2 wk. Before taking a sub-sample for the measurements, bottles were gently inverted by hand to distribute cells. Specific growth rates were calculated using cell counts (see below) during logarithmic growth.

Cell size measurements. Light microscopy was used to estimate the cell size of living cells in mid-logarithmic growth. Using an eyepiece micrometer, which was calibrated with a stage micrometer at 1000-fold magnification, 15 to 20 cells were measured to the nearest $0.5 \mu\text{m}$. The values were averaged and 1 standard deviation was calculated.

Cell enumerations and examinations for bacterial contamination. Depending on cell density, 0.25 to 5 ml of culture was preserved and stained with acridine orange (Sherr et al. 1993) before filtering onto 0.2 or $0.8 \mu\text{m}$ black stained polycarbonate filters (Poretics, diameter 25 mm). Cells were enumerated by counting 20 squared grids at 400-fold magnification (equivalent to a filter area of $0.24 \times 0.24 \text{ mm}$) using an epifluorescence microscope (Nikon Diaphot, blue excitation). Cell numbers in the individual grids were averaged and the number of cells per ml of cell culture was calculated. Additionally, the same staining method was used to inspect the cultures for bacterial contamination (Hobbie et al. 1977). At 1000-fold magnification, at least 20 fields of vision were examined for bacterial cells. Also, cell suspensions were plated onto 1% peptone-seawater-agar and incubated for several days in the dark. Bacterial contamination was not detected with either method.

Chlorophyll *a* measurements. On every sampling day, an aliquot of cell culture (10 ml) was filtered with a low vacuum (less than 100 mm Hg) onto a glass fibre filter (Whatman GF/F), which was then individually wrapped in aluminium foil and stored at -20°C prior to analysis. Chl *a* was extracted for 24 h at 4°C in the dark using 6 ml 90% acetone and measured with a fluorometer (Kleinfeld TCF) which was calibrated with 20 to $200 \text{ ng chl } a \text{ cell}^{-1}$ during logarithmic growth were averaged and 1 standard deviation was calculated.

Particulate DMSP measurements. Duplicate samples of cell culture (10 or 20 ml) were filtered with a

low vacuum (less than 100 mm Hg) onto Whatman GF/F filters which were directly transferred into glass vials (25 ml volume). Ice-cold 10 M NaOH (5 ml) was added before closing the vials gas-tight with a teflon-coated silicone septum and a screw cap. During a 24 h dark incubation the DMSP completely hydrolyses to DMS, acrylate and H_2O (Challenger 1959). An aliquot (50 to $250 \mu\text{l}$) of the gas phase was taken with a gas-tight syringe and injected into a gas chromatograph (Shimadzu GC-9A) equipped with a flame photometric detector and a column packed with Chromosorb 101 (Macherey-Nagel). At a column temperature of 190°C and a carrier gas flow of 60 ml min^{-1} nitrogen, DMS eluted at 0.9 min. Peak areas were quantified relative to DMSP standards of 0.12 to $12 \mu\text{M}$ in 10 M NaOH. Other analytical details were the same as those reported by Steinke & Kirst (1996). The results from the duplicate measurements were averaged and particulate DMSP per cell and internal DMSP concentration calculated for every sampling day. The values during logarithmic growth were then averaged and 1 standard deviation was calculated.

DMSP lyase activity measurements. *Emiliana huxleyi* cell culture in late exponential growth (1 l) was concentrated to 50 ml by centrifugation for 20 min at $3500 \times g$ and 15°C . After a second centrifugation for 10 min at $20000 \times g$ and 4°C , the resulting pellet was resuspended in 3 ml buffer containing 160 mM citric acid/phosphate pH 6, with 500 mM NaCl, 20 mM CaCl_2 , 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (Complete Cocktail, Boehringer, 1 tablet 50 ml^{-1}). Cells were homogenised on ice using ultrasound (2 times 5 s; Branson Sonifier Cell Disruptor B-15). DMSP lyase activity was stable in frozen crude extracts so that $500 \mu\text{l}$ aliquots were stored at -20°C until activity measurements were conducted.

Prior to all enzyme experiments, the crude extracts of 373 and 379 were diluted 1:100 and 1:10, respectively, with the test buffer used in the assay. DMSP lyase activity measurements were performed in duplicates (if not stated otherwise) in 1.8 ml glass vials capped with teflon-coated silicone septae. After mixing $245 \mu\text{l}$ of test buffer with $50 \mu\text{l}$ crude extract, we monitored the possible outgassing of DMS from the extracts for 10 min. Thereafter, the enzymatic reaction was started by the addition of $5 \mu\text{l}$ 1.2 M DMSP (final concentration 20 mM). Depending on DMSP lyase activity the DMSP cleavage was followed for 10 to 120 min at 30°C . A heated control (45 min at 90°C proved to be sufficient) was used to quantify abiotic DMS production. Rates of DMS production were calculated and duplicate measurements averaged.

NaCl and pH requirements of DMSP lyase. To determine the NaCl requirements of DMSP lyase, a buffer with 160 mM citric acid/phosphate pH 6 con-

taining 1 mM DTT and protease inhibitor cocktail was adjusted to 0 to 2 M NaCl using a 5 M NaCl stock solution in citric acid/phosphate buffer. After NaCl additions, the test buffer was titrated to pH 6 using 1 M HCl.

The effect of pH on DMSP lyase activity was investigated with a series of citric acid/phosphate buffer at pH 2, 3, 4, 5, 6, 7, and 8 (no duplicate measurements). 500 mM NaCl was added to this buffer to allow for measurements with *Emiliania huxleyi* strains requiring NaCl for DMSP lyase activity. After each experiment the pH was checked to exclude possible pH changes during the measurements.

Synthesis of DMSP chloride. Dimethyl sulfide (100 ml, Riedel-de Haen), 100 ml acrylic acid (Merck) and 100 ml benzaldehyde (Merck), all analysis grade, were mixed on ice in a round-bottomed flask and bubbled for 10 min with hydrochloric acid vapour. The DMSP chloride (DMSP-Cl) which precipitated was harvested on a glass frit and rinsed with 50 ml of benzaldehyde. After freeze-drying the DMSP-Cl for 24 h, it was dissolved in boiling ethanol and re-crystallised by slow cooling to -20°C . This procedure was repeated a second time before the DMSP-Cl was analysed for purity by NMR-spectroscopy. Commercially prepared DMSP-Cl (Research Plus Inc., New Jersey, USA) was used as a standard.

RESULTS

Growth experiments

Cell numbers and particulate DMSP increased exponentially for at least 5 d in all strains as observed previously (Wolfe & Steinke 1996). Specific growth rate (μ) varied from 0.62 to 0.82 d^{-1} , resulting in final cell densities of 3.5×10^5 to 7×10^5 cells ml^{-1} (Table 2). On a per cell basis, the DMSP concentration was lowest in 1516 (3.6 fmol DMSP cell^{-1}) and averaged 10.8 fmol DMSP cell^{-1} in 370, 374, 379 and strain L. The highest DMSP concentrations were observed in 373 (18.9 fmol DMSP cell^{-1}) (Table 2).

Cell diameters in living cells ranged from 4.4 to $5.3\ \mu\text{m}$ as observed with a light microscope, resulting in calculated cell volumes (assuming spherical cells) of 44 to $78 \times 10^{-12}\text{ cm}^3$ (Table 2). Therefore, intracellular DMSP concentrations are low in 1516 (50 mM), higher in strain L (157 mM) and average 219 mM in the other strains (Table 2). These values were higher than previously reported for 370 and 373 (113 and 107 mM, respectively) (Wolfe & Steinke 1996). Different culture conditions (different batches of natural seawater, light quality and quantity, light-dark cycles of 18:6 vs 16:8 h, reduced iron concentration in f/2 medium) may have resulted in different physiological responses with

Table 2. *Emiliania huxleyi*. Comparison of growth, cell, chl a and DMSP characteristics for 6 strains

Parameter	<i>Emiliania huxleyi</i> strain					
	370	373	374	379	1516	L
Specific growth rate (μ , d^{-1})	0.80	0.72	0.82	0.67	0.62	0.82
Final cell density (10^5 cells ml^{-1}) ^a	6.1	4.2	3.5	6.9	6.8	7.0
Cell diameter (μm) ^b						
Arithmetic mean	4.4 (2–5)	5.3 (4–8)	4.9 (4–8)	4.8 (3–5)	5.2 (4–10)	4.7
Standard deviation	0.5	0.5	0.4	0.6	1.0	0.6
n	20	15	20	20	20	20
Cell volume (10^{-12} cm^3)						
Arithmetic mean	44	78	62	58	73	55
Standard deviation	14	22	15	25	60	26
n	20	15	20	20	20	20
Chlorophyll a (pg cell^{-1})						
Arithmetic mean	0.16	0.24	0.20	0.19	0.08	0.14
Standard deviation	0.03	0.06	0.04	0.06	0.02	0.03
n	6	5	5	6	6	6
DMSP (fmol cell^{-1})						
Arithmetic mean	10.2	18.9	13.1	11.2	3.6	8.7
Standard deviation	2.9	5.3	3.0	2.0	1.3	1.7
n	8	8	8	9	8	8
Intracellular DMSP concentration (mM)						
Arithmetic mean	232	242	211	192	50	157
Standard deviation	65	67	49	35	18	31
n	8	8	8	9	8	8

^aMaximum cell density in stationary phase

^bValues from CCMP homepage (<http://ccmp.bigelow.org/>) in parentheses

higher cellular DMSP concentrations. However, it may be possible that calibration variance between sulfur measurements in our 2 laboratories could have contributed to the difference. Chl *a* values ranged from 0.08 to 0.24 $\mu\text{g cell}^{-1}$ (Table 2), which is similar to values reported previously for *Emiliana huxleyi* (Pond & Harris 1996).

DMSP lyase activities

We previously showed that DMSP lyase activity per cell is constant over the growth cycle in 370 and 373 (Wolfe & Steinke 1996). Therefore, it can be assumed that growth stage does not affect DMSP lyase activity per cell in *Emiliana huxleyi*, and that DMSP lyase activities reported in the present study reflect cell activities independent of cell growth stage.

Activity of DMSP lyase showed high strain-specific variability. At 30°C, pH 6 and optimal additions of NaCl for each strain, DMSP cleavage varied over more than 3 orders of magnitude from 0.002 to 12.5 $\text{fmol DMS cell}^{-1} \text{min}^{-1}$ (Fig. 1).

In 374, DMS production rate was very low but 370, 1516 and strain L showed a higher DMS production of 0.01 to 0.03 $\text{fmol DMS cell}^{-1} \text{min}^{-1}$ (Fig. 1). Compared to these production rates, in 373 and 379 DMSP lyase activities were more than 100-fold higher (6.1 to 12.5 $\text{fmol DMS cell}^{-1} \text{min}^{-1}$).

Intracellular DMSP concentration did not directly correlate with DMSP lyase activity in investigated

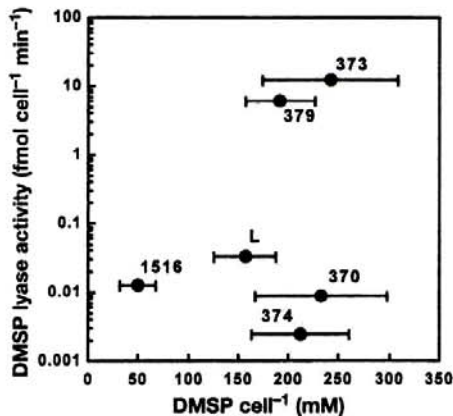


Fig. 1. *Emiliana huxleyi*. *In vitro* DMSP lyase activities versus intracellular DMSP concentrations in 6 strains. DMSP lyase activities are for pH 6 and optimal NaCl concentration as follows: 0 M (374); 0.25 M (373); 0.5 M (379); 1 M (370, L); 2 M (1516). Data shown represent average values. Error bars indicate standard deviation (x-axis, see Table 2). The range of data on the y-axis ($n = 2$) is smaller than the indicated data points

strains. Although DMSP concentrations were very similar in 4 strains (370, 373, 374, 379), 370 and 374 showed low DMSP lyase activity, whereas in 373 and 379 the activity was high.

Effect of NaCl additions on DMSP lyase activities

Additions of NaCl resulted in increased DMSP lyase activities in 370, 1516 and strain L (Fig. 2B), whereas 373 and 374 showed reduced DMS production with increasing NaCl concentrations (Fig. 2A).

Strains L and 370 had maximum activity with 1 M NaCl added but in 1516 DMSP lyase activity increased up to 2 M NaCl. In contrast to these findings, NaCl additions to 373 and 374 inhibited lyase activity to 57 and 64% of optimal activity, respectively. Strain 379 was relatively insensitive to NaCl additions but activity was slightly increased with 0.5 to 1 M NaCl added (no measurement at 2 M NaCl).

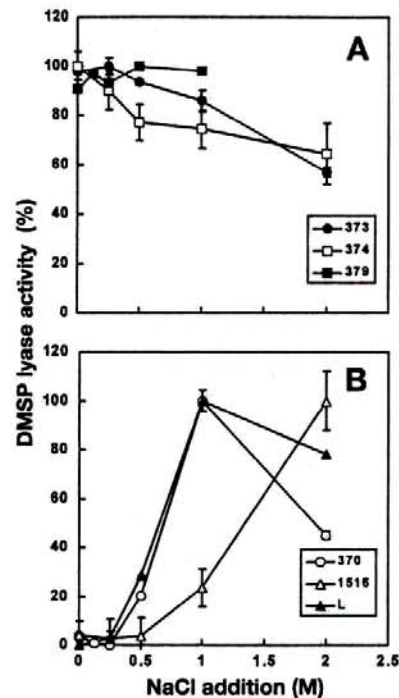


Fig. 2. *Emiliana huxleyi*. DMSP lyase activities in 6 strains with various additions of NaCl. Maximum DMSP lyase activities (100%) of individual strains are as presented in Fig. 1. (A) Strains with halotolerant DMSP lyases (373, 374 and 379). (B) Strains with halophilic DMSP lyases (370, 1516 and L). Data shown represent average values. Error bars indicate range of data ($n = 2$)

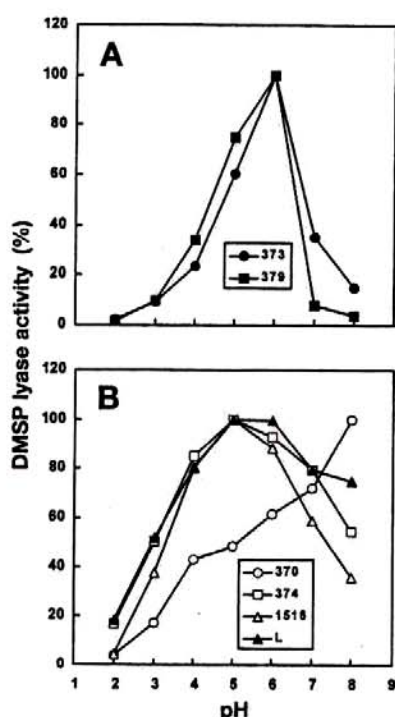


Fig. 3. *Emiliana huxleyi*. DMSP lyase activities in 6 strains at various pH levels in citric acid/phosphate buffer, at optimal NaCl concentration as given for Fig. 1. (A) Strains with high DMSP lyase activities (373 and 379). (B) Strains with low DMSP lyase activities (370, 374, 1516 and L)

pH optima

Various pH levels revealed further intraspecific differences in DMSP lyase activities. The 2 strains with high lyase activity (373 and 379) had a narrow pH optimum around pH 6 (Fig. 3A). In contrast, the low activity strains either had a pH optimum around pH 5 with substantial activity (above 80% of maximum) at pH 4 and pH 6 (374, 1516 and L) (Fig. 3B) or showed increasing activity with increasing pH (370), similar to the DMSP lyase from *Phaeocystis* sp. (Stefels & Dijkhuizen 1996).

DISCUSSION

There have been few isozyme studies in marine phytoplankton taxa, although several authors have compared total cell protein patterns (electrophoretic banding) within a phytoplankton species (Gallagher 1982, Beam & Himes 1987). The work presented here provides information about intraspecific DMSP lyase

variation in *Emiliana huxleyi*. On the basis of our results we suggest hypotheses concerning the relationship between DMSP lyases in *E. huxleyi* and enzymes with the same function in other taxa, the conditions under which DMS production might occur, and possible functions of the DMSP cleavage reaction.

Intraspecific DMSP lyase variation

The *Emiliana huxleyi* strains exhibited much more variability in DMSP lyase activity than in intracellular DMSP concentration, and there was no correlation between the two. Based on *in vitro* activity and pH and NaCl optima, the DMSP lyase isozymes studied appear to fall into at least 2 groups. Strains 373 and 379 had high activities, roughly 100-fold per cell higher than the other strains (Fig. 1), exhibited sharp pH optima at 6 with less than 40% of maximal activity at pH 4 and 7 (Fig. 3A), and showed no NaCl requirement but inhibition with addition of more than 500 mM NaCl (Fig. 2A). These 2 strains were isolated from widely separated geographic regions (Sargasso Sea and English Channel, respectively) (Table 1) and also showed phenotypic variation such as cell size (Table 2) and lipid composition (Wolfe unpubl. data).

The other strains had much lower DMSP lyase activities (Fig. 1) but showed marked differences in requirements for *in vitro* enzyme activity. Strains 370, L and 1516 required high NaCl concentrations for optimal activity (Fig. 2B), while strain 374 showed no NaCl requirement but 20 to 30% inhibition at NaCl concentrations above 250 mM. Strains 374, L and 1516 showed broad pH maxima at 5 and retained about 50% DMSP lyase activity over pH 3 to 8 (Fig. 3B), while strain 370 showed increasing activity with increasing pH. Therefore, the assay conditions used to generate the data shown in Fig. 1 (pH 6, optimal NaCl concentration) result in an underestimation of the DMSP lyase activity in 370 (0.009 fmol DMS cell⁻¹ min⁻¹). During the pH experiments, an activity of 0.02 fmol DMS cell⁻¹ min⁻¹ was measured at pH 8.

Intra-strain variation of DMSP lyase activities was only small. Over more than a 1 yr investigation period, the general pattern of high-lyase strains (373 and 379) versus strains with low DMSP lyase activity remained unchanged.

On the basis of the differing pH and NaCl requirements, we hypothesize that structural differences among the DMSP lyase isozymes could contribute to activity variation. Additionally, preliminary attempts to isolate DMSP lyase enzymes from *Emiliana huxleyi* indicated varying solubilities: after centrifugation (105 000 × g) of crude extracts from 373 and 379, recovery in the supernatant was 4 and 54% of total activity,

respectively. Further purification steps with 379 indicated precipitation of DMSP lyase at 10 to 30% ammonium sulfate (hydrophobic protein), and incomplete binding to ion-exchange and hydrophobic-interaction chromatography columns. This partially purified DMSP lyase showed maximum activity at 35°C, which was reduced to 84% at 30°C (Steinke pers. obs.).

Confirmation of structural differences will require protein purification and sequencing. The only DMSP lyases partially sequenced to date are from the marine bacteria *Pseudomonas doudoroffii* and *Alcaligenes* sp. strain M3A (deSouza & Yoch 1996). However, we cannot dismiss the possibility that variable activities in *E. huxleyi* may be due to differing amounts of DMSP lyase per cell. Also, enzyme stability in the extracts may vary from strain to strain. Nevertheless, once the extracts were prepared and frozen at -20°C, DMSP lyase activity remained constant over many months.

Since bacterial DMSP lyases may be involved in DMS production, all experimental procedures were carried out with axenic cultures using sterile techniques. Nevertheless, sterility tests carried out may not be suitable to recognize intracellular bacteria. Therefore, the potential of contamination from such sources may be a cause of false interpretations that needs to be eliminated in future studies.

The presence of various DMSP lyase isozymes in *Emiliana huxleyi* is consistent with other recently published information concerning genetic diversity within this species (Young & Westbroek 1991, Medlin et al. 1996) resulting in various morphotypes (van Bleijswijk et al. 1991) and altered cell physiology and growth characteristics (Brand 1982, van Bleijswijk et al. 1994). How DMSP lyase variation relates to other phenotypic or genotypic factors will require further characterisation of strains.

Table 3. Comparison of algal DMSP lyases in crude or cell-free extracts (purified enzymes noted; ND: not determined)

Class Species	Maximum activity Temperature (°C)	pH	Cofactor requirements	Location	Source
Rhodophyceae					
<i>Polysiphonia lanosa</i>	ND ^a	5.1	Reduced thiol groups ^b	Bound to subcellular fraction	Cantoni & Anderson (1956)
<i>Polysiphonia paniculata</i> ^c	ND ^d	ND ^e	Mg ²⁺ , Ca ²⁺	Membrane bound (vacuole?)	Nishiguchi & Goff (1995)
Chlorophyceae					
<i>Enteromorpha clathrata</i>	25	6.2–6.4	None ^f	Membrane bound	Steinke & Kirst (1996)
<i>Ulva lactuca</i>	ND	8.5	ND	ND	Diaz & Taylor (1994)
<i>Ulva curvata</i> ^g	ND	8	75 mM NaCl	Partially membrane bound	de Souza et al. (1996)
Haptophyceae					
<i>Phaeocystis</i> sp.	≥20 ^h	≥10.5	DTT ≥1 mM	Outer cell surface	Stefels & Dijkhuizen (1996), Stefels & van Boekel (1993)
<i>Emiliana huxleyi</i> CCMP 370	ND	≥8	DTT ≥1 mM NaCl = 1 M	Membrane bound, inside cells	Wolfe & Steinke (1996), this study
CCMP 373	ND	6	No NaCl	Membrane bound, inside cells	Wolfe & Steinke (1996), this study
CCMP 374	ND	5	No NaCl	ND	This study
CCMP 379	35 ⁱ	6	No NaCl	Membrane bound ^{l,k}	This study
CCMP 1516		5	NaCl ≥2 M	ND	This study
Strain L		5	NaCl = 1 M	ND	This study
Dinophyceae					
<i>Cryptocodinium</i> (<i>Gyrodinium</i>) <i>cohnii</i>	27	6.0–6.5	≥400 mM NaCl	ND ^l	Kadota & Ishida (1968)

^aAll experiments were carried out at 25°C
^bGlutathione (6 mM) was used in this study
^cPurified enzyme: larger subunit 92.6 kDa ($V_{max} = 1.62 \mu\text{mol DMS min}^{-1} \text{mg}^{-1} \text{protein}$, $K_m = 73 \mu\text{M}$) and 2 smaller subunits of 36.5 and 34.2 kDa
^dSamples were incubated at 12°C
^eAll experiments were carried out at pH 6.8
^fCofactors tested: ADP, ATP, NAD, NADH, NADP, NADPH at 5 mM and NaCl, MgCl₂ and CaCl₂ at 5 mM and 500 mM
^gPurified enzyme: 1 soluble and 3 membrane-bound monomers, all 78 kDa (soluble monomer: $V_{max} = 86 \mu\text{mol DMS min}^{-1} \text{mg}^{-1} \text{protein}$, $K_m = 520 \mu\text{M}$)
^hIn whole cells
ⁱSteinke pers. obs.
^kEnzyme precipitated at 10 to 30% ammonium sulfate (highly hydrophobic)
^lEnzyme precipitated at 25 to 50% ammonium sulfate (hydrophobic)

Comparison with DMSP lyases from other algae

Table 3 summarises the DMSP lyase behaviour in crude *Emiliana huxleyi* extracts with other algal DMSP lyase enzymes which have been characterised or isolated.

Our findings characterise the DMSP lyases from strains L, 370 and 1516 as halophilic enzymes, comparable with other halotolerant and halophilic enzymes in marine organisms (Bylund et al. 1991, Vegavillasante et al. 1993). With the exception of the dinoflagellate *Cryptothecodinium* (*Gyrodinium*) *cohnii*, these *Emiliana huxleyi* strains are the only others to have DMSP lyases with a significant salt requirement. The DMSP lyase of *Polysiphonia paniculata* required 20 to 40 μM Mg^{2+} or Ca^{2+} ions for optimal activity with little effect above this concentration (Nishiguchi & Goff 1995), and *Ulva curvata* required 75 mM NaCl to stimulate DMSP cleavage, but activity was reduced by 40% at 600 mM NaCl (de Souza et al. 1996).

Of the few algal DMSP lyases examined to date many require acidic pH for optimal activity. A preference for acidic pH was found in the macroalgae *Polysiphonia lanosa* (Cantoni & Anderson 1956), *Enteromorpha clathrata* (Steinke & Kirst 1996) and the heterotrophic dinoflagellate *Cryptothecodinium cohnii* (Kadota & Ishida 1968). All *Emiliana huxleyi* strains except strain 370 showed similar response. However, there are also DMSP lyases which are active under alkaline conditions, including those from *Ulva lactuca* (Diaz & Taylor 1994) and *U. curvata* (de Souza et al. 1996), and the haptophyte *Phaeocystis* sp. (Stefels & Dijkhuizen 1996). The latter showed increasing DMSP lyase activity with increasing pH (to a maximum value at pH 10.5, the highest pH tested in the study) similar to the pattern showed for strain 370 (Fig. 3B).

The widespread occurrence of the DMSP lyase enzyme, and the variations in its behaviour, suggest a broad family of DMSP lyase isozymes, possibly with several fundamental forms. Bacterial DMSP lyases are a good example, since they show considerable variation (Yoch et al. 1997), have been more fully characterised and may offer some insight into possible algal DMSP lyase location and function. The cytoplasmic DMSP lyase from *Pseudomonas doudoroffii* (de Souza & Yoch 1996) required 600 mM salt for optimal activity and showed an acidic pH optimum, while the periplasmic or cell-surface DMSP lyase from *Alcaligenes* strain M3A (de Souza & Yoch 1995b) showed no salt requirement and an alkaline pH optimum. The DMSP lyase from the haptophyte *Phaeocystis* sp. is also thought to be a cell-surface enzyme, and also shows no salt requirement and high pH optimum (Stefels & Dijkhuizen 1996). It is therefore feasible that the enzymes in *Emiliana*

huxleyi differ not only in behaviour, but in cellular location. Two studies have found evidence for both membrane-bound and soluble DMSP lyase activity within an organism (Nishiguchi & Goff 1995, de Souza et al. 1996). Hence, it cannot be ruled out that DMSP lyase activities reported in this study result from a mixture of various isozymes present in 1 *E. huxleyi* strain.

There is also evidence for variation in DMSP lyase function at the genus level among macroalgae. In a study investigating 21 algal strains (Steinke et al. 1996), the closely related macroalgal species *Enteromorpha intestinalis*, *E. clathrata* and *E. compressa* showed high DMSP lyase activities of 10 to 100 nmol DMS mg^{-1} cell protein min^{-1} and intracellular DMSP concentrations of 25 to 90 mmol DMSP kg^{-1} fresh weight. Activities in 2 strains of *E. bulbosa* were about 1000-fold lower, averaging 0.01 nmol DMS mg^{-1} cell protein min^{-1} despite a similar intracellular DMSP concentration of 20 to 50 mmol DMSP kg^{-1} fresh weight. Although the enzyme assay used by Steinke et al. (1996) was optimised to test for DMSP lyase in *E. clathrata*, the results indicated the presence of DMSP lyase isozymes with various total activities and possibly also differences in requirements for optimal activity. The diversity seen here within *Emiliana huxleyi* suggests that DMSP lyase isozyme variants may also be common in microalgae.

Implications for DMSP lyase activity and DMS production *in vivo*

During exponential and stationary growth of *Emiliana huxleyi*, we found only little production of DMS by axenic cultures (Wolfe & Steinke 1996), despite consistent *in vitro* DMSP lyase activity per cell over the growth period. This implies that in all strains this reaction is strongly repressed. We previously suggested that physical separation of the DMSP lyase and DMSP might be a mechanism, based on the activation of this reaction following cell lysis (Wolfe & Steinke 1996). However, the reaction might also be highly regulated *in vivo* by the different NaCl and pH requirements *in situ*, depending on the cellular location of the enzyme. Fluorescent dye measurements of internal pH in *E. huxleyi* suggest cytoplasmic values around 7, with chloroplast pH as high as 8 (Dixon et al. 1989). Since alkaline pH reduces DMSP lyase activity in most strains except for 370, this might explain why DMS production is so low *in vivo*. Interestingly, we previously found that strain 370 produced relatively high *in vivo* DMS compared to strain 373, despite the much lower activity of its enzyme in crude extracts (Wolfe & Steinke 1996).

Possible functions of DMSP cleavage reaction

The role of the DMSP cleaving reaction in phytoplankton or macroalgal physiology is still unknown. DMSP is well recognised to function as a compatible solute, so the degradation of internal osmotica following external salt stress is the assumed natural function of this reaction. However, there is little if any evidence that cellular DMSP is involved in short-term osmotic adjustment but may change with long-term stress and especially under high salinities (Dickson & Kirst 1986, Kirst 1990). The role of the cleavage products has not been critically examined. DMS, a small, polar molecule, would almost certainly diffuse rapidly from cells the size of *Emiliana huxleyi*. Acrylate and a proton would most likely remain. The metabolism of acrylate by algae has not been studied, and it is not clear what function it might serve.

Recently, we suggested that this reaction, which does not appear to proceed under *in vivo* growth conditions, might be stimulated by cell lysis, as, for example, following grazing by protozoan herbivores. We found that such grazing resulted in increased production of DMS and the amount of DMS produced was consistent with relative *in vitro* DMSP lyase activity in the investigated strain (Wolfe & Steinke 1996). Also, high lyase strains such as 379 were grazed at lower rates than low lyase strains (Wolfe et al. 1997). Based on the feeding preferences of marine protozoan herbivores, Wolfe et al. (1997) concluded that this reaction may potentially be involved in a grazing-activated chemical defence mechanism by producing concentrated acrylate.

The protistan digestion process is initiated by fusion of acidosomes with the food vacuole, resulting in a decrease of vacuolar pH, thereby maximising the activity of hydrolytic enzymes (Hausmann & Hülsmann 1996). Our data suggest that the DMSP lyase enzyme could maintain activity within the protozoan food vacuole following ingestion. In the ciliate *Paramecium*, the pH of the food vacuole is decreased to pH 3 only 4 to 8 min after ingestion (Fok et al. 1982), but the process may take significantly longer in other predators (Dodge & Crawford 1974, Öpik & Flynn 1989). Additionally, it is a common occurrence for small prey organisms to remain active for a limited time after ingestion (Grula & Bovee 1977). If not all prey is immediately digested and still metabolically active after ingestion by a protist, the temporal gradient of pH and ionic conditions experienced by the ingested prey cell may allow optimal activity of *Emiliana huxleyi* DMSP lyases. Even a short period of high lyase activity during the digestion process may convert most of the algal DMSP to DMS and acrylate: Wolfe & Steinke (1996) calculated cleavage of roughly 60% of the prey DMSP

during only 3 to 5 min after ingestion of strain 373 by *Oxyrrhis marina*, resulting in average DMS, and hence acrylate production of 65 mM.

Conclusions

In *Emiliana huxleyi*, DMSP lyase isozymes with various requirements for optimal activity resulted in strain-specific DMS production rates *in vitro*. The specific rates were not constant but varied during an investigation period of several months. Nevertheless, the general pattern of high- and low-activity strains remained true over more than a 1 yr cultivation period. There were pronounced differences among DMSP lyase characteristics from different strains. We suggest that the strains with high activity have structurally different DMSP lyases—which then may result in higher DMS production rates—rather than simply having higher DMSP lyase titers per cell. However, only amino acid sequencing of purified DMSP lyase would ultimately explain structural differences.

Assuming that DMSP lyases are still active after loss of cell integrity, the enzyme requirements demonstrated here may be important clues to the fate of dissolved DMSP in seawater. Algal DMSP lyases probably compete with bacterial pathways for DMSP utilisation but we do not know for how long free algal DMSP lyases remain active.

The cellular location of DMSP and DMSP lyases in *Emiliana huxleyi* needs further investigation. There is evidence that DMSP lyases in *E. huxleyi* are membrane-bound (Wolfe & Steinke 1996, Steinke pers. obs.) but what organelles are involved in enzyme-substrate segregation is still unknown. Finally, the activation of DMS production after cell ingestion by herbivorous protists (Wolfe et al. 1997) and the fate of acrylate, which is produced during this process, is an attractive task for future research. Acrylate production may affect competition and species succession in microbial communities, whereas knowledge on DMS production pathways is essential for our understanding of the oceanic and atmospheric sulfur cycle and its implications for global climate.

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