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Comparative functional characteristics of DMSP lyases extracted from polar and temperate *Phaeocystis* species

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ABSTRACT: Members of the marine phytoplankton genus *Phaeocystis* (Prymnesiophyceae) produce large amounts of the intracellular osmolyte DMSP and they are known to also produce lyase enzymes that cleave DMSP into the biogeochemically important trace gas DMS. The functional characteristics of DMSP lyase activity in *Phaeocystis* spp. are not well known. We characterized DMSP lyase activity in extracts from 2 ecologically important species from this genus, the mesophile *P. globosa* (strain CCMP629) and the psychrophile *P. antarctica* (strain CCMP1374). Results from whole cell extracts showed that both algal species were potent producers of DMSP lyase, with Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) values of 1.77 mM and 17.3 nmol DMS min^{-1} mg $protein^{-1}$, respectively, for *P. globosa*, and 2.31 mM and 28.2 nmol DMS min^{-1} mg $protein^{-1}$, respectively, for *P. antarctica*. The optimal DMSP lyase activity was recorded at pH 4 and 30°C for *P. globosa*, and at pH 5 and 20°C for *P. antarctica*. The half-life of the DMSP lyase of *P. globosa* was 210 min at 25°C, which was longer than that of the *P. antarctica* enzyme (61.9 min). First-order kinetic analysis of DMSP lyase thermal denaturation demonstrated that the activation energy, free energy, enthalpy and entropy of denaturation in *P. antarctica* extracts were lower than for *P. globosa* extracts, confirming that the *P. antarctica* DMSP lyase was more thermolabile than the lyase from the temperate strain. Inhibitor tests with metals, a chelator (EDTA) and a serine binding agent (PMSF) suggested that the DMSP lyases from both *Phaeocystis* species were metalloenzymes with serine and sulfhydryl groups at the active site. The acidic pH optima for the *Phaeocystis* strains are consistent with findings from other Prymnesiophyceae, and we speculate that this may reflect adaptation to an acidic sub-cellular location for the DMSP lyase.

KEY WORDS: Dimethylsulfide · Dimethylsulfoniopropionate · DMSP · DMSP lyase · Prymnesiophyte · Psychrophile

INTRODUCTION

DMSP is a secondary metabolite synthesized by a wide variety of marine algae (reviewed in Keller et al. 1989). DMSP has been linked to different physiological functions, such as osmoregulation (Dickson & Kirst 1987), cryoprotection (Kirst et al. 1991, Karsten et al. 1996), oxidative stress protection (Sunda et al. 2002), methyl donor (Ishida 1968) and overflow metabolite for excess reducing power during photosynthesis (Stefels 2000). DMSP degradation in marine environments occurs via 2 different biochemical pathways (reviewed in Curson et al. 2011, Moran et al. 2012). In the first pathway, the DMSP is cleaved

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via the enzyme DMSP demethylase to 3-(methylmercapto)propionic acid (MMPA). In a subsequent reaction, MMPA either undergoes demethylation to form 3-mercaptopropionic acid and/or demethiolation to form methanethiol (Reisch et al. 2011). In the second pathway, DMSP lyase (EC 4.4.1.3) catalyzes the lysis of DMSP into dimethylsulfide (DMS), acrylate and a proton. There is worldwide interest in evaluating the biochemical and molecular mechanisms involved in the regulation of enzymatic cleavage of DMSP to DMS because DMS is an important component of the global sulfur cycle and a potential climate-active trace gas. DMS is supersaturated in all surface ocean waters and, once transferred to the atmosphere, it rapidly oxidizes to dimethylsulfoxide, methanesulfonate (MSA) or sulfate. Sulfur-containing, acidic aerosols derived from these compounds can affect the Earth's climate system by scattering incoming sunlight and contributing to the population of aerosol particles that act as cloud condensation nuclei (Charlson et al. 1987, Simó 2001).

DMSP lyases are the key enzymes responsible for the biogenic production of DMS in marine waters. They have been found in marine algae, including phytoplankton (Steinke et al. 1996, 1998), and in a variety of different bacteria (de Souza & Yoch 1995a) in which 6 different DMSP lyase proteins (DddD, DddL, DddP, DddQ, DddW and DddY) have been identified (Todd et al. 2007, 2009, 2011, 2012, Curson et al. 2008, 2011, Kirkwood et al. 2010). All these proteins except DddD catalyze cleavage of DMSP into acrylate and DMS. The DddD protein produces 3-hydoxypropionate instead of acrylate. Most of the genes encoding bacterial DMSP lyases are prone to horizontal gene transfers, and in the case of *dddP*, the gene has been subjected to interdomain horizontal gene transfer between bacteria and Ascomycete fungi (Todd et al. 2009). While DMSP lyases from bacteria have been relatively well characterized at the genetic level, much less is known about phytoplankton DMSP lyases. Published studies of phytoplankton DMSP lyases have shown that their occurrence and properties are often species- and strain-specific (Steinke et al. 1996, 1998, Niki et al. 2000, Yost & Mitchelmore 2009, Franklin et al. 2010), but few species have been well characterized.

Phaeocystis (Prymnesiophyceae) is a cosmopolitan group of marine phytoplankton that comprises 6 different species, including colony-forming *P. globosa*, *P. antarctica* and *P. pouchetii*, and unicellular *P. cordata*, *P. jahnii* and *P. scrobiculata* (Zingone et al. 1999). Colonial *Phaeocystis* species form massive blooms in both temperate and polar regions and substantially impact the yield of commercially important marine products (Schoemann et al. 2005). *Phaeocystis* blooms play a major role in the biogeochemical cycle of carbon and sulfur by fixing a large amount of carbon dioxide and by producing high concentrations of DMSP and DMS (Stefels & van Leeuwe 1998, van Duyl et al. 1998, Arrigo et al. 1999, DiTullio et al. 2003), and by providing an important substrate for bacterial carbon demand (Rellinger et al. 2009).

Despite the importance of *Phaeocystis* species in global biogeochemical cycles, climate dynamics and coastal fisheries, very few studies have been performed to characterize the DMSP lyase activity of bloom-forming *Phaeocystis* (Stefels & van Boekel 1993, Stefels et al. 1995, Stefels & Dijkhuizen 1996). The objective of the present study was to compare the functional (i.e. DMSP cleaving) properties of DMSP lyase extracted from 2 different, but closely related *Phaeocystis* species: the mesophile *P. globosa* and the psychrophile *P. antarctica*. Our findings define the significant properties of DMSP lyase in these ecologically important species that will be useful in future investigations into the ecophysiology of DMSP cleavage in temperate and polar regions of the world oceans.

MATERIALS AND METHODS

Phaeocystis culture

P. globosa (CCMP 629), originally isolated from the subtropical North Atlantic (~23° N, 75° W; typical temperature range 20 to 30°C) and *P. antarctica* (CCMP 1374), isolated from the Ross Sea (77.8° S, 163° E; –1.8 to 3.0°C), were obtained from The Provasoli-Guillard Center for the Cultivation of Marine Phytoplankton (CCMP, Maine, USA; now the National Center for Marine Algae and Microbiota [NCMA]). The batch cultivation of the algal species was carried out in duplicate in 600 ml polycarbonate tissue culture flasks with 0.2 µm vent caps (Corning), containing 500 ml f/2 medium (Guillard 1975). The incubation temperature was 20 and 1°C for *P. globosa* and *P. antarctica*, respectively, and cultures were grown under fluorescent light (133 µEinstein m^{-2} s^{-1}) with a 12:12 h light:dark cycle. Algal cultures were harvested in the exponential growth phase by centrifugation at 3000 × *g* (4°C) for 15 min. The supernatant fluid was discarded and the resulting pellets were used for the extraction of DMSP lyase. Chlorophyll (chl) *a* concentrations at the time of harvest were

0.56 ± 0.05 µg ml^{-1} for *P. globosa* and 0.47 ± 0.03 µg ml^{-1} for *P. antarctica* . The chl *a* concentration was determined fluorometrically as described previously (Parsons et al. 1984). Both phytoplankton species were predominantly in the colonial cell form at the time of harvest.

DMSP lyase extraction

The extraction of DMSP lyase was performed as reported previously with some modifications (Harada & Kiene 2012). Briefly, algal pellets from each flask were resuspended in 5 ml of ice-cold 200 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl, 2 mM dithiothreitol (DTT) and 0.1% (v/v) Triton X-100, followed by vigorous vortex mixing and centrifugation at 3500 × *g* (4°C) for 15 min. The supernatant was collected and concentrated using an Amicon Ultra 10000 MWCO centrifugal filter device (Millipore). As the standard deviation of the DMSP lyase activities of the replicate cultures of each algal species were <10% of the mean values, the concentrated fractions of each algal species were pooled together. The pooled fractions were diluted with 10 ml of 25 mM Tris-HCl buffer (pH 7.5), divided into 0.5 ml aliquots and stored at –20°C. The aliquots were used for characterization studies. There was no loss in enzyme activity detected during the 4 wk period during which characterization studies were performed.

DMSP lyase assay

DMSP lyase activity was defined as the rate of conversion of DMSP to DMS. DMSP lyase activity was assessed at 30 and 20°C for *P. globosa* and *P. antarctica*, respectively, in sealed 10 ml serum vials containing a standard assay mixture of 146 µl assay buffer (200 mM phosphate-citrate buffer adjusted to pH 4 for *P. globosa* and pH 5 for *P. antarctica*) and 50 µl of enzyme (~120 µg protein ml^{-1}). The reaction was started by the addition of 4 µl of 250 mM DMSP·HCl aqueous solution (5 mM final concentration), and the resulting DMS in the headspace of the vial was quantified in a 30 min period using a gas chromatograph equipped with a flame photometric detector (Steinke et al. 2000, Harada et al. 2004). Appropriate blanks were also prepared to correct for abiotic DMS production from DMSP hydrolysis. For these controls, 50 µl heat-treated enzyme preparation (95°C for 30 min in a sealed tube, and rapidly cooled to 4°C) was added in place of the original enzyme solution. All assays were repeated twice and data are presented as mean ± SD. One unit of specific enzyme activity (U) was defined as 1 nmol of DMS produced min^{-1} mg protein^{-1}. Protein was quantified using the Bio-Rad Bradford protein assay kit with bovine serum albumin as the standard.

Contribution of bacterial DMSP lyase

A subsample (0.5 ml) of each *Phaeocystis* culture was filtered through a Whatman GF/C filter (25 mm diameter, 1.2 µm nominal pore size) with gentle vacuum, followed by washing with 0.5 ml f/2 medium to rinse through bacteria. The resulting filtrate was filtered again through a 0.22 µm pore size polycarbonate filter (25 mm diameter, Millipore) using gentle filtration. Both GF/C and polycarbonate filters were placed into separate 14 ml serum vials containing 980 µl of assay buffer (200 mM phosphate-citrate buffer adjusted to pH 4 for *P. globosa* and pH 5 for *P. antarctica*) supplemented with 500 mM NaCl, 2 mM DTT and 0.1% (v/v) Triton X-100. The reaction was started by the addition of 20 µl of 250 mM DMSP·HCl aqueous solution (5 mM final concentration), and the resulting DMS in the headspace of the vial was quantified as described in the DMSP lyase assay section. The abiotic corrections of DMS were made in a similar fashion with sterile f/2 medium. Bacterial abundance in the GF/C filtrate was quantified using DAPI staining as described previously (Porter & Feig 1980).

DMSP lyase: pH and temperature dependence

To determine the optimum pH for DMSP lyase activity in *Phaeocystis globosa* and *P. antarctica* extracts, the enzyme assay was performed with buffers of different pH added to the assay mixture: 200 mM phosphate-citrate (pH 3 to 6), 200 mM Tris-HCl (pH 7, 7.5 and 8) or 200 mM borate (pH 9 and 10). The temperature dependence of lyase activity was assayed from 5 to 50°C at 5°C increments. We also carried out pH and thermal stability studies in which the enzyme was exposed to a non-optimal pH (or temperature in a separate experiment) and then returned to the standard condition (pH 4 and 30°C for *P. globosa*, and pH 5 and 20°C for *P. antarctica*). The pH stability tests were carried out by pre-incubation of the DMSP lyase solution at 20°C for *P. antarctica* or 25°C for *P. globosa* in one of the above-mentioned buffers at different pHs ranging from 3 to 10 for

15 min. The reaction mixture was then returned to pH 4 and 5 for *P. globosa* and *P. antarctica*, respectively, and the DMSP lyase activity was assayed. For the temperature stability tests, lyase preparations were pre-incubated for 30 min at different temperatures between 5 and 45°C (with 5°C increments), before the residual enzyme activity was assayed at optimum temperatures, as described in the DMSP lyase assay section.

Determination of kinetic constants

To determine the Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) for DMSP lyase extracted from the 2 different *Phaeocystis* species, the enzyme activity was measured as described in the DMSP lyase assay section by using DMSP substrate at different concentrations (0.1, 0.5, 1, 2.5, 5, 7.5, 10, 20 mM). The values of K_m and V_{max} were determined with Eadie-Hofstee plots, with V_{max} normalized to protein.

Effect of NaCl

The effect of NaCl on DMSP lyase activity for both algal species was examined by adding aqueous NaCl (final added concentrations of 0.086 to 0.86 M in 0.086 M increments) to the assay buffer (200 mM phosphate-citrate pH 4 buffer for *Phaeocystis globosa* and pH 5 buffer for *P. antarctica*). Then 50 µl of enzyme extract and 4 µl of DMSP were added to 146 µl of the ionic-strength-adjusted buffer solution, and the enzyme activity assayed as described in the DMSP lyase assay section.

Effect of DMSP analogs, metal ions and organic chemicals

The effect of different DMSP analogs (2-methyl DMSP, 2-chloro DMSP, dimethylsulfonioacetate, choline-*o*-sulfate and glycine betaine) was determined by incubating the enzyme solution with the individual analog (final concentration 20 mM) for 10 min at 20 or 25°C for *Phaeocystis antarctica* or *P. globosa*, respectively. After a 10 min pre-incubation with the DMSP analog, the lyase enzyme activity was determined for each sample through the addition of 5 mM DMSP (final concentration) to the assay mixture. The enzyme activity was determined as described in the DMSP lyase assay section and compared to the control activity in the assay mixture without the added DMSP analog. Similarly, metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}) and several organic chemicals, EDTA, DTT (dithiothreitol), PMSF (phenylmethanesulfonylfluoride) and pCMB (parachloromercuribenzoate), were tested at 0.1 and 1.0 mM. All the metal ions were in the chloride form and analytical reagent (ACS) grade. DTT, PMSF and pCMB were molecular biology grade. Separate controls were also prepared containing either the individual metal ion or organic chemical but with no added DMSP. Assays were repeated twice (n = 2) and data are presented as the mean ± SD. Differences were determined by calculating p-values using a one-tailed *t*-test (Microsoft Excel 2010).

Thermodynamics of DMSP lyase

The kinetics of thermal denaturation of DMSP lyase in extracts from both algal species were studied by incubating the enzyme solution at different temperatures (20 to 35°C in 5°C increments) for 60 min. Samples were removed at 15 min intervals, rapidly cooled in ice for 10 min and assayed for DMSP lyase activity using standard assay conditions (DMSP lyase assay section). The value of the first-order denaturation constant (K_d) was determined at each temperature by measuring the slope of the plot of ln (A/A_0) versus incubation time (t), where A_0 = initial enzyme specific activity, and A = measured enzyme specific activity after heat treatment. The half-life of the enzyme ($t_{1/2}$, min) was calculated using Eq. (1), assuming first-order kinetic decay:

$$t_{1/2} = \ln 2 / K_d \quad (1)$$

The activation energy of denaturation (E_d) was estimated from the slope ($-E_d/R$) of an Arrhenius plot of ln(K_d) versus the reciprocal of absolute temperature [$1/T$ (K^{-1})], where R (gas constant) = 8.314 J mol^{-1} K^{-1}. Values for changes in free energy (ΔG_d), enthalpy (ΔH_d) and entropy (ΔS_d) of denaturation were determined using Eqs. (2), (3) and (4), respectively:

$$\Delta G_d = -RT \ln[(K_d h) / (K_b T)] \quad (2)$$

where h (Planck constant) = 6.626×10^{-34} J s, and K_b (Boltzmann constant) = 1.381×10^{-23} J K^{-1}.

$$\Delta H_d = E_d - RT \quad (3)$$

$$\Delta S_d = (\Delta H_d - \Delta G_d)/T \quad (4)$$

All kinetics measurements were performed twice and the mean values are reported. In all cases, SDs of the mean values were <15%.

RESULTS AND DISCUSSION

The *Phaeocystis* cultures we used contained bacteria, but at relatively low abundances; 2.36 ± 0.19 × 10^5 cells ml^{-1} and 2.45 ± 0.22 × 10^4 cells ml^{-1} in the <1.2 µm size fractions (GF/C filtrate) of the *P. globosa and P. antarctica* cultures, respectively. The DMSP lyase activities were compared between the particles retained on GF/C filters (>1.2 µm; phytoplankton fraction) and those passing through the GF/C and subsequently retained on a 0.22 µm filter (bacterial fraction). For the *P. globosa* culture, the activity in the phytoplankton fraction was 17.3 ± 0.94 nmol min^{-1} ml^{-1} whereas it was 0.33 ± 0.02 nmol min^{-1} ml^{-1} in the bacterial fraction. For the *P. antarctica* culture, the values were 19.7 ± 2.06 and 0.29 ± 0.014 nmol min^{-1} ml^{-1} in the phytoplankton fraction and the bacterial fraction, respectively. Thus, >98% of the DMSP lyase activity was associated with the phytoplankton cells. This finding is consistent with previous studies that have shown that the majority of DMSP lyase activity is associated with phytoplankton size classes in seawater and phytoplankton cultures (Steinke et al. 2002, Yost & Mitchelmore 2009, Harada & Kiene 2012). While some bacteria may have been attached to the *Phaeocystis* cells, our preliminary studies on the de novo peptide sequencing of the partially purified *Phaeocystis* DMSP lyases indicated lack of homology with the 6 known proteins of bacterial DMSP lyases (B. R. Mohapatra et al. unpubl. data). Thus, the extracted lyase activities that we report on below are likely to reflect those of the *Phaeocystis* enzymes.

The effect of pH on the DMSP lyase activity in extracts from *Phaeocystis globosa* and *P. antarctica* indicated that the enzymes of both algal species retained >70% of the maximum enzyme activity over a broad pH range (4.0 to 7.5) (Fig. 1). The optimum pH of the lyase activity was clearly acidic and nearly the same for both species, with an optimum rate at pH 4 for *P. globosa* (11.7 ± 1.66 U) and pH 5 for *P. antarctica* (19 ± 2.7 U) at 30°C and 20°C, respectively. The optimum pHs observed here are much lower than the pH 10.5 optimum reported for *Phaeocystis* sp. strain K (likely *P. globosa*) by Stefels & Dijkhuizen (1996), but are within the range of that previously reported for *in vitro* DMSP lyase activity in other prymnesiophyte extracts, including 3 different strains of *Gephyrocapsa oceanica* (pH 5) (Franklin et al. 2010) and 4 strains of *Emiliania huxleyi* (pH 4 to 6) (Steinke et al. 1998, 2000). The lyases from *P. globosa* and *P. antarctica* also had lower pH optima than found in the green macroalga *Ulva curvata* (pH 8) (de Souza et al. 1996), or in the bacteria *Roseovarius nubinhibens* (pH 6) (Kirkwood et al. 2010) and *Alcaligenes* sp. strain M3A (pH 8) (de Souza & Yoch 1995a). For the *Phaeocystis* strains studied here, DMSP lyase activity declined substantially at assay pHs >7.5, and at pH 9 the activity was diminished by >80% from the optimum.

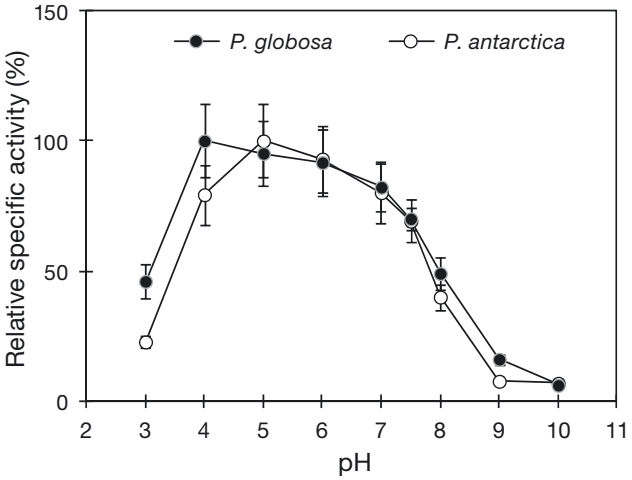

Fig. 1. *Phaeocystis globosa* and *P. antarctica*. Effect of pH on DMSP lyase activity (mean ± SD, n = 2). Relative specific activity of 100% = 11.7 ± 1.66 and 18.9 ± 2.7 nmol DMS min^{-1} mg $protein^{-1}$ for *P. globosa* and *P. antarctica*, respectively

Results of the pH stability study, in which the DMSP lyase enzyme was exposed to a test pH for 15 min, then returned to its optimum pH, revealed that the enzymes of both *Phaeocystis globosa* and *P. antarctica* were relatively stable in the pH range between 4 and 7.5, returning to >70% of maximum activity after exposure to a sub-optimal pH. The stability test also showed that lyase activity was lost rapidly at pH 3 (>60% loss) and above pH 8 (>80% loss), and the loss in activity at these or other pHs was not recovered when the assay solution pH was returned to the optimal pH (Fig. 2).

Collectively, the results of the pH tests suggest that the DMSP lyase enzymes in *Phaeocystis globosa* and *P. antarctica* function optimally at the low pH characteristic of some intracellular environments (e.g. secretory vesicles, chloroplast lumen) rather than the cell surface environment exposed to seawater (pH ~8). Relatively little is known about the cellular location of DMSP lyases in algae or their physiological functions. Recently it was found that acidification of seawater or culture samples containing colonial *Phaeocystis* sp. to pH ~1 caused rapid losses of intracellular DMSP through conversion to DMS (del Valle et al. 2011). The authors concluded that DMSP lyases remained

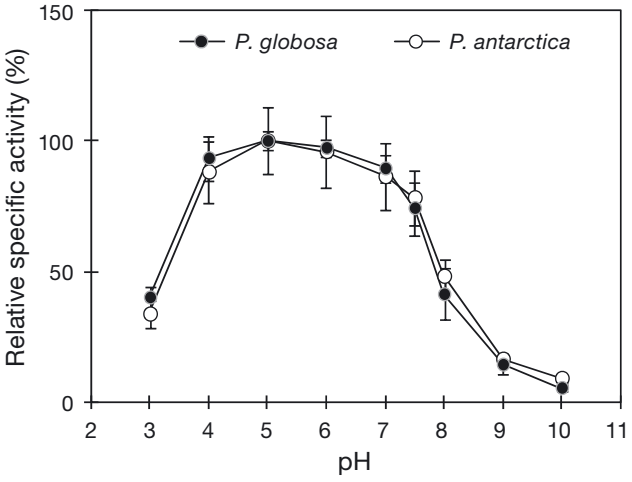

Fig. 2. *Phaeocystis globosa* and *P. antarctica*. pH stability study of DMSP lyase (mean ± SD, n = 2). pH stability tests were carried out by pre-incubation of the DMSP lyase solution at 20 (*P. antarctica*) or 25°C (*P. globosa*) in buffers at different pHs ranging from 3 to 10 for 15 min. The reaction mixture was then returned to pH 4 (*P. globosa*) and 5 (*P. antarctica*), and the DMSP lyase activity was assayed. Relative specific activity of 100% = 11.3 ± 0.42 and 18.2 ± 2.34 nmol DMS min^{-1} mg $protein^{-1}$ for *P. globosa* and *P. antarctica*, respectively

active for several minutes following acidification before being inactivated by the low pH (~1). The high tolerance towards acidity of the DMSP lyase in *P. globosa* and *P. antarctica* that we observed here (25 to 50% of maximum activity at pH 3) is consistent with the findings of del Valle et al. (2011). Recently, Orellana et al. (2011) speculated that DMSP lyases could be present in acidic secretory vesicles of *P. antarctica*, where both DMSP and DMS are associated with condensed polysaccharides that ultimately expand to form mucous polymers upon release to alkaline seawater. Another potential sub-cellular location with low pH is the chloroplast lumen. DMSP lyase has been proposed to be part of an antioxidant system in phytoplankton (Sunda et al. 2002) where cleavage of DMSP near the site of reactive oxygen species production (e.g. chloroplasts) could be beneficial to cells. It will be interesting in future work to test whether DMSP lyases are specifically adapted to function in such acidic sub-cellular locations.

DMSP lyase activity in extracts of *Phaeocystis globosa* and *P. antarctica* showed sharp and distinct temperature optima with maximum enzyme activities at 30 and 20°C, respectively (Fig. 3a). At 40°C, the DMSP lyase of *P. globosa* and *P. antarctica* exhibited only 38 and 18% of maximum enzyme activity. Nearly complete inactivation of the enzyme activity was observed above 45°C. The activation energy of catalysis (E_a) of DMSP lyases of *P. globosa* and *P. antarctica*, calculated from an Arrhenius plot, were 70.1 and 71.1 kJ mol^{-1}, respectively (Fig. 3b). Previous temperature dependence studies on the DMSP lyases of different algal and bacterial species documented variation in temperature optima. The temperature optimum was reported as 25, 27 and 35°C for the algal species *Enteromorpha clatharata* (Steinke & Kirst 1996), *Cryptheocodinium cohnii* (Kadota & Ishida 1968) and *Emiliania huxleyi* CCMP379 (Steinke et al. 1998), respectively, and 60°C for the bacterium *Roseovarius*

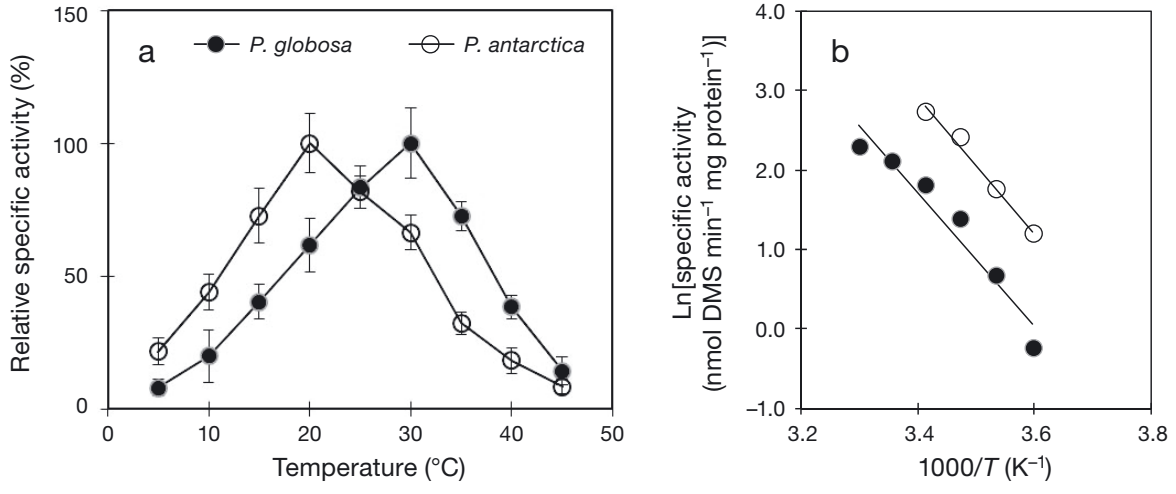

Fig. 3. *Phaeocystis globosa* and *P. antarctica*. (a) Temperature dependence of DMSP lyase activity (mean ± SD, n = 2). Relative specific activity of 100% = 9.86 ± 1.31 and 15.3 ± 2.21 nmol DMS min^{-1} mg $protein^{-1}$ for *P. globosa* and *P. antarctica*, respectively. (b) Arrhenius plot for determination of the activation energy of catalysis of DMSP lyase. Lines: linear regression of mean value of duplicate measurements at different temperatures (T). Linear regression analyses equations: $y = -8.42x + 30.36$, $r^2 = 0.94$ for *P. globosa*; and $y = -8.56x + 32$, $r^2 = 0.98$ for *P. antarctica*

nubinhibens (Kirkwood et al. 2010). The different temperature dependence of various bacterial and algal species might be attributed to differences in the phenotypic and/or genotypic traits of the organisms which are likely influenced by environmental conditions in their growth habitat. In this regard, it is not surprising that the DMSP lyase from the polar strain *P. antarctica* had a lower optimum temperature and higher activity at low temperature (3.2 ± 0.4 U at 5°C) than did the temperate strain *P. globosa* (0.8 ± 0.1 U at 5°C). The observed shift to a lower temperature optimum, but with higher specific lyase activity at the lowest temperature in *P. antarctica* (natural habitat <5°C), is typical of enzyme adaptation in psychrophiles (Struvay & Feller 2012). Thermal stability of the isolated DMSP lyase from both *P. globosa* and *P. antarctica* was also investigated by pre-incubation of the extracted enzyme preparations at 11 different temperatures ranging from 5 to 45°C in steps of 5°C, and measuring the residual activity at the optimum temperatures of 30°C for *P. globosa* and 20°C for *P. antarctica* (Fig. 4). This test revealed that the DMSP lyase of *P. globosa* was more thermostable than that of *P. antarctica*. At 30°C, the *P. globosa* lyase retained 89 ± 13% of its enzyme activity; however, only 57 ± 9% of the *P. antarctica* DMSP lyase activity was retained at the same temperature.

The thermal denaturation process of DMSP lyase of both *Phaeocystis globosa* and *P. antarctica* followed first-order kinetics. The enzyme of *P. globosa* exhibited a $t_{1/2}$ of 210 and 30.1 min at 25 and 35°C, respectively (Table 1). In contrast, the DMSP lyase of *P. antarctica* had a $t_{1/2}$ of 61.9 and 14.6 min, respectively, at the same temperatures. The E_d of DMSP lyase was estimated from an Arrhenius plot as 135 kJ mol^{-1} for *P. globosa* and 106 kJ mol^{-1} for *P. antarctica*. These $t_{1/2}$ and E_d values suggested that the DMSP lyase of *P. globosa* was more thermostable than the *P. antarctica* lyase, again not surprising considering the polar habitat of *P. antarctica*. The E_d values were higher than the DMSP lyase E_a, indicating the requirement of higher energy to initiate the thermal denaturation compared to catalysis.

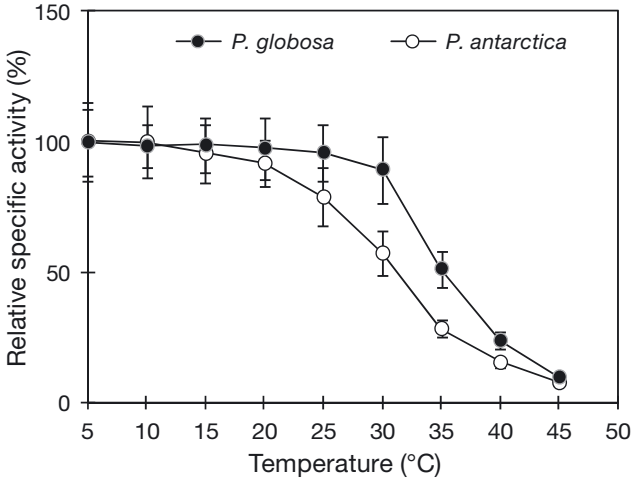

Fig. 4. *Phaeocystis globosa* and *P. antarctica*. Thermostability of DMSP lyase (mean ± SD, n = 2). Relative specific activity of 100% = 8.59 ± 1.39 and 14.2 ± 2.11 nmol DMS min^{-1} mg protein^{-1} for *P. globosa* and *P. antarctica*, respectively

In order to gain more knowledge on the mechanism of thermal denaturation of the DMSP lyase, the thermodynamic parameters such as ΔG_d, ΔH_d and ΔS_d of denaturation were determined (Table 1). The ΔG_d and ΔH_d values of thermal denaturation of DMSP lyase of both algal species declined with increases in temperature, suggesting a considerable change in enzyme conformation during the process of denaturation and a requirement of lesser energy to denature the enzyme at higher temperatures. Lower values of ΔG_d and ΔH_d for *Phaeocystis antarctica* lyase compared to *P. globosa* lyase indicated that the former was more thermolabile. Moreover, the positive ΔH_d and ΔS_d values obtained for both lyases provided supportive evidence that thermal denaturation of the enzymes is initiated by disruption of non-covalent linkages, which increases the disorder or entropy during denaturation (reviewed in Nosoh & Sekiguchi 1990, Vieille & Zeikus 1996). To our knowledge, the present study is the first to estimate the thermodynamic properties of DMSP lyase, and therefore it is not possible to compare our values with other studies.

We used Eadie-Hofstee plots to determine K_m and V_{max} for DMSP lyase extracted from the 2 different *Phaeocystis* species (data not shown). Under the opti-

Table 1. *Phaeocystis globosa* and *P. antarctica*. Half-life ($t_{½}$) and thermodynamic parameters for denaturation of DMSP lyase at different temperatures. ΔG_d: free energy; ΔH_d: enthalpy; ΔS_d: entropy of denaturation

Species Temp (°C)	$t_{½}$ (min)	ΔG_d (kJ mol^{-1})	ΔH_d (kJ mol^{-1})	ΔS_d (J K^{-1} mol^{-1})
P. globosa				
20	462	87.56	132.80	154.43
25	210	87.14	132.76	153.10
30	91.2	86.54	132.72	152.41
35	30.1	85.17	132.68	154.23
P. antarctica				
20	116	84.17	103.15	64.75
25	61.9	84.11	103.11	63.75
30	26.9	83.47	103.07	64.67
35	14.6	83.31	103.03	63.99

mal assay conditions with DMSP as the substrate, the lyase of *P. globosa* (pH 4 and 30°C) and *P. antarctica* (pH 5 and 20°C) followed Michaelis-Menten saturation kinetics with K_m values of 1.77 and 2.31 mM and V_{max} values of 17.3 and 28.2 U, for *P. globosa* and *P. antarctica*, respectively. K_m values ranging from 0.072 to 14 mM were reported for the *in vitro* DMSP lyase from several algae including *Phaeocystis* strain K (2.25 mM) (Stefels & Dijkhuizen 1996), *Polysiphonia paniculata* (0.072 mM) (Nishiguchi & Goff 1995) and *Ulva curvata* (0.52 mM) (de Souza et al. 1996), and bacteria including *Alcaligenes* sp. (1.4 mM) (de Souza & Yoch 1995a) and *Roseovarius nubinhibens* (13.8 ± 5.5 mM) (Kirkwood et al. 2010). Intracellular DMSP concentrations in *Phaeocystis* sp. are typically in the 100 to 300 mM range (Stefels & van Leeuwe 1998). These concentrations are well above the K_m values that we determined, suggesting that lyase activity in *Phaeocystis* sp. could operate close to saturation *in vivo* if the enzyme is exposed to those high intracellular concentrations. But evidence from several studies suggests that *in vivo* lyase activity operates well below maximal potential activity in healthy phytoplankton cells (Wolfe & Steinke 1996, Wolfe et al. 2002, Harada et al. 2004), indicating that lyase activity must be regulated within the cells. Unfortunately, the mechanism of DMSP lyase regulation remains largely unknown, although factors such as grazing-induced cell disruptions, high light exposure and oxidative stress have been suggested to up-regulate lyase activity (Wolfe & Steinke 1996, Sunda et al. 2002, Galí et al. 2011).

The presence of NaCl affected DMSP lyase activity similarly for both *Phaeocystis globosa* and *P. antarctica* (Fig. 5). Optimal catalytic activity was found at 0.34 and 0.43 M NaCl for *P. globosa* and *P. antarctica* DMSP lyases, respectively (Fig. 5). For comparison, seawater with a salinity of 35 ppt has a NaCl concentration of ~0.5 M. Above 0.6 M the lyase activity sharply declined and the enzyme lost >50% of the activity. The optimal NaCl concentrations for DMSP lyase *in vitro* activity in the 2 *Phaeocystis* species was similar to that of several different strains of the prymnesiophyte *Emiliania huxleyi* (0.5 to 1.0 M NaCl for CCMP 370, 379, 1516; Steinke et al. 1998), but much higher than found for the macroalgae *Enteromorpha clatharata* (0.075 M NaCl; Steinke & Kirst 1996) and *Ulva curvata* (0.075 M NaCl; de Souza et al. 1996).

The relative catalytic efficacy of DMSP lyase isolated from *Phaeocystis globosa* and *P. antarctica* was assessed against different DMSP analogs (Table 2). For both organisms, the DMSP lyase activity was not significantly inhibited by 20 mM 2-methyl-DMSP (80.9 ± 8.9 and 89.1 ± 10.1% of the control, with p = 0.114 and 0.210, for *P. globosa* and *P. antarctica*, respectively), but was partially inhibited by 2-chloro-DMSP (57.9 ± 8 and 78.2 ± 7.1% of the control, with p = 0.003 and 0.013, for *P. globosa* and *P. antarctica*, respectively). Choline-*o*-sulfate was strongly inhibitory to lyase activity in both species (5.3 to 7.09% of controls, p < 0.029). Dimethylsulfonioacetate, one carbon shorter than DMSP, had no significant effect on lyase activity. Glycine betaine, a compound known to inhibit transmembrane transport of DMSP in marine bacteria (Kiene 1998), did not inhibit DMSP lyase

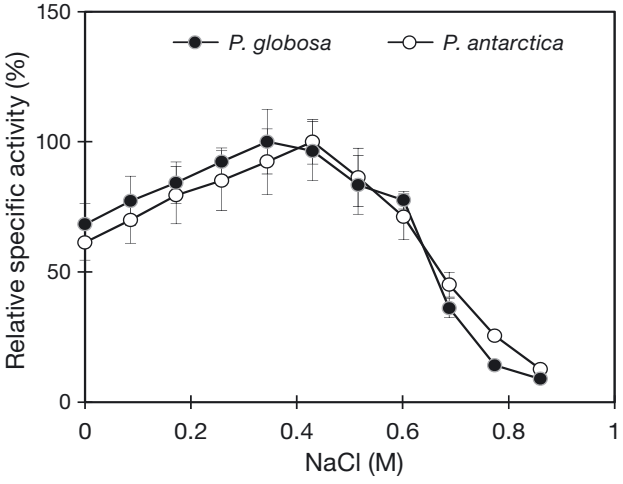

Fig. 5. *Phaeocystis globosa* and *P. antarctica*. Effect of NaCl concentration on DMSP lyase activity (mean ± SD, n = 2). Relative specific activity of 100% = 11.9 ± 1.48 and 22.6 ± 1.93 nmol DMS min^{-1} mg $protein^{-1}$ for *P. globosa* and *P. antarctica*, respectively

Table 2. *Phaeocystis globosa* and *P. antarctica*. Effect of various DMSP analogs on DMSP lyase activity expressed as a percentage of the activity retained when DMSP was added to the assay mixtures lacking the DMSP analog. DMSP analogs (20 mM) were pre-incubated in the DMSP lyase assay mixture from *P. globosa* or *P. antarctica* for 10 min at 25 and 20°C, respectively. The residual enzyme activity was subsequently assayed with 5 mM DMSP as the substrate

DMSP analog	*P. globosa* (%)	p	*P. antarctica* (%)	p
2-methyl-DMSP	80.9 ± 8.9	0.114	89.2 ± 10.1	0.210
2-chloro-DMSP	57.9 ± 8	0.035	78.2 ± 7.1	0.086
Dimethylsulfonioacetate	91.7 ± 12.4	0.284	93.5 ± 13.2	0.326
Choline-*o*-sulfate	45.3 ± 6.6	0.026	51.2 ± 6	0.029
Glycine betaine	95.5 ± 10.7	0.366	94.3 ± 4	0.305

activity appreciably, a result consistent with previous findings in bacteria (de Souza & Yoch 1995b). Bacic & Yoch (1998) observed induction of *in vivo* DMSP lyase activity in the fungus *Fusarium lateritium* by choline, glycine betaine and dimethylglycine, but no induction by dimethylselenoniumpropionate (DMSeP). We did not test specifically for induction of DMSP lyase activity in the *Phaeocystis* species, but we observed lyase activity in all *P. globosa* and *P. antarctica* cultures tested suggesting it is present at relatively high levels under the growth conditions we used.

The effect of various metal ions and organic chemicals on the extracted DMSP lyases were studied for both algal species (Table 3). DMSP lyase activities from both *Phaeocystis* species were not affected by the major divalent cations present in seawater, Ca^{2+} and Mg^{2+}; however, the activities were strongly inhibited by Cu^{2+}, Mn^{2+} and Ni^{2+}. The metal chelating agent, EDTA, also inhibited lyase activity, suggesting that the extracted DMSP lyases of both the algal species were metalloenzymes. Additionally, inhibition of the lyase activity by PMSF and pCMB points towards the participation of both serine and sulfhydryl (–SH) groups, respectively, at the active site of the enzyme. The thiol binding reagent pCMB has been used previously to inhibit DMSP lyase activity in *Phaeocystis* sp. (Stefels & Dijkhuizen 1996, del Valle et al. 2011). The non-inhibition of enzyme activity by DTT provided additional evidence of the involvement of an –SH group at the active site as suggested by Stefels & Dijkhuizen (1996). Metal ion and inhibitor studies have also been performed for DMSP lyases isolated from other algal and bacterial species. The proteins of bacterial DMSP lyase, DddL, DddQ and DddW contain metal binding cupin pockets (Curson et al. 2008, Todd et al. 2011, 2012). However, the DddP protein of the bacterium *Roseovarius nubinhibens* was not affected by EDTA, indicating no requirement of a metal cofactor for DMSP lyase activity (Kirkwood et al. 2010). It was reported that the *in vitro* DMSP lyase activity in the alga *Polysiphonia paniculata* (Nishiguchi & Goff 1995) was partially inhibited by EDTA, PMSF and pCMB, and was not affected by Ca^{2+} and Mg^{2+}, similar to what we found with the 2 *Phaeocystis* species.

In conclusion, the DMSP lyases extracted from mesophilic *Phaeocystis globosa* and psychrophilic *P. antarctica* showed very similar functional characteristics. The main differences were lower pH optimum in *P. globosa* (pH 4) compared to *P. antarctica* (pH 5) and higher temperature optimum (and thermal stability) in *P. globosa* compared to *P. antarctica.* Further structural and genetic characterization will be required to determine the homology of the DMSP lyase of these 2 algal species. The DMSP lyases from the 2 *Phaeocystis* species tested function optimally at acidic pH and this is similar to what has been shown previously for several other prymnesiophytes. This raises the possibility that DMSP lyase in these organisms is adapted to function in a locally-acidic environment, but further work will be needed to determine the *in vivo* location and physiological function of DMSP lyase in these organisms. Our study has added important new information about the functional characteristics of DMSP lyase in *Phaeocystis* species that are globally important contributors to DMSP and DMS production in temperate and polar oceans. Future studies of DMSP lyase will benefit from purification of the enzyme and identification of gene(s) encoding these proteins.

Table 3. *Phaeocystis globosa* and *P. antarctica*. Effect of different metal ions and organic chemicals on DMSP lyase activity expressed as % enzyme activity retained. Individual metal ions or organic compounds, at either 0.1 or 1.0 mM, were pre-incubated with the DMSP lyase assay mixture from *P. globosa* or *P. antarctica* for 10 min at 25 and 20°C, respectively. The residual enzyme activity was subsequently assayed with 5 mM DMSP as substrate. DTT: dithiothreitol; pCMB: para-chloromercuribenzoate; PMSF: phenylmethanesulfonylflouride

Metal ions and chemicals	*P. globosa*				*P. antarctica*			
	0.1 mM (%)	p	1 mM (%)	p	0.1 mM (%)	p	1 mM (%)	p
Ca^{2+}	98.2 ± 12.4	0.445	108 ± 11	0.253	104 ± 13	0.373	97.2 ± 11	0.397
Cu^{2+}	56.4 ± 6.8	0.023	9.1 ± 0.9	0.024	78.3 ± 7.7	0.054	27.2 ± 7.7	0.006
Mg^{2+}	100 ± 11	0.500	104 ± 13	0.376	100 ± 12	0.499	95.1 ± 4.1	0.271
Mn^{2+}	42.3 ± 4	0.024	23.2 ± 2.7	0.022	58.2 ± 8.4	0.018	36.1 ± 5.2	0.009
Ni^{2+}	54.1 ± 5.9	0.024	27.1 ± 7.5	0.009	72.2 ± 5.9	0.034	48.2 ± 7.4	0.029
EDTA	13.5 ± 1.1	0.024	10.5 ± 1.3	0.023	22.1 ± 2.8	0.014	16.3 ± 2.1	0.016
DTT	103 ± 10	0.389	97.6 ± 8.5	0.412	110 ± 9	0.176	107 ± 8	0.239
pCMB	18.2 ± 2.4	0.022	9.2 ± 1.4	0.023	26.1 ± 3.6	0.012	14.2 ± 1.2	0.019
PMSF	18.3 ± 2.4	0.023	10.3 ± 1.3	0.023	29.1 ± 3.8	0.012	16.3 ± 1.7	0.018

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