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Methanesulfonate supports growth as the sole sulfur source for the marine diatom *Thalassiosira pseudonana* NCMA 1335

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ABSTRACT: The marine diatom *Thalassiosira pseudonana* NCMA 1335 produces dimethylsulfoniopropionate (DMSP), a compound thought to be the start of an antioxidant system in some marine algae that ends with methanesulfonate ($CH_3SO_3^-$, MS). We demonstrate that *T. pseudonana* is able to use MS as a sole sulfur source, which implies a capacity to recycle sulfur derived from MS for growth. Growth on MS-only cultures was similar to controls (0.25 ± 0.05 d^{-1} versus 0.33 ± 0.04 d^{-1}, respectively) but resulted in lower total biomass (2.4 ± 0.3 × 10^6 cells ml^{-1} versus 5.7 ± 0.5 × 10^6 cells ml^{-1}). Growth on trifluoromethanesulfonate was extremely limited. DMSP content was not significantly affected when grown on MS, but cellular formaldehyde and sulfite contents were significantly higher, suggesting that MS metabolism is similar to that of bacteria and yeast. These results are the first demonstration of *T. pseudonana* being able to grow on a sulfonate as the exclusive sulfur source and hint at a rich sulfur metabolism that has hitherto been unexplored and which may play a role in the biogeochemical cycling of sulfur. These results also underscore the potential for DMSP and its derived compounds to act as an antioxidant system.

KEY WORDS: Nutrient dynamics · Biogeochemistry · Dimethylsulfoniopropionate · Dimethylsulfide

INTRODUCTION

Methanesulfonate (MS) is a critical component of the global sulfur cycle in that it represents the penultimate step in the re-mineralization of sulfur derived from dimethylsulfide (DMS). MS is a major product of atmospheric oxidation of DMS (Barnes et al. 2006), comprising approximately 50% of the total atmospheric DMS loss (Kelly 1996). After removal from the atmosphere via deposition, remineralization of MS has been found to occur by the activity of soil microbes (Baker et al. 1991) as well as other bacteria (Kelly & Baker 1990, Kelly et al. 1993, Kahnert & Kertesz 2000). Despite this global picture, the synthesis and metabolism of sulfonates at the cellular level have not been adequately investigated in eukaryotic organisms, particularly marine phytoplankton, many of which are sources of DMS and oxidized sulfur (Keller et al. 1989, Hatton & Wilson 2007).

Many marine algae produce large quantities of dimethylsulfoniopropionate (DMSP), which is the biochemical precursor to DMS. While the physiological roles of these compounds are uncertain, it has been suggested that DMS, DMSP, and dimethylsulfoxide (DMSO) may serve as an antioxidant system because of the relatively high reactivity of DMS and DMSO towards reactive oxygen species (Sunda et al. 2002). Previous work has focused on DMS and DMSO and has not addressed the ultimate fate of DMSP-derived sulfur. After DMSO is oxidized in

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aqueous solution, it forms methanesulfinic acid (MSI) (Bardouki et al. 2002). MSI in turn, is rapidly oxidized to form MS, which is relatively stable toward oxidation (Bardouki et al. 2002, Barnes et al. 2006). Thus, it is possible that under oxidative stress conditions, MS may accumulate in algal cells if DMSP and its breakdown products are present (Spiese 2010). Several mechanisms to eliminate MS and similar sulfonates exist, but have not been specifically studied in DMSP-producing organisms.

Chlorella vulgaris has been shown to metabolize MS and other alkylsulfonates when sulfur-starved (Biedlingmaier & Schmidt 1983, 1986, Biedlingmaier et al. 1986). *Saccharomyces cerevisiae* is also able to cleave the carbon-sulfur bond in sulfonate (Hogan et al. 1999). For both of these organisms, sulfonate metabolism appears to be related to sulfur limitation, a condition unlikely for marine eukaryotes. Despite this, marine phytoplankton retain some sulfur starvation genes, as significant physiological changes have been observed in the marine prymnesiophyte *Emiliania huxleyi* when sulfur concentrations were reduced (Bochenek et al. 2013). These included reduced DMSP content and increases in sulfur assimilation enzyme transcripts, demonstrating a retained ability to cope with sulfur restriction.

This study aimed to determine whether *Thalassiosira pseudonana* has the capability to utilize MS as a sulfur source, which would lend support to the DMSP antioxidant hypothesis, although not directly confirm such a physiological role. This study is the first to demonstrate utilization of sulfonate-derived sulfur for growth in a marine eukaryote. *T. pseudonana*, a DMSP-producing diatom, was shown to be able to use MS for growth. Trifluoromethanesulfonate (Tf) was able to support extremely limited growth when supplied as the sole sulfur source. The presence of an MS metabolic pathway may close an antioxidant cycle within the cell that begins with DMSP (Sunda et al. 2002). These findings also indicate that marine algae may play a role in the remineralization of organic sulfur in the environment.

MATERIALS AND METHODS

Abbreviations

To clarify the range of compounds described herein, compounds are reported with the appropriate chemical formula and are abbreviated as follows: methanesulfonate, $CH_3SO_3^-$ (MS); trifluoromethanesulfonate, $CF_3SO_3^-$ (Tf), dimethylsulfoniopropionate, $(CH_3)_2S^+CH_2CH_2CO_2^-$ (DMSP); dimethylsulfide, CH_3SCH_3 (DMS); dimethylsulfoxide, $CH_3S(O)CH_3$ (DMSO); carbonyl fluoride, CF_2O (no abbreviation); formaldehyde, CH_2O (no abbreviation); trifluoromethanol, CF_3OH (no abbreviation); methanol, CH_3OH (no abbreviation).

Chemicals

Sodium methanesulfonate (98%) and sodium trifluoromethanesulfonate (98%) were purchased from Alfa Aesar. 2,4-dinitrophenylhydrazine (DNPH) was obtained from Sigma Aldrich and was recrystallized from 70% acetonitrile before use (Nuccio et al. 1995). All other compounds were purchased from commercial sources and used without further purification. All solutions were prepared in Milli-Q water (18.2 MΩ·cm).

Algal culturing

Axenic cultures of *Thalassiosira pseudonana* NCMA 1335 were obtained from the National Center for Marine Algae and Microbiota (Boothbay Harbor, ME, USA) and maintained in artificial seawater (Parsons 1984) amended with standard f/2 nutrients. To control the sulfur source, sulfate was excluded from the artificial seawater and trace metals solution. Cultures were manipulated under sterile conditions and were tested for bacterial contamination using fluorescein diacetate staining (Agustí & Sánchez 2002). Triplicate batch cultures (10 to 30 ml) were grown in 25 cm^2 canted neck vented (0.2 µm membrane) culture flasks at 21 ± 1°C under 65 µmol photons m^{-2} s^{-1} lighting on a 14 h light:10 h dark cycle. Relative culture density was measured by absorbance at 680 nm on a BioTek Synergy H1 microplate spectrophotometer in a 96-well microplate (200 µl in each well) (Spiese & Tatarkov 2014). Absolute culture density and cell volume were determined using a Beckman Coulter Z2 Counter.

For each treatment group of triplicate flasks, sulfur was added as either Na_2SO_4 (control), $NaCH_3SO_3$ (MS), or $NaCF_3SO_3$ (Tf). Tf was added to examine the role of chemical structure on metabolism and to differentiate between potential pathways. A negative control was made by addition of NaCl. All solutions were from autoclaved 0.25 M stocks (1 ml per 10 ml of culture medium) to a final concentration in each case of 25 mM. Sulfate was assayed by addition of saturated (3.4 M) $SrCl_2$ ($K_{sp} = 3.27 \times 10^{-7}$) and obser-

vation of visible precipitation. In cultures other than positive controls, sulfate was present at ≤0.2 µM. To assess whether cultures grown on MS were experiencing sulfur limitation, 9 parallel batch cultures grown on MS were cultured for 14 d (stationary phase). Three cultures each were amended with either sulfate or glucose (25 mM final concentration for both) or left unamended. Glucose was added to examine the potential for energy limitation due to sulfite inhibition of photosynthesis (*vide infra*). Cultures were then incubated an additional 7 d, and the absorbance at 680 nm was measured. Sulfate-replete cultures were also determined over the same time frame. Glucose was chosen to determine whether produced sulfite was inhibiting photosynthesis or respiration (Sheridan 1978).

Chemical analysis

Total DMSP was determined as DMS released after addition of 100 µl 2 M NaOH to 1 ml of sample in a 14 ml vial sealed with a PTFE-faced butyl rubber septum to duplicate aliquots from each culture flask. Samples were allowed to react at room temperature in the dark for >24 h. Samples were analyzed by headspace gas chromatography (500 µl fixed volume). A Shimadzu GC2010 Plus equipped with a flame photometric detector with sulfur-selective filters was used for separation and detection. The column used was a 30 m × 0.32 mm × 5.0 µm Rtx-1 (Restek) with He carrier gas (67.5 cm s^{-1} flow at 60.0°C). Standards were prepared identically to samples from authentic DMSP, synthesized according to the method of Chambers et al. (1987).

Total formaldehyde (particulate + dissolved) was quantified by HPLC. Raw culture (2 ml) was collected from triplicate flasks and stored at −80°C until analysis. Samples were treated with 0.1 ml of DNPH (1 mg ml^{-1}) and allowed to react for 1 h in the dark. DNPH was previously recrystallized from 70% aqueous aceto-nitrile, prepared according to the method of Nuccio et al. (1995). Separation was performed using an Agilent 1100 series HPLC with a Zorbax ODS column (4.6 mm × 15 cm, 5 µm particle diameter) and diode array detection (370 nm). Analytes were eluted under the following conditions: 0 to 3 min 55% methanol; 3 to 13 min linear increase to 90% methanol; 13 to 20 min isocratic 90% methanol.

Particulate sulfite was determined according to the method of Vairavamurthy & Mopper (1990), with modifications. Triplicate cultures were filtered using GF/F, and the filters were submerged in 1 ml of deionized water. Samples were then reacted with 50 µl of 2 mmol l^{-1} 2,2′-dithiobis(nitrobenzoic acid) (DTNB) in acetonitrile for >5 min and stored at −20°C until analysis. DTNB derivatives were separated by HPLC using a 15 cm × 4.5 mm Zorbax C_{18} column (5 µm particle diameter) with a mobile phase comprised of (A) 7.5 mM tetrabutylammonium hydrogen sulfate in 0.05 M sodium acetate, pH 3.05 and (B) acetonitrile. The gradient elution was 0 to 1 min isocratic 25% B, 1 to 11 min linear increase to 55% B, then return to initial composition from 11 to 13 min with a 5 min isocratic delay before the next sample. Detection was by absorbance at 320 nm.

Toxicity

To assay the toxicity of formaldehyde or sulfite produced by algae from MS, cultures grown using sulfate were dosed extracellularly with varying amounts of either formaldehyde (0 to 100 µM) or sodium sulfite (0 to 1 mM) on the day of inoculation and allowed to grow for 96 h under previously described conditions. Culture density was measured using absorbance at 680 nm (A), normalized to the density in the control cultures (A_0). The concentration inhibiting growth by 50% (IC_{50}) determined according to:

$$\frac{A}{A_0} = \frac{a}{1+\left(\frac{[\text{compound}]}{IC_{50}}\right)^b} \quad (1)$$

where a and b are empirical constants, and [compound] refers to the applied concentration of either formaldehyde or sulfite ion. Nonlinear fitting to Eq. (1) was performed using SigmaPlot 12.

Bioinformatics

Amino acid sequences of known MS metabolizing enzymes were subjected to HMMER analysis (Finn et al. 2011) against the reference proteome of *T. pseudonana* (*taxid:35128* and *taxid:296543*) (Armbrust et al. 2004) to identify putative MS protein homologs in the *T. pseudonana* proteome. The comparison sequences utilized for this analysis were as follows: *Escherichia coli* SsuD, *E. coli* TauD, *Methylsulfonamonas methylovara* MsmABCDEFGH and *Psedomonas aeruginosa* MsuABCD. All MS-metabolizing enzyme sequences were obtained from published sources or from the UniProt database (www.uniprot.org). Putative *T. pseudonana* MS-metabolizing proteins identified by HMMER were

Table 1. Growth rate, biomass and cell size for *Thalassiosira pseudonana* utilizing different sulfur sources. Growth rates were determined from the linear fit of $\log_2 A_{680}$ versus time for the first 6 d post innoculation in each series and are expressed with their standard error. Culture density and cell diameters were measured in triplicate and are expressed with their standard deviations. nd: not determined; *significantly different from control ($p < 0.00001$); **significantly different from both control and negative control ($p < 0.00001$). MS: methanesulfonate; Tf: trifluoromethanesulfonate

	Specific growth rate (d^{-1})	Mean cell diameter (µm)	Culture density (10^6 cells ml^{-1})
Positive control	0.33 ± 0.04	4.7 ± 0.4	5.7 ± 0.5
MS	0.27 ± 0.05	5.1 ± 0.6	2.4 ± 0.3*
Tf	0.05 ± 0.01**	nd	nd
Negative control	0.010 ± 0.003*	nd	nd

subjected to standard BLASTp analysis to ascertain whether homologues exist in other DMSP-producing phytoplankton such as *Emiliania huxleyi* and *Thalassiosira oceanica*. Sequences for phytoplankton were obtained from Joint Genome Institute (www.jgi.org).

RESULTS

Growth on sulfonates

Algal cultures of *Thalassiosira pseudonana* were grown using 2 sulfonates as the sole sulfur source to determine whether the alga was able to acquire and metabolize the sulfur. Cultures grew well when either sulfate (positive control) or methanesulfonate (MS) was present as the sole sulfur source (0.33 ± 0.04 d^{-1} and 0.27 ± 0.05 d^{-1}, respectively, $\log_2$ growth rates during exponential phase; Table 1). Growth on trifluoromethanesulfonate (Tf) was low and significantly less than control (0.05 ± 0.01 d^{-1}, $p < 0.00001$ versus control). Negative controls (no added S) showed essentially no growth (0.010 ± 0.003 d^{-1}, $p < 0.00001$ versus control). Negative controls and Tf cultures were significantly different from each other ($p < 0.0001$), indicating that Tf was able to support some growth, albeit extremely inhibited.

Despite the similar growth rates in sulfate and MS cultures, the absolute cell density at Day 8 was significantly lower in MS cultures (2.4 ± 0.3 × 10^6 cells ml^{-1}, $p < 0.00001$; Table 1) versus positive controls (5.7 ± 0.5 × 10^6 cells ml^{-1}). This is due to a longer exponential growth phase in Control cultures versus that for MS cultures, leading to overall lower cell density. No significant difference was observed between positive controls and MS cultures with respect to cell size (Table 1).

MS metabolites

The major products of MS metabolism are expected to be formaldehyde and sulfite, similar to yeast and bacteria (Kelly et al. 1994, Kelly & Murrell 1999). To confirm this, both particulate sulfite and total formaldehyde concentrations were measured in control and MS cultures. For formaldehyde, aliquots of culture were collected and frozen at –80°C 3 d after the onset of stationary phase (Day 11 for MS, Day 15 for control) to ensure that the cultures were in the same phase of growth. Control cultures showed low total (dissolved + particulate) formaldehyde concentrations (158 ± 7 nM; 0.09 ± 0.03 fmol $cell^{-1}$) (Fig. 1), which is approximately the same as observed in axenic cultures of *Skeletonema costatum* and *Heterocapsa pygmaea* (Nuccio et al. 1995). In cultures grown on MS as a sole sulfur source, however, the total formaldehyde concentration was significantly higher (279 ± 7 nM; 0.22 ± 0.02 fmol $cell^{-1}$; $p < 0.0001$). For Tf cultures, if oxidation of $CF_3SO_3^-$ is analogous to bacterial metabolism of $CH_3SO_3^-$, carbonyl fluoride (CF_2O) would be produced, which rapidly hydrolyzes in water to form CO_2 and HF (2 equiv.). However, oxidation at that carbon is unlikely because no hydrogens can be abstracted for oxygen reduction. Rather, production of trifluoromethanol (CF_3OH) and sulfite by direct substitution is a more likely scenario (*vide infra*). CF_3OH can also hydro-

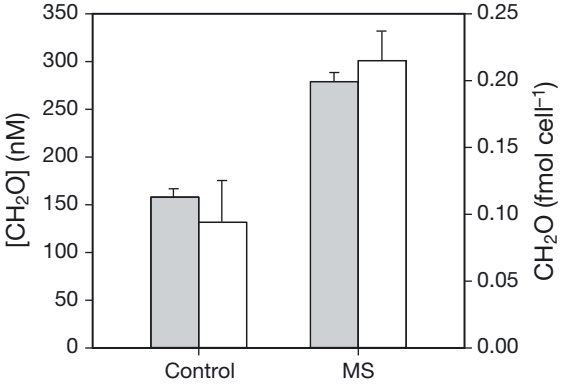

Fig. 1. Total formaldehyde production by *Thalassiosira pseudonana* cultures using either sulfate (control) or methanesulfonate (MS) as sole sulfur source. Gray bars indicate total CH_2O concentration (left axis), and open bars indicate cell-normalized CH_2O (right axis). Both data sets represent the mean of triplicate measurements, and error bars represent 1 standard deviation

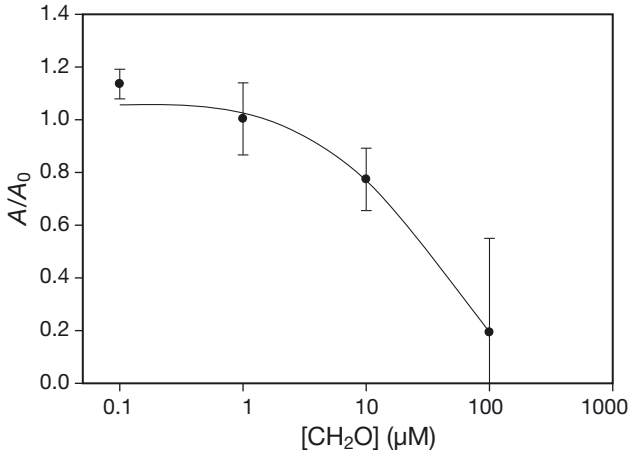

Fig. 2. Formaldehyde toxicity in *Thalassiosira pseudonana*. Absorbance was determined after 96 h exposure to formaldehyde, normalized to the control value. The line denotes the best fit to Eq. (1), with $a = 1.06 \pm 0.05$, $b = 1.1 \pm 0.2$, and $IC_{50} = 25 \pm 7$ µM ($p = 0.018$, $F_{2,2} = 53.48$). Points denote the mean of 6 replicates, and error bars represent 1 standard error

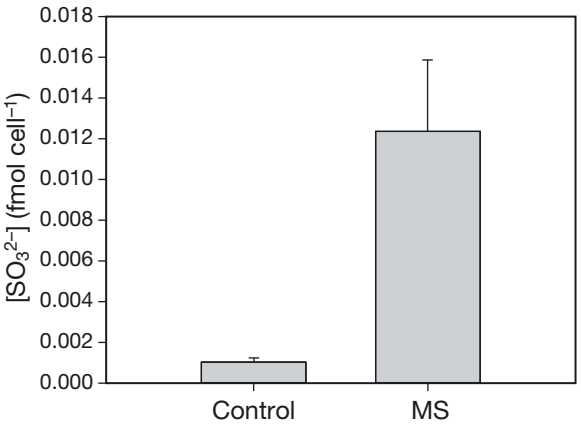

Fig. 3. Cellular sulfite concentrations in *Thalassiosira pseudonana* cultures grown under sulfate-replete conditions (Control) or on MS as sole sulfur source (MS). Bars represent mean of triplicate cultures, and error bars represent 1 standard deviation

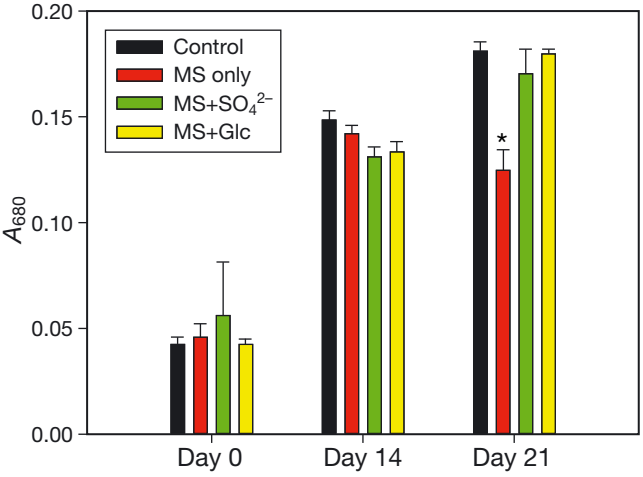

Fig. 4. Change in absorbance at 680 nm in cultures of *Thalassiosira pseudonana* utilizing sulfate exclusively (Control), MS exclusively (MS only), MS with sulfate added on Day 14 (MS+SO_4^{2-}), or MS with glucose added on Day 14 (MS+Glc). Sulfate or glucose was added to appropriate cultures on Day 14. Bars represent the mean of triplicate cultures, and error bars represent 1 standard deviation. Only MS cultures without other additions on Day 21 were statistically significant from controls, indicated by an asterisk ($p = 0.0034$)

lyze, forming CF_2O and HF. Toxicity of HF is a likely explanation for reduced growth rates in Tf cultures versus Control or MS cultures.

Formaldehyde toxicity was determined in sulfate-replete cultures of *T. pseudonana* after an exposure period of 4 d. Formaldehyde was added from stock solutions in media via sterile filtration to final concentrations ranging from 0.1 to 100 µM. Up to 1 µM, no significant differences were noted in mean absorbance at 680 nm, indicating no inhibition of growth (Fig. 2). At 100 µM, however, the cultures showed almost complete growth inhibition. From the data, the concentration inhibiting growth by half (IC_{50}) was 25 ± 7 µM. As this IC_{50} is 3 orders of magnitude higher than the observed total formaldehyde concentrations, toxicity due to formaldehyde production is unlikely to be the cause of growth inhibition in MS cultures.

Particulate sulfite concentrations were significantly higher in MS cultures (0.012 ± 0.004 fmol cell^{-1}) versus controls (0.0010 ± 0.0002 fmol cell^{-1}, $p = 0.0304$) (Fig. 3), indicative of increased sulfite production, which is expected if sulfite is the major sulfur product of MS metabolism. The observed intracellular sulfite concentrations (particulate sulfite per unit biovolume) in MS cultures were approximately 95 µM. In previous studies, no physiological effects were found at concentrations below approximately 4 mM (Sheridan 1978, Singh & Singh 1984), suggesting that MS cultures are not significantly inhibited by sulfite. However, *T. pseudonana* may have different sensitivity to sulfite than *Chlorococcum* sp.

A toxicity experiment similar to that with formaldehyde was conducted, with extracellular sulfite concentrations ranging from 0 to 1 mM. After 96 h, no differences in absorbance were noted for *T. pseudonana*, indicating little toxicity at these concentrations of extracellular sulfite (Fig. 4). Accordingly, the best fit to Eq. (1) was not statistically significant for any of the parameters ($p > 0.1$). It should be noted, however, that the sulfite was added extracellularly, and the levels do not necessarily reflect intracellular concentrations.

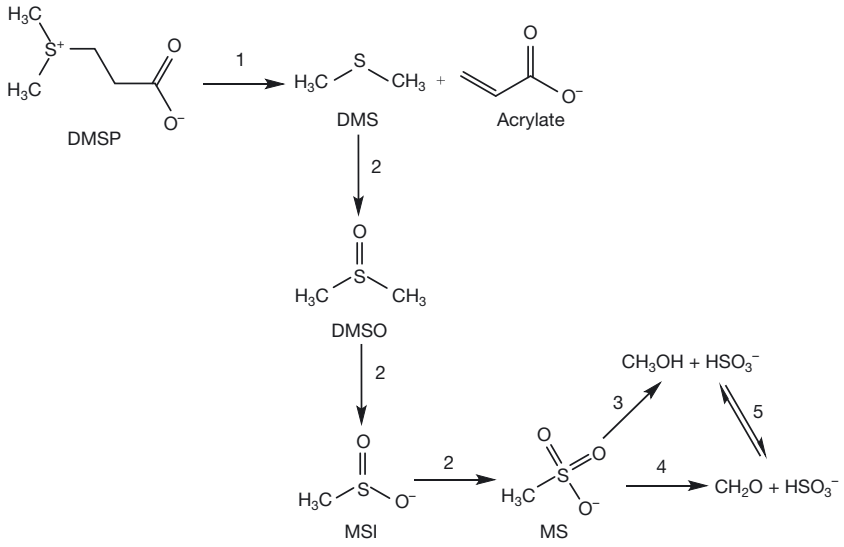

Fig. 5. Oxidative pathway from DMSP to sulfate including known or suspected metabolic pathways involving MS. Reactions: (1) DMSP lyase or reactive oxygen species (ROS) (Steinke et al. 1996, Sunda et al. 2002); (2) ROS (Bardouki et al. 2002, Sunda et al. 2002); (3) hydrolysis of MS to methanol and sulfite (Kelly & Murrell 1999); (4) oxidation of MS to formaldehyde and sulfite (Kelly et al. 1994, Eichhorn et al. 1999, Kelly & Murrell 1999); (5) alcohol dehydrogenase

Sulfur limitation

To examine whether MS-grown cultures were limited by bioavailable sulfur, cultures were tested for sulfur starvation by amending MS cultures in stationary phase (culture Day 14) with either 25 mM sulfate or 25 mM glucose. Glucose was added to differentiate between sulfur limitation (growth in sulfate-amended cultures but not glucose-amended cultures) and inhibition due to energy limitation (growth in all amended cultures). After an additional 7 d, cultures amended with sulfate or glucose showed increased biomass, while unamended MS cultures declined (Fig. 5). Sulfate- and glucose-amended cultures were not statistically different from positive controls ($p = 0.1773$ and 0.6398 for sulfate- and glucose-amended cultures, respectively), while unamended MS cultures had a significantly lower absorbance versus controls ($p = 0.0034$). This indicates that cultures metabolizing MS were somehow energy-limited, possibly due to intracellular sulfite inhibition of photosynthesis or respiration (Sheridan 1978).

DMSP content

DMSP is often the dominant sulfur-containing molecule in marine algal cells (Keller et al. 1989, 1999), although diatoms are not thought of as exceptional DMSP producers. However, because DMSP production correlates with stress (Sunda et al. 2002, Bucciarelli & Sunda 2003), we examined the DMSP content of cultures grown on MS as the sole sulfur source. In MS cultures, DMSP content was not significantly different than in the control (2.1 ± 0.3 mM versus 2.9 ± 0.8 mM in controls, n = 6 each), indicating that there is no sulfur limitation present.

Bioinformatics

To elucidate possible enzymatic pathways used by *T. pseudonana* to metabolize MS, published sequences for 11 known proteins involved in MS metabolism were compared to the entire *T. pseudonana* genome. Bacterial MS metabolism is regulated by a number of operons, including *Escherichia coli ssuABCDE* and *tauABCD*, *Methylsulfonamonas methylovora msmABCDEFGH*, and *Pseudomonas aeruginosa msuABCD*. Ssu had no significant homology to any *T. pseudonana* proteins. Similarly, Tau and Msu showed no homologous domains either. The MS-metabolizing protein MsmA (hydroxylase large subunit) in *M. methylovora* showed the best comparison to *T. pseudonana* across all genes. MsmA had 25.6% identity and 48.0% similarity to the *T. pseudonana* protein B8LEA7, likely due to the presence of a Rieske domain (Table 2). MsmD had close homology to *T. pseudonana* protein A0T0N0, which carries a ferredoxin domain. MsmG, an ABC transporter, showed significant homology to multiple proteins within the *T. pseudonana* genome, suggesting a large family of ABC transporter protein family exists within the *T. pseduonana* genome. Although ABC transporters have been shown to be sulfate transporters (van der Ploeg et al. 2001), these genes are functionally distinct from the genes likely to regulate MS metabolism. It should be noted that the sequence similarity results presented here are limited due to the quality and level of annotation present in the *T. pseudonana* genome, which may explain the lack of homology to other components of the *msm* operon.

MS metabolism has also been noted in the budding yeast *Saccharomyces cerevisiae* and linked to open reading frame YLL057c (Hogan et al. 1999). However, no homologs to YLL057c were identified in the

Table 2. Summary of sequence similarity between MS metabolism proteins and similar sequences in *Thalassiosira pseudonana*

Bacterium	Bacterial protein	*T. pseudonana* protein	% identity	Function
Methylosulfonomonas methylavora	MsmA	B8LEA7	25.6%	Metal binding (Rieske-type)
	MsmD	A0T0N0	29.3%	Redox (ferridoxin)
	MsmG	Multiple	Variable	Transport (ABC transport domain)
Escherichia coli	SsuB	Multiple	Variable	Transport (ABC transport domain)

T. pseudonana genome when subjected to HMMER analysis at sequence and hit thresholds ≤10.

Identification of candidate MS metabolizing proteins (B8LEA7 and A0T0N0) in *T. pseudonana* allows for identification of similar proteins in other DMSP-producing marine phytoplankton, such as the prymnesiophyte *Emiliania huxleyi* and the diatom *Thalassiosira oceanica*.

Comparing these sequences via BLASTp to entries in the UniProt database yielded a number of homologous proteins. *E. huxleyi* and *T. oceanica* each were found to have proteins homologous to *T. pseudonana* B8LEA7 (R1CU38 and R1F4E4 for *E. huxleyi* and K0SWV2 for *T. oceanica*). Each of these had *E*-values ≤ 2.5×10^{-42}. *T. pseudonana* protein A0T0N0 also had homologs in *T. oceanica* (K0RP06) and in *E. huxleyi* (JGI ID 264994), identified as chloroplast ferridoxins ($E = 3.8 \times 10^{-40}$).

DISCUSSION

This study reports MS utilization by a marine alga for the first time. This is a highly significant result in that *Thalassiosira pseudonana*, as a DMSP-producing alga, theoretically has the capacity to form MS from compounds already present in the cell — DMSP, DMS, and DMSO (Simó et al. 1998, Hatton & Wilson 2007, Sunda et al. 2007). Abiotic production of MS from DMSO has been demonstrated in aqueous solution (Bardouki et al. 2002), as has abiotic production from DMSP (Spiese 2010). Direct production *in vivo*, however, has not been demonstrated in any phytoplankton species. This may be due to rapid loss of DMS or DMSO from the cell (Lavoie et al. 2016, Spiese et al. 2016). If the oxidation of DMS(O) is slow relative to diffusive loss, then MS would not form. Diffusive loss of DMSO occurs with a first order rate constant of ~0.4 s^{-1}, while the pseudo-first order rate constant for oxidation by OH radical could range from 10^{-8} to 10^{-2} s^{-1}, depending on the concentration of OH (Lavoie et al. 2016). Therefore, oxidation appears to only compete with diffusion during instances of increased levels of reactive oxygen species (e.g. oxidative stress), and thus, MS may only be present in the cell during these times. Regardless, the cell appears to have the capacity to recycle sulfur that has become oxidized from the DMSP pool. This is a key step in an antioxidant system starting with DMSP and proceeding through DMS and DMSO to methanesulfinic acid to MS, and from there to sulfate (Fig. 5). The presence of MS metabolism in *T. pseudonana* does not preclude active export from the cell of MS or any other DMSP-derived species, but it suggests that recycling of sulfur may play a role in removing the end products of DMSP-based antioxidant activity.

By demonstrating growth on MS by *T. pseudonana*, we are able to conclude that MS is metabolized into a form of sulfur that can be incorporated into biomolecules. While we propose formaldehyde as the carbon product of MSA degradation, it should be noted that its production may also be tied to production of methanol (Kelly & Murrell 1999):

$$CH_3SO_3^- + H_2O \rightarrow CH_3OH + HSO_3^- \quad (2)$$

Methanol is metabolized in most cases to formaldehyde and from there to formate. However, alcohol dehydrogenase is a reversible enzyme, allowing reduction of formaldehyde to form methanol. Therefore, although formaldehyde is proposed as the major carbon product, methanol is still a possibility. Metabolism of Tf allows for differentiation of the 2 pathways. The initial step for Tf degradation would be either oxidation to form CF_2O or substitution to form trifluoromethanol (CF_3OH). Given that Tf oxidation involves cleavage of a strong C-F bond and that no hydrogens exist to complete the reduction of O_2 (*vide infra*), it is unlikely that direct oxidation of Tf occurs, suggesting that it is metabolized through the substitution pathway. CF_3OH decomposes at room temperature to form CF_2O (Klöter & Seppelt 1979) and may explain the apparent toxicity of Tf. Although these results do not definitively prove substitution as the initial metabolic step, they do suggest that sulfonate degradation may proceed via substitution at least in some cases.

The sulfur product of MS metabolism is sulfite. In this regard, MS metabolism in *T. pseudonana* appears to be similar to MS metabolism in the bacterium *Methylosulfonomonas methylovora*. This result is supported by the observation of a homologue to MsmA, the hydroxylase subunit of the MS-metabolizing complex in *M. methylavora*. Sequences homologous to *msmA* were observed in both marine and estuarine microbial (bacteria and archaea) communities (Henriques & de Marco 2015, Henriques et al. 2016). Metabolism of MS in *M. methylovora* is an NADH-dependent pathway (Kelly et al. 1994, Eichhorn et al. 1999, Kelly & Murrell 1999):

$$CH_3SO_3^- + NADH + O_2 + H^+ \rightarrow CH_2O + HSO_3^- + NAD^+ + H_2O \quad (3)$$

Identification of similar proteins based on sequence similarity in multiple species of DMSP-producing phytoplankton suggests that MS metabolism could be a common feature for these algae. It should be noted here that within the genome of *Emiliana huxleyi*, there are homologous proteins with good identity ($E \leq 10^{-10}$) to other MS-metabolizing domains, such as TauD from *Escherichia coli* and *Saccharomyces cerevisiae* YLL057c, which are absent in *T. pseudonana*. It is possible that because *E. huxleyi* produces DMSP to a much greater extent than *T. pseudonana* (Keller et al. 1989), MS metabolism may utilize additional pathways in that species.

T. pseudonana was shown to use MS as its sole sulfur source, but with an accompanying limitation in energy, as evidenced by increased growth when presented with glucose. MS resists C-S bond breakage, in contrast to other sulfonates (Kelly & Murrell 1999 and references therein). One possible reason for this energy limitation is the additional requirement to process sulfite produced by MS catabolism. Kelly & Murrell (1999) suggest that sulfite will be transformed into sulfate either by enzymatic action or abiotically in solution. Incorporation of the produced sulfate is an energy-intensive process, which may explain at least some of the observations from glucose-amended cultures.

This report of utilization of MS by *T. pseudonana* also expands our understanding of sulfur metabolism in marine phytoplankton. Seawater is approximately 25 mM SO_4^{2-}, and therefore, sulfur starvation and the need to utilize alternative sulfur sources are extremely unlikely. *S. cerevisiae* showed a clear preference for sulfate over sulfonate sulfur, as did *C. fusca* (Biedlingmaier et al. 1986, Uria-Nickelsen et al. 1993). Nevertheless, *E. huxleyi* has been shown to adapt to low sulfate concentration by lowering DMSP concentrations, presumably to shift sulfur into cysteine and methionine, among other essential sulfur compounds (Bochenek et al. 2013). In contrast, *T. pseudonana* maintained cellular DMSP content while using MS. Sustained growth with the addition of glucose indicates that *T. pseudonana* does not in fact experience sulfur limitation while growing on MS. Rather, the sulfite produced from MS metabolism may inhibit photosynthesis or respiration. While no toxic effects were observed over 96 h at dissolved phase concentrations ≤1 mM, intracellular concentrations may be substantially different than dissolved, and the lower intracellular pH (~7.5) versus seawater (~8.0) may result in a higher proportion of sulfite as either HSO_3^- or SO_3^{2-}. Further experiments are warranted to determine the mechanism for sulfur incorporation from MS and other sulfonates and whether this incorporation results in reduced respiration or photosynthesis.

The ability for the marine diatom *T. pseudonana* to utilize MS as its sole sulfur source suggests that marine phytoplankton may play a role in closing the sulfur cycle with respect to DMS-derived sulfur. This activity further points to the potential for DMSP and its derived compounds to be part of a proposed antioxidant system because the internal mechanism for recycling oxidized sulfur is present. Further work on this metabolic pathway is necessary, however, to elucidate end products and to determine regulating factors, including the extent to which sulfate is preferred as a substrate over MS or other sulfonates.

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