Vitamin B1 ecophysiology of marine picoeukaryotic algae: Strain-specific differences and a new role for bacteria in vitamin cycling

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Abstract

We confirmed multiple picoeukaryotic algae, Ostreococcus, Micromonas, and Pelagomonas spp., as thiamine (vitamin B1) auxotrophs in laboratory experiments with axenic cultures. Examined strains have half saturation growth constants ($K_s$) for B1 between 1.26 and 6.22 pmol B1 L$^{-1}$, which is higher than reported seawater concentrations. Minimum B1 cell quotas for Ostreococcus and Micromonas spp. are high ($2.20 \times 10^{-8} - 4.46 \times 10^{-8}$ pmol B1 cell$^{-1}$) relative to other B1 auxotrophic phytoplankton, potentially making them B1 rich prey for zooplankton and significant B1 reservoirs in oligotrophic marine habitats. Ostreococcus and Micromonas genomes are nonuniformly missing portions of the B1 biosynthesis pathway. Given their gene repertoires, Ostreococcus lucimarinus CCE9901 and Ostreococcus tauri OTH95 are expected to salvage B1 from externally provided 4-methyl-5-thiazoleethanol (HET) and 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP). However, in culture, neither could use HET plus HMP instead of B1, highlighting current limitations of genome-based prediction of B1 salvaging by picoeukaryotic algae. HMP and phosphorylated B1 use varied amongst tested strains and notably all Prasinophytes tested could not use HMP. B1-limited O. lucimarinus CCE9901 could not grow on added thiamine diphosphate (TDP), a phosphorylated B1 form. However, in co-culture with Pseudoalteromonas sp. TW7, a bacterium known to exhibit phosphatase activity, O. lucimarinus CCE9901 exhibited increased growth following TDP additions. This demonstrates that bacteria influence vitamin B1 availability beyond de novo synthesis and consumption; they can also serve as conduits that chemically alter, but not completely degrade or retain B1 analogs (e.g., TDP), and make them accessible to a broader range of microbes.

Vitamin B1 (called B1 herein), also known as thiamine, is required by all cells due to its role as a cofactor in the form of thiamine pyrophosphate (also called thiamine diphosphate [TDP]) for enzymes critical to carbon and amino acid metabolism (Jurgenson et al. 2009). Additionally, certain riboswitches, untranslated sections of messenger ribonucleic acid (RNA) capable of regulating gene expression, are activated by binding TDP, B1, and moieties (parts of the thiamine molecule, e.g., thiazole and pyrimidine; Moulin et al. 2013). Notably, this is one of the few documented riboswitch systems in eukaryotes, and appears to be important in regulating thiamine metabolism in marine microbes (McRose et al. 2014). In the euphotic upper ocean, thiamine concentrations appear to be low relative to macronutrients, e.g., pmol L$^{-1}$ or below (Sañudo-Wilhelmy et al. 2012). Despite the scarcity of B1 in the upper ocean, especially the oligotrophic regions where published B1 concentrations are < 0.05 pmol L$^{-1}$ (Barada et al. 2013), several marine plankton (eukaryotes and prokaryotes) persist as B1 auxotrophs, unable to synthesize their own vitamin B1. Surveys of the occurrence of vitamin auxotrophy within phytoplankton noted that a large percentage (~80%) of prymnesiophytes were B1 auxotrophs (Provasoli and Carlucci 1974; Croft et al. 2006). In contrast, a majority of diatoms produce B1 (Provasoli and Carlucci 1974), and both B1 producing and auxotrophic bacteria are present in the ocean (MacLeod et al. 1954; Burkholder and Lewis 1968).

Many new algal strains have been isolated as the pioneering surveys of auxotrophy (Provasoli and Carlucci 1974), including multiple picoeukaryotic algal strains (less than three micrometer in cell diameter). There is increasing
evidence, based largely on genome analysis, that several key picoeukaryotic algae are B1 auxotrophs (Palenik et al. 2007; Worden et al. 2009; Bertrand and Allen 2012; McRose et al. 2014) but also based on a small set of experiments with axenic cultures (e.g., A ureococcus anophagefferens CCMP1984, Micromonas spp.; Tang et al. 2010; Bertrand and Allen 2012; McRose et al. 2014). Picoeukaryotic phytoplankton are important contributors to marine primary production and phytoplankton biomass (Li 1994; Worden et al. 2004). Also some B1 auxotrophic picoeukaryotic and larger eukaryotic algae are harmful algal bloom (HAB)-associated species, e.g., A. anophagefferens and Phaeocystis globosa (Peperzak 2000; Tang et al. 2010). Thus, vitamin B1 availability may influence primary production rates, marine algal community structure, and water quality by influencing the growth and activity of picoeukaryotic phytoplankton.

Several studies found positive correlations between primary productivity rates and additions of B1 or increases in natural B1 seawater concentrations. B1 additions to subarctic N. Pacific seawater stimulated bulk primary productivity (Natarajan 1970), a significant positive correlation between net primary productivity and B1 concentrations was found at one location off La Jolla, California (Carlucci and Silbernagel 1966). This seawater base was then supplemented with vitamin B1 (Kc) and maximum (Kmax) saturation growth constants, their ability to utilize B1 chemical analogs (moieties and phosphorylated forms), and their respective minimal B1 cell quotas. Lastly, we examined if the presence of a simple microbial population (Gammaproteobacterium Pseudo monas sp. TW7) would enable a B1 auxotrophic algae (O. lucimarisinus CC9901) to utilize a phosphorylated B1 analog it cannot use in isolation.

Methods

M. pusilla CCMP487, Ostreococcus spp., and P. lutheri cultures were grown on modified F/2 medium (Guillard 1975) plus sodium selenite and sodium citrate (as formulated by Palenik unpubl.). The seawater base was twice 0.22 μm filtered, microwaved and treated with activated charcoal to remove organics as previously described (Carlucci and Silbernagel 1966). This seawater base was then supplemented with autoclaved F/2 nitrate and phosphate solutions as well as 0.2 μm filter-sterilized stocks of the trace metal mix, sodium selenite (87 nmol L⁻¹ final), and sodium citrate (50 μmol L⁻¹ final). P. calceolata CCMP1756 cultures were grown in Aquil synthetic seawater supplemented with F/2 concentrations of filter-sterilized nitrate, phosphorus, and trace metal mix. For all cultures, filter-sterilized vitamin B12 (cobalamin) and B7 (biotin; 184 pmol L⁻¹ and 1 nmol L⁻¹ final respective concentrations) solutions were added individually so that B1 could be added later at varying concentrations (medium without B1 is noted as F/2 – B1). Before use, the growth medium lacking B1 was tested for bacterial contaminants by adding one to two milliliter of ZoBell bacterial growth medium (ZoBell 1941). Micromonas, Ostreococcus, and P. lutheri cultures were grown in five milliliter polystyrene fal con tubes (Becton Dickinson) at ~ 20°C, without shaking and under ~ 160 μmol quanta m⁻² s⁻¹ of constant light from T5 28 W fluorescent white light bulbs, unless noted
Ostreococcus sp. CCE1301 were obtained using an antibiotic cultures of cleic acid gene phylogeny (Palenik, data not shown). Axenic nia and is closer in relation to CCE1301 was isolated from 100 m waters off La Jolla, Califor- isolates of phototrophic bacteria (Bidle and Azam 2001) were maintained in exponential growth on minimal B1 medium for at least five transfers then transferred 1:10 to F/ 2 – B1 medium until growth declined relative to a parallel positive control culture amended with 1 nmol B1 L⁻¹. The culture exhibiting early B1 growth limitation was transferred into a series of cultures containing a range of B1 concen- trations (8–10 total cultures, from 0 to 100 pmol B1 L⁻¹ final concentration). Instantaneous growth (µ) was determined using the formula µ = ln (Bf/Bi)/T where Bf = final biomass, Bi = initial biomass, and T = time. Analysis of maximum instantaneous growth rate vs. B1 concentration was done using the Michealis–Menten analysis option within the soft- ware package Prism (GraphPad Software). Maximum growth constants were estimated based on the maximum growth rate calculated by the Michealis–Menten analysis. The mini- mal B1 cell quota was determined using the difference between maximum yields of mid-range B1 amendments (0.5–100 pmol L⁻¹), which reached stationary phase well before higher B1 additions.

For cell biovolume estimates, first M. pusilla CCMP487, O. lucimarinus CCE9901, and Ostreococcus sp. CCE1301 were obtained using an antibiotic cocktail of streptomycin, chloramphenicol, and cefotaxime at respective final concentrations of 10 µg mL⁻¹, 1 µg mL⁻¹, and 80 µg mL⁻¹ or only cefotaxime treatments at a final concentration of 80 µg mL⁻¹. Antibiotic treatments were discontinued or deemed unnecessary after confirming cultures as bacteria free by epifluorescence microscopy using 4′,6-diame- indinino-2-phenylindole staining, and by an absence of bacterial growth in flasks containing two milliliter ZoBell bacterial liquid medium and one milliliter of the tested algal culture. Axenic cultures were always transferred to fresh medium at a ratio of 1:10, algal culture to fresh medium.

Vitamins and chemical analogs (moieties or phosphorylated forms) used in experiments were obtained from Sigma, Spectrum, Alfa Aesar, Tokyo Chemical Industry, MP Biome- dicals or Calbiochem at high performance liquid chromatog- raphy (HPLC) grade, > 98% pure, except for HMP which was > 90% pure based on nuclear magnetic resonance and was purchased from VitasMlab. Working stocks of chemicals were dissolved in autoclaved Milli-Q water except for chloro- amphenicol, which was dissolved entirely in ethanol.

Algal biomass in cultures was monitored based on in vivo Chlorophyll a (Chl a) fluorescence measured using an Aquafluor portable fluorometer (Model 8000-010), or bench top fluorometer AU-10, (Turner Designs). In vivo Chl a fluorescence and algal cell abundances were linearly correlated for O. lucimarinus CCE9901, Ostreococcus sp. CCE1301, M. pusilla CCMP487, and P. calceolata CCMP1756 over the measured fluorescence range (data not shown). Prior to limitation experiments, all algal cul- tures were maintained in exponential phase on F/2 – B1 medium as described above plus ~ 1 nmol B1 L⁻¹. All experiments were conducted in triplicate, unless noted otherwise.

Growth kinetic constants and minimal B1 cell quota (pmol B1 per cell) were determined for each alga from two or three separate gradient addition experiments. Cultures were grown with continuous light: dark regime were exposed to ~ 72 µmol quanta m⁻² s⁻¹ of the same white light. Cultures were vortexed and inverted at least once a day. Pelagomonas cultures were grown in 50 mL acid washed glass tubes, vortexed daily, and exposed to a 14 h:10 h light:dark regime with daytime light levels of ~ 70 µmol quanta m⁻² s⁻¹.

Ostreococcus tauri OTH95, M. pusilla CCMP487, and P. lutheri CCMP1325 were obtained from the National Center for Marine Algae and Microbiota (NCMA). Axenic cultures of P. lutheri CCMP1325 and P. calceolata CCMP1756 were obtained from NCMA. O. lucimarinus CCE9901 was originally isolated from waters off La Jolla, California (Price et al. 1989; Kirchman 2000; Worden et al. 2004). Ostreococcus sp. CCE1301 was isolated from 100 m waters off La Jolla, Califor- nia and is closer in relation to Ostreococcus sp. CCE9901 and RCC356 than O. tauri OTH95 based on 18S ribosomal ribonu- cleic acid gene phylogeny (Palenik, data not shown). Axenic cultures of M. pusilla CCMP487, O. lucimarinus CCE9901, and Ostreococcus sp. CCE1301 were obtained using an antibiotic cocktail of streptomycin, chloramphenicol, and cefotaxime at respective final concentrations of 10 µg mL⁻¹, 1 µg mL⁻¹, and 80 µg mL⁻¹ or only cefotaxime treatments at a final concentration of 80 µg mL⁻¹. Antibiotic treatments were discontinued or deemed unnecessary after confirming cultures as bacteria free by epifluorescence microscopy using 4′,6-diamindinino-2-phenylindole staining, and by an absence of bacterial growth in flasks containing two milliliter ZoBell bacterial liquid medium and one milliliter of the tested algal culture. Axenic cultures were always transferred to fresh medium at a ratio of 1:10, algal culture to fresh medium.

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O. lucimarins CCE9901 growth in co-cultures reached exponential to early stationary phase based on Chl a fluorescence, the single co-cultures were transferred at a ratio of 1:10 (e.g., 200 µL of culture to 1800 µL of medium) to new medium (either F/2 + NH4 – B1 or F/2 + NH4 + 1 nmol L⁻¹ TDP where appropriate). In vivo Chl a fluorescence of all co-cultures over seven transfers was measured daily as described above using an Aquafluor portable fluorometer. After six transfers of the B1-limited co-culture, aliquots (50 µL) of the co-culture were transferred and spread on multiple ZoBell agar plates (1% agarose). Lawns of TW7 were visible on all ZoBell plates after two to three days, while no colonies appeared on a negative (medium only added) control ZoBell plate.

Hidden Markov Model (HMM) search (Eddy 1998) was used to identify TDP-dependent enzymes in peptide sequence files. HMM models from each of the eight TDP-dependent superfamilies were downloaded from the Thiamine diphosphate dependent Enzyme Engineering Database (TEED; http://www.teed.uni-stuttgart.de) and concatenated into one HMM file for searching against peptide data. Peptide data were downloaded from the Joint Genome Institute (JGI), the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) website (http://camera.calit2.net/mmetsp/) or Genoscope (https://bioinformatics.psb.ugent.be/gdb/bathyccoccus/). Protein sequences with a domain score > 20 were considered positive hits and then dereplicated based on protein sequence identifier (ID). Replicates with the highest domain score were used in the analysis.

B1 biosynthesis proteins in O. lucimarins CCE9901 and O. tauri OTH95 were identified using blastp and searching known reference proteins (e.g., ThiM from Bacillus subtilis, Uniprot ID E8VEP7_BACST) against Ostreococcus genomes via the National Center for Biotechnology Information (NCBI) website. Positive hits (an alignment score > 20) were then verified using blastp searches against the UniProtKB protein database via the Uniprot website.

Results

B1 auxotrophy, growth kinetics, and minimum cell quota of tested strains

All axenic algal cultures exhibited growth limitation following multiple transfers to F/2 – B1 medium without vitamin B1 addition. In the growth kinetics experiments, cultures exhibited maximum growth rates ranging from 0 d⁻¹ to ~ 1.13 d⁻¹ depending on the amendment of B1. Plots of maximum growth rate vs. vitamin B1 amendment yielded a typical Michaelis–Menten hyperbolic curve for each algal strain (Fig. 1; data shown only for O. lucimarins CCE9901). A Michaelis–Menten model strongly represented changes in growth rate vs. B1 amendment concentrations (e.g., $r^2 \geq 0.84$). The half-saturation growth constants ($K_s$) were 1.26–6.22 pmol B1 L⁻¹ for all strains tested. The maximum saturation growth constants ($K_{max}$) were ~ 150 pmol B1 L⁻¹ or less (Table 1), with 1 nmol B1 L⁻¹ being the maximum addition (data not shown).

The minimum vitamin B1 cell quota for strains ranged from $2.20 \times 10^{-8}$ pmol B1 cell⁻¹ + $1.89 \times 10^{-8}$ pmol B1 cell⁻¹ to $4.46 \times 10^{-8}$ pmol B1 cell⁻¹ + $1.09 \times 10^{-8}$ pmol B1 cell⁻¹. O. lucimarins CCE9901 cultures grown under a 14 h : 10 h light : dark regime exhibited a comparable quota to that of cultures exposed to continuous higher irradiance (~ $160 \mu$mol quanta m⁻² s⁻¹), $2.20 \times 10^{-8}$ pmol B1 cell⁻¹ + $1.89 \times 10^{-8}$ pmol B1 cell⁻¹ vs. $2.72 \times 10^{-8}$ pmol B1 cell⁻¹ + $1.70 \times 10^{-8}$ pmol B1 cell⁻¹ (Table 1).

Utilization of vitamin B1 chemical analogs

Based on complete genome sequences, O. lucimarins CCE9901 and O. tauri OTH95 each possess partial vitamin B1 biosynthesis pathways (Bertrand and Allen 2012; McRose et al. 2014). Both lack ThiC or Thi5, proteins required for de novo HMP production, but possess ThiM and Th1 (Fig. 2), which are involved in thiazole and pyrimidine modification as well as condensation of moieties to yield thiamine monophosphate (TMP; Rapala-Kozik et al. 2007; Jurgenson et al. 2014). As a result, we hypothesized that both could generate TMP and TDP from thiazole and pyrimidine precursors 4-methyl-5-thiazoleethanol (HET) and HMP (Fig. 2). We tested in culture experiments if HMP and HET (and each individual compound) could satisfy the B1 requirement of O. lucimarins CCE9901 and O. tauri OTH95, as well as other B1 auxotropic algae whose genome sequences are not currently available. Single or combined additions of HET and HMP, did not support the growth of Osteococcus spp. or M. pusilla.
Table 1. Minimum vitamin B1 cell quotas, half saturation growth constants ($K_s$), maximum saturation growth constants ($K_{max}$), and maximum growth rates ($\mu_{max}$) for several B1 auxotrophic eukaryotic phytoplankton. All numeric data pertain to vitamin B1 additions except for *P. calceolata* CCMP1756 data noted with an HMP superscript, in which case, HMP was the added substrate instead of B1 and it is assumed that each HMP molecule is completely converted to a B1 molecule within the cell. OTH95 data are noted with two asterisks, as cultures were found postexperiment to contain bacterial contaminants. *P. lutheri* biovolume was determined in this study by microscopy ($8.27 \times 10^{-28} \mu L$ minimum, $7.12 \times 10^{-26} \mu L$ maximum, see Methods). Data from this study are in bold. Additional *P. lutheri* data are from Carlucci and Silbernagel (1966) and all other data are from Tang et al. (2010). For quota estimates the average value plus minus one standard deviation is presented. L:D = data for *O. lucimarinus* CCE9901 cultures grown under a light:dark regime. Util. = utilization of a provided B1 substrate indicated by growth (N = no, Y = yes). B1 = phosphorlated B1 compounds thiamine monophosphate and thiamine diphosphate. HMP = 4-amino-5-hydroxymethyl-2-methylpyrimidine. HET = hydroxyethylthiazole. nd = not determined.

| Organism | Class, Order | B1 + P Util.? | HMP and/or HET Util.? | B1 per biovolume (pmol B1 $\mu L^{-1}$) | B1 per cell (pmol B1 cell$^{-1}$) | $K_s$ (pmol L$^{-1}$) | $K_{max}$ (pmol L$^{-1}$) | $\mu_{max}$ (d$^{-1}$) |
|----------|--------------|---------------|----------------------|----------------------------------------|----------------------------------|----------------------|----------------------|----------------------|
| *O. lucimarinus* CCE9901 | Prasinophyceae, Mamiellales | N | N | 67.1±57.6 | 2.20±08±1.89±08 | 1.26±0.13 | -50 | 1.06±0.02 |
| *O. lucimarinus* CCE9901 (L:D) | Prasinophyceae, Mamiellales | N | N | nd | 2.72±08±1.70±08 | (n=12) | nd | nd |
| *O. tauri* OTH95 | Prasinophyceae, Mamiellales | N** | N** | nd | nd | nd | nd | nd |
| *Ostreococcus* sp. CCE1301 | Prasinophyceae, Mamiellales | Y | N | 89.7±16.5 | 3.58±10±08±12 | 6.22±0.97 | -150 | 1.07±0.03 |
| *M. pusilla* CCMP487 | Prasinophyceae, Mamiellales | Y | N | 72.82±16.5 | 2.55±10±09±14 | 4.88±0.65 | -150 | 0.77±0.02 |
| *P. calceolata* CCMP1756 | Pelagophyceae, Pelagomonadales | Y | HMP | 6.52±1.56 | 4.46±10±08±12 | 2.38±0.57 | -100 | 0.67±0.03 |
| *P. calceolata* CCMP1756 HMP | Pelagophyceae, Pelagomonadales | Y | HMP | 5.24±1.07 | 3.59±10±10±12 | 3.46±0.85 | -100 HMP | 0.72±0.03 |
| *P. lutheri* CCMP4325 | Pavlovophyceae, Pavlovelas | Y | HMP | 10.04±11.65 | 8.30±10±07 | | | |
| *A. anophagefferens* CCMP1984 | Pelagophyceae, Pelagomonadales | nd | nd | 1.16 | 6.52±10±09 | 5.94±1.36 | nd | nd |
| *Proorocentrum* minimum CCMP696 | Dinophyceae, Prorocentrales | nd | nd | 0.545 | 6.27±10±07 | 86.3±3.76 | nd | nd |
| *Scripsiella trochoidea* MS1 | Dinophyceae, Peridiniales | nd | nd | 0.285 | 2.41±10±06 | 131±30.2 | nd | nd |
| *Gyrodinium aureolum* KA6 | Dinophyceae, Dinotrichales | nd | nd | 5.65 | 1.94±10±05 | 96.9±4.01 | nd | nd |
| *Rhodomonas salina* CCMP1319 | Cryptophyceae, Pyrenomonadales | nd | nd | 3.43 | 3.02±10±07 | 184±81.5 | nd | nd |
CCMP487; HMP alone supported the growth of \textit{P. calceolata} CCMP1756 and the larger alga \textit{P. lutheri} CCMP1325 (Fig. 3).

Phosphorylated B1 is generated within cells and presumably becomes available in the water column due to cell mortality, excretion, or diffusion. The majority of strains examined (all except \textit{O. lucimarinus} CCE9901 and \textit{O. tauri} OTH95) grew on TMP or TDP as sole B1 sources (Fig. 4). The \textit{F/2} growth medium is phosphate replete (0.36 mmol PO$_4$ L$^{-1}$), which we hypothesized could inhibit phosphatase activity, and furthermore, dephosphorylation of TMP or TDP to B1. Comparable levels of phosphate cause inhibition of alkaline phosphatase activity in certain algae (Dyhrman and Ruttenberg 2006). To test for this inhibition, \textit{O. lucimarinus} CCE9901 was grown on \textit{F/2} medium without typical phosphate amendment, containing only phosphorous from the seawater base, and TDP as a B1 source. Phosphate concentrations from surface waters off the Scripps Pier, La Jolla, California, are typically $<0.8$ mmol L$^{-1}$ (Tai and Pale- nik 2009). Reduced phosphate concentrations did not enable \textit{O. lucimarinus} CCE9901 to grow on TDP (Fig. 5A).

Growth of CCE9901 in co-cultures with \textit{Pseudomonas} sp. TW7 following TDP amendment

In co-culture with TW7, B1-limited \textit{O. lucimarinus} CCE9901 grew in response to the addition of 1 nmol TDP L$^{-1}$ and ultimately reached $\sim 10\times$ its maximum biomass (in vivo Chl $a$ fluorescence) observed in un-amended control cocultures (Fig. 5B). \textit{O. lucimarinus} CCE9901 exhibited net...
positive growth in B1-limited co-cultures with TW7 over multiple transfers (to B1-deplete medium), but maximum O. lucimarinus CCE9901 biomass remained low relative to co-cultures amended with TDP (Fig. 5B). Axenic O. lucimarinus CCE9901 cultures transferred successively to B1-deplete medium or medium supplemented with TDP showed no net growth, and in vivo Chl \( a \) fluorescence became undetectable after one to two transfers (data not shown).

**Discussion**

**Physiological and ecological implications of picoeukaryotic algae vitamin B1 \( K_s \) values**

Picoeukaryotic algal strains examined in this study must possess high-affinity B1 uptake systems to explain their very low \( K_s \) (again, half-saturation growth constant) values (1.26–6.22 pmol B1 L\(^{-1}\); Table 1). They also possess relatively low \( K_{\text{max}} \) values (\( \sim 150 \) pmol B1 L\(^{-1}\)), indicating they have singularly prioritized maintenance of a high-affinity acquisition lifestyle, rather than also possessing a low-affinity system, which is often concomitant with a higher maximum growth rate. Other larger bloom forming B1-auxotrophic algae presumably posses lower affinity systems based on their higher \( K_s \) values (Table 1; Tang et al. 2010).

It is unknown what proteins are critical to high affinity transport of B1 in our tested picocyanobacteria, nor any other algae to the best of our knowledge. Notably, A. anophagefferens CCMP1984 also has a relatively low \( K_s \) value, \( \sim 6 \) pmol L\(^{-1}\), \( r^2 = 0.71 \); Tang et al. 2010), suggesting other picoeukaryotic algae beyond those tested in this study possess high affinity B1 uptake systems. A high affinity, adenosine triphosphate (ATP)-driven thiamine transport system is a logical candidate for bringing B1 into the cell against the dramatic B1 concentration gradient present between seawater (\(< 0.05 \) pmol B1 L\(^{-1}\) to \( \sim 100 \) pmol B1 L\(^{-1}\)) and the inside of a picoeukaryotic phytoplankton cell (24.6–32.2 \( \mu \)mol B1 L\(^{-1}\); based on the minimum yield quota for CCE9901, Table 1). Possible candidate proteins have been identified via sequence analysis (Worden et al. 2009), but are yet to be characterized in the laboratory.

The low \( K_s \) values of picoeukaryotic phytoplankton make them highly competitive for B1 in our tested picocyanobacteria, nor any other algae to the best of our knowledge. Notably, A. anophagefferens CCMP1984 also has a relatively low \( K_s \) value, \( \sim 6 \) pmol L\(^{-1}\), \( r^2 = 0.71 \); Tang et al. 2010), suggesting other picoeukaryotic algae beyond those tested in this study possess high affinity B1 uptake systems. A high affinity, adenosine triphosphate (ATP)-driven thiamine transport system is a logical candidate for bringing B1 into the cell against the dramatic B1 concentration gradient present between seawater (\(< 0.05 \) pmol B1 L\(^{-1}\) to \( \sim 100 \) pmol B1 L\(^{-1}\)) and the inside of a picoeukaryotic phytoplankton cell (24.6–32.2 \( \mu \)mol B1 L\(^{-1}\); based on the minimum yield quota for CCE9901, Table 1). Possible candidate proteins have been identified via sequence analysis (Worden et al. 2009), but are yet to be characterized in the laboratory.

The low \( K_s \) values of picoeukaryotic phytoplankton make them highly competitive for B1 at low concentrations. However, their \( K_s \) values are high relative to published B1 concentrations from oligotrophic euphotic waters (Fig. 6), making it feasible that some picoeukaryotic phytoplankton experience growth limitation due to B1 availability in such habitats. There is broad interest in characterizing nutrient limitation of picophytoplankton populations (eukaryotes and cyanobacteria) in the pelagic ocean. Primarily, “classic”
**Fig. 4.** Maximum yields of B1-limited algal cultures grown on B1 or TMP or TDP (all added at a final concentration of 1 nmol L\(^{-1}\)). Means of triplicate cultures are presented; error bars represent one standard deviation from the mean and where not visible, fall within the bounds of the line. All cultures were exposed to \(\sim 160 \mu\text{mol quanta m}^{-2} \text{s}^{-1}\) white light.

**Fig. 5.** *O. lucimarinus* CCE9901 is unable to utilize TDP under reduced PO\(_4\) concentrations, but begins to utilize TDP in co-culture with *Pseudomonas* sp. TW7. (A) Maximum Chl a fluorescence of *O. lucimarinus* CCE9901 cultures grown on F/2 medium with varying additions of B1, TDP and phosphate concentrations. B1 or TDP was added at a final concentration of 1 nmol L\(^{-1}\). Filter-sterilized (0.2 \(\mu\)m) sodium dihydrogen phosphate monohydrate stock was added at a final concentration of \(\sim 36 \mu\text{mol L}^{-1}\) (noted as + PO\(_4\)) or at varying concentrations (as noted). An *O. lucimarinus* CCE9901 culture in F/2 medium \(-\text{PO}_4\) \(-\text{B1}\) in exponential growth was used to inoculate the first grow-out experiment (larger plot). Maximum yield data for cultures transferred a second time (1 : 10, culture to medium) to respective media (ensuring phosphate concentrations and B1 concentrations were sufficiently low in the starting F/2 \(-\text{PO}_4\) \(-\text{B1}\) medium) are presented on the inset plot. All cultures exhibited positive net growth before the second transfer. Data presented are from triplicate cultures; error bars are plotted (although not visible in most cases as they fall within the bounds of the line) and represent one standard deviation from the mean. (B) *O. lucimarinus* CCE9901 growth increases in co-cultures with TW7 following the addition of TDP. TDP (1 nmol TDP L\(^{-1}\)) was added (where applicable) immediately following the transfer of co-cultures. Values for \(-\text{B1} + \text{TW7}\) and \(+\text{TD} + \text{TW7}\) treatments are the means of maximum Chl a fluorescence for individual co-cultures across seven consecutive transfers \((n = 8)\). The data for \(+\text{TD} + \text{TW7}\) is from Fig. 4, where TDP was added to axenic *O. lucimarinus* CCE9901 cultures \((n = 3)\). Error bars represent one standard deviation from the mean.
macronutrients (N, P) and Fe have been considered as growth-limiting nutrients for picophytoplankton (Mann and Chisholm 2000; Davey et al. 2008). Representative picocyanobacterial (Synechococcus and Prochlorococcus) genomes possess complete B1 biosynthesis pathways (Sanudo-Wilhelmy et al. 2014) and are presumed not to depend on environmental B1 sources. In stark contrast, multiple picoeukaryotic phytoplankton depend on exogenous B1 (Table 1; Croft et al. 2006; Bertrand and Allen 2012; McRose et al. 2014). Picoeukaryotic phytoplankton cells are present throughout the oligotrophic pelagic ocean (Li 1994; Zubkov et al. 1998), where B1 concentrations are reported to be as low as < 0.05 pmol L\(^{-1}\) (Barada et al. 2013). Considering our presented \(K_s\) data alongside published B1 concentrations from euphotic open-ocean waters, B1-associated growth limitation of pico-phytoplankton in B1-deplete euphotic waters deserves further investigation.

**Ostreococcus** and **Micromonas** spp. have relatively high minimum B1 quotas

The minimum B1 cell quotas (pmol B1 µL\(^{-1}\) biovolume) for **Ostreococcus** and **Micromonas** spp. examined in this study were notably higher than that of *P. calceolata* CCMP1756, *A. anophagefferens* CCMP1984, and dinoflagellates (Table 1). Why there is a large difference based on biovolume remains unclear. Thiamine itself exhibits antioxidant activity in vitro (Lukienko et al. 2000) and is linked to hydrogen peroxide accumulation in plants (Ahn et al. 2007). Possibly thiamine is a more important component of cellular oxidative stress defense in these green algae than in other phytoplankton. Alternatively, there may be differences in the demand for B1 as an enzyme cofactor that may help explain these quota differences. A variety of TDP-requiring enzymes, e.g., oxidoreductases, dehydrogenases, and decarboxylases, belong to described superfamilies based on amino acid sequence and predicted function (Widmann et al. 2010). *O. lucimarinus* CCE9901, *P. calceolata* CCMP1756, and *A. anophagefferens* CCMP1984 possess a similar total number of TDP-requiring enzymes (11, 10, and 10, respectively), but there is a difference in the number belonging to each superfamily (Table 2). *O. lucimarinus* CCE9901 possesses a more consistent number of TDP-requiring enzymes across superfamilies where as *P. calceolata* CCMP1756 and *A. anophagefferens* CCMP1984 have more ketoacid dehydrogenases (PK2) than \(\alpha\)-ketoglutarate dehydrogenases (KDH) and decarboxylases (DC; Table 2). These patterns are largely consistent among related *Mamielales* and *Pelagomonadales* based on currently available genome and transcriptome data (Table 2). However, to thoroughly evaluate cofactor associated B1 demands of these small algae (especially vs. antioxidant demand), abundances of TDP-requiring enzymes need to be estimated per cell between strains.

Using our minimum quota estimates (Table 1), **Ostreococcus** and **Micromonas** spp. seem to be vitamin B1 rich prey for planktonic grazers based on B1 per biovolume (Table 1). Picoeukaryotic algae are desirable prey in the ocean and thought to be periodically grazed at rates > 1 d\(^{-1}\) (Worden et al. 2004). Primarily picoeukaryotic algae have been considered in terms of their carbon contribution to higher trophic levels (Worden et al. 2004), but likely they also serve as key sources of vitamins for predators, particularly vitamin auxotrophic grazers that lack high-affinity vitamin transporters.

The relatively high picoeukaryotic algal demand may at times significantly decrease B1 concentrations in oligotrophic coastal waters like those off southern CA, as described below. However, availability of B1 analogs may change the potential for B1 limitation of the overall community. At the San Pedro Ocean Time Series (SPOTS), off Los Angeles, California), **Ostreococcus** abundances reach upward of 8.2 \times 10^4 cells mL\(^{-1}\) in surface waters, ~ 3 \times 10^5 cells mL\(^{-1}\) near the deep Chl \(a\) max, and other B1 auxotrophic picoeukaryotic phytoplankton besides **Ostreococcus** sp. are expected to occur at SPOTS (Countway and Caron 2006). A concentration of 8.2 \times 10^4 B1 auxotrophic picoeukaryotic cells mL\(^{-1}\)
Table 2. Abundances and types of TDP-requiring enzymes in small (approximately less than three micrometers in diameter) eukaryotic algae belonging to the Mamielloales or Pelagomonadales. Individual TDP-requiring enzyme superfamilies classified by the TEED database are as follows: \( \text{KDH} = \alpha \)-ketoglutarate dehydrogenase, \( \text{K} \) (type 1 or 2) = 2-ketoacid dehydrogenases, \( \text{TK} \) = transketolase and \( \text{DC} \) = decarboxylases. The average (avg.) ± one standard deviation (SD) for total TDP-requiring (req.) enzymes and each superfamily are included in bold, in the last two rows. Transcriptomes or genomes were obtained from the Joint Genome Institute (JGI), the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (http://camera.calit2.net/mmetsp/) or Genoscope (https://bioinformatics.psb.ugent.be/gdb/bathycoccus/) websites.

| Organism                        | Order        | Transcripome or genome version | TDP Req. Enzymes | KDH | K1 | K2 | TK | DC |
|---------------------------------|--------------|--------------------------------|------------------|-----|----|----|----|----|
| O. lucimarinus CCE9901          | Mamielloales | jgi/Ost9901.3                 | 11               | 3   | 0  | 3  | 2  | 3  |
| O. tauri OTH95                  | Mamielloales | jgi/Ott4a                    | 10               | 2   | 0  | 3  | 2  | 3  |
| O. prasinos RCC99000             | Mamielloales | MMETSP0933                   | 10               | 2   | 0  | 2  | 4  |
| O. lucimarinus Clade-A-BCC118000| Mamielloales | MMETSP0939                   | 11               | 3   | 0  | 1  | 3  | 4  |
| Micromonas sp. CCMP1545          | Mamielloales | jgi/MicpuC3                  | 10               | 2   | 0  | 3  | 2  | 3  |
| Micromonas sp. RCC299            | Mamielloales | jgi/MicpuN3                  | 10               | 2   | 0  | 3  | 2  | 3  |
| Micromonas sp. CCMP1646          | Mamielloales | MMETSP1080                   | 16               | 6   | 0  | 2  | 4  | 4  |
| Bathycoccus prasinos RCC1105     | Mamielloales | bathy14mar2011               | 10               | 2   | 0  | 3  | 2  | 3  |
| P. caeleolata CCMP1756           | Pelagomonadales | MMETSP0887                 | 10               | 0   | 0  | 5  | 2  | 3  |
| P. caeleolata RCC596             | Pelagomonadales | MMETSP1328                 | 10               | 1   | 0  | 6  | 2  | 1  |
| A. anophagefferens CCMP1850      | Pelagomonadales | MMETSP0914                 | 11               | 1   | 0  | 6  | 2  | 2  |
| A. anophagefferens CCMP1984      | Pelagomonadales | jgi/Auran1                  | 10               | 1   | 0  | 6  | 2  | 1  |
| Mamielloales avg. ± SD           | 11.2 ± 2.07  | 2.75 ± 1.39                  | 0               | 2.5 ± 0.76     | 2.38 ± 0.74    | 3.38 ± 0.52    |
| Pelagomonadales avg. ± SD        | 10.25 ± 0.5  | 0.75 ± 0.5                   | 0               | 5.75 ± 0.5     | 2.00 ± 0.0      | 1.75 ± 0.96    |
B1 (B1 + P) as a B1 source. In contrast, none utilized the provided B1 moieties to satisfy their B1 requirements (Table 1); it may be that these strains utilize alternative pyrimidine and thiazole compounds rather than HET and HMP to salvage B1. The inability of O. lucimarinus CCE9901 (and possibly other Prasinophytes) to use TMP or TDP is not simply due to phosphatase inhibition by high phosphate concentrations in our test medium (~36 μmol PO₄ L⁻¹ in F/2; Fig. 5). O. lucimarinus CCE9901 presumably lacks extracellular phosphatases that can dephosphorylate TDP or the appropriate proteins for direct TDP uptake. Furthermore, TMP and TDP utilization was not inhibited relative to B1 utilization at high phosphate concentrations in M. pusilla CCMP487 and P. calceolata CCMP1756 suggesting that dephosphorylation is not a prerequisite for uptake of TDP and TDP in these strains. Contrasting with the Prasinophytes, P. calceolata CCMP1756, and P. lutheri CCMP1325 (belonging to classes Pelagophyceae and Pavlovophyceae, respectively) utilized HMP and B1 + P (Table 1). As these strains are able to use HMP, it is expected that they possess a complete thiazole biosynthesis pathway; this has yet to be genetically identified or characterized. Given the recent demonstration of HMP utilization by E. huxleyi, these data suggest that HMP use by marine algae may be widespread (McRose et al. 2014). Additionally, P. lutheri CCMP1325 was used previously to estimate B1 concentrations in seawater samples (Carlucci and Silbernagel 1966). P. lutheri-associated B1 concentration estimates can now be understood to include (at least) B1 + P and HMP and thus overestimate B1 concentrations.

TDP is considered to be an important active form of B1 that serves as an enzyme cofactor and/or riboswitch ligand (Jurgenson et al. 2009). Presumably, obtaining B1 + P, especially TDP, from the environment is advantageous, enabling conservation of intracellular resources required for de novo TDP biosynthesis or salvaging (e.g., enzyme synthesis, ATP, P). Some model and marine bacteria can use TDP as a B1 source (Burkholder and Lewis 1968; Webb et al. 1998). Considering intracellular concentrations of TDP can reach ~x1.3–2 that of B1 in larger phytoplankton (Pinto et al. 2003) and marine plankton mortality is expected to be high (Sherr and Sherr 1994; Suttle 1994), B1 + P might be present in seawater at significant concentrations despite potentially fast degradation or dephosphorylation rates, either of which remain incompletely characterized.

The utilization and availability of B1 analogs could be key in determining the persistence of particular auxotrophic picoeukaryotic algae in regions of the euphotic ocean, especially as B1 concentrations are apparently very low in oligotrophic euphotic waters (e.g., < 0.05 pmol B1 L⁻¹; Sañudo-Wilhelmy et al. 2012; Barada et al. 2013). B1 Kₚ values are comparable to HMP Kₛ values for at least one B1 auxotrophic algae (P. calceolata CCMP1756; Table 1) and initial measurements of one moiety (HMP) indicate it is present at concentrations equivalent to or greater than concentrations of B1 in seawater (Carini et al. 2014). Although, the advantages obtained by picoeukaryotic phytoplankton using B1 analogs are yet to be elucidated, but presuming that analogs exist at environmentally relevant concentration in seawater, like HMP, we speculate about their ecological effects.

The HMP demand by picoeukaryotic algae (like P. calceolata CCMP1756 and E. huxleyi) potentially affect the growth of B1 auxotrophic populations with obligate HMP requirements, like Pelagibacter ubique HTCC1062 and other representatives of the abundant SAR11 bacterioplankton clade (Carini et al. 2014). Additional estimates of HMP concentrations in the euphotic ocean, HMP turnover rates, and further characterization of HMP uptake and/or growth kinetics of algal and bacterial populations will provide an improved understanding of competition for HMP amongst plankton in the upper ocean. We did find that P. calceolata CCMP1756 utilized HMP at a comparable capacity to that of B1, making it an equivalent resource to B1 for this organism (Table 1).

In B1-deplete waters, B1 analogs could promote the growth of select portions of the microbial community, e.g., promoting phytoplankton like P. calceolata CCMP1756 over phytoplankton like O. lucimarinus CCE9901, as the two appear to not salvage the same compounds. It is currently unknown if HAB-associated B1 auxotrophic species, e.g., A. anophagefferens spp. and P. globosa, are capable of salvaging analogs for B1 biosynthesis. If so, the success and persistence of HAB-associated B1 auxotrophic populations could depend on their ability to use B1 analog pools.

Clearly B1 analogs deserve further consideration as B1 sources for auxotrophic plankton (algae, bacteria, and zooplankton) in the ocean. B1 has been a primary focus of recent research largely due to the development of chemical methods to estimate concentrations of the B1 molecule in seawater (Sañudo-Wilhelmy et al. 2012; Barada et al. 2013). More recently, HMP concentrations were estimated and it appears to serve a critical role in sustaining the growth of several representatives of the SAR11 bacterioplankton clade (Carini et al. 2014). B1 analogs beyond HMP exist in nature, e.g., TMP, TDP, and could also play key roles in B1 cycling, microbial interactions, and the ecology of B1 auxotrophic plankton.

Coculturing of O. lucimarinus CCE9901 with TW7 implicates bacteria as conduits in B1 cycling

Knowing that O. lucimarinus CCE9901 could not utilize TDP as a sole B1 source, we determined that a simple consortium with a B1 prototrophic (synthesizing) bacterium (TW7), known to exhibit extracellular or periplasmic phosphatase activity (Chichester et al. 2008) enabled O. lucimarinus CCE9901 to grow on the phosphorylated B1 analog TDP (Fig. 5B). It is likely that the added TDP was dephosphorylated to B1 by TW7-derived phosphatases, then utilized by O. lucimarinus CCE9901. This result demonstrates that bacteria can increase vitamin availability beyond de novo synthesis.
and also it suggests that B1 auxotrophic plankton like O. lucimarinus CCE9901, that are unable to utilize B1 + P (and HMP, HET) alone, ultimately depend on other microorganisms to modify B1 + P to an utilizable form. These are newly described functional interactions within marine microbial consortia that may be critical in marine B1 cycling, depending on modification and turnover rates of B1 analogs in seawater and the B1 physiologies of ecologically important B1 auxotrophs, e.g., SAR11 and SAR86 clade representatives (Dupont et al. 2011; Carini et al. 2014).

Bacteria are critical remineralizers of organic compounds in the ocean (Kirchman 2000) and they are traditionally viewed as “producers” of vitamins (especially B12; Haines and Guillard 1974). More recently, they have been proposed as top competitors in terms of uptake for available vitamins in seawater (specifically B1 and B12; Koch et al. 2012), although how this relates to net vitamin availability is unknown. Our results suggest an additional role for bacteria as conduits that do not extensively remineralize a vitamin analog (TDP) but rather chemically modify it and make it available to microbes that cannot use the analog in isolation. We anticipate B1 and analogs play key roles in interactions between B1 auxotrophic algae and B1 prototrophic microbes and in the survival of B1 auxotrophic picoeukaryotic algae in the sea. Here we have demonstrated one instance where a B1 analog (TDP) fits into a simple microbial interaction, primarily benefiting a B1 auxotrophic picoeukaryotic alga.

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