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ORIGINAL ARTICLE

# Discovery of a SAR11 growth requirement for thiamin's pyrimidine precursor and its distribution in the Sargasso Sea

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**Vitamin traffic, the production of organic growth factors by some microbial community members and their use by other taxa, is being scrutinized as a potential explanation for the variation and highly connected behavior observed in ocean plankton by community network analysis. Thiamin (vitamin $B_1$), a cofactor in many essential biochemical reactions that modify carbon–carbon bonds of organic compounds, is distributed in complex patterns at subpicomolar concentrations in the marine surface layer (0–300 m). Sequenced genomes from organisms belonging to the abundant and ubiquitous SAR11 clade of marine chemoheterotrophic bacteria contain genes coding for a complete thiamin biosynthetic pathway, except for *thiC*, encoding the 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) synthase, which is required for *de novo* synthesis of thiamin's pyrimidine moiety. Here we demonstrate that the SAR11 isolate '*Candidatus* Pelagibacter ubique', strain HTCC1062, is auxotrophic for the thiamin precursor HMP, and cannot use exogenous thiamin for growth. In culture, strain HTCC1062 required 0.7 zeptomoles per cell (ca. 400 HMP molecules per cell). Measurements of dissolved HMP in the Sargasso Sea surface layer showed that HMP ranged from undetectable (detection limit: 2.4 pM) to 35.7 pM, with maximum concentrations coincident with the deep chlorophyll maximum. In culture, some marine cyanobacteria, microalgae and bacteria exuded HMP, and in the Western Sargasso Sea, HMP profiles changed between the morning and evening, suggesting a dynamic biological flux from producers to consumers.**

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## Introduction

Thiamin (vitamin $B_1$) is an essential coenzyme found in proteins that catalyze crucial transformations of carbon in all living systems. Specifically, thiamin is an essential cofactor for enzymes of the tricarboxylic acid cycle, the non-oxidative portion of the pentose phosphate pathway, the Calvin cycle and for enzymes required for the biosynthesis of branched-chain amino acids and isoprenoids (via the non-mevalonate pathway) (Lengeler *et al.*, 1999). The pathways, enzymes and regulation of *de novo* thiamin synthesis and salvage have been the topic of extensive research in bacteria, yeasts and some microalgae (Winkler and Breaker, 2005; Croft *et al.*, 2007; Jurgenson *et al.*, 2009). In all organisms capable of *de novo* thiamin biosynthesis, the formation of thiamin monophosphate (ThP) results from the enzyme-catalyzed linkage of two separately synthesized moieties: 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate and 4-methyl-5-(2-phosphoethyl)-thiazole (Figure 1). Phosphorylation of ThP yields the active thiamin coenzyme, thiamin diphosphate (ThPP) (Figure 1) (reviewed in Jurgenson *et al.*, 2009).

Renewed interest in vitamin distributions in marine ecosystems has been driven by the development of

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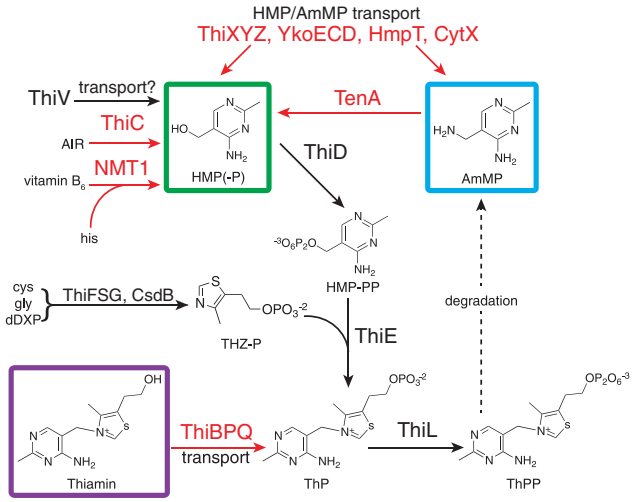


**Figure 1** Simplified illustration of thiamin metabolism in *Ca.* P. ubique. Black colored lines and enzyme abbreviations represent reactions and enzymes encoded by the *Ca.* P. ubique genome. Red colored lines and enzyme abbreviations represent reactions and enzymes that are absent from the *Ca.* P. ubique genome. AIR, aminoimidazole ribotide; AmMP, 4-amino-5-amino-methyl-2-methylpyrimidine; cys, cysteine; dDXP, 1-deoxy-D-xylulose 5-phosphate; gly, glycine; his, histidine; HMP(-P), 4-amino-5-hydroxymethyl-2-methylpyrimidine (-phosphate); HMP-PP, 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate; ThP, thiamin monophosphate; ThPP, thiamin diphosphate; THZ-P, 4-methyl-5-(2-phosphoethyl)-thiazole.

more sensitive analytical techniques to measure vitamin concentrations (Sañudo-Wilhelmy *et al.*, 2012) and a greater appreciation of the importance of trace compounds to plankton productivity. Whereas the sources, distributions and speciation of trace metals have been extensively researched as they pertain to ocean productivity (reviewed in Morel and Price, 2003), relatively little is known about vitamin biogeochemistry or the affect of vitamins on the structure and composition of planktonic communities. Direct measurements of B-vitamin concentrations in coastal ocean systems found picomolar concentrations and complex patterns in the distributions of several vitamins, including thiamin (Sañudo-Wilhelmy *et al.*, 2012; Barada *et al.*, 2013). In bottle experiments, iron and B-vitamins, particularly vitamin $B_{12}$, acted synergistically to increase phytoplankton and bacterial productivity, suggesting colimitation (Panzeca *et al.*, 2006; Bertrand *et al.*, 2007). Supporting the view that the exchange of vitamins between species is important, adaptive strategies for coping with low vitamin concentrations have been identified in diatoms (Bertrand *et al.*, 2012). Furthermore, there is evidence that some marine bacteria produce vitamin $B_{12}$ that is used by phytoplankton (Croft *et al.*, 2005).

Thiamin is a particularly interesting vitamin because the genomes of many environmentally abundant microorganisms do not encode for complete, canonical thiamin biosynthetic pathways (Bertrand and Allen, 2012; Helliwell *et al.*, 2013), suggesting that auxotrophy is common. The distribution of thiamin biosynthetic genes in algal genomes does not correlate well with phylogeny, an indication that thiamin metabolism has evolved and diversified in response to selective pressures that vary with habitat (reviewed in Croft *et al.*, 2006; Helliwell *et al.*, 2013). The evolution of thiamin metabolism in phytoplankton is likely complex, as evidenced by the ability of some strains to use the thiamin moieties 4-methyl-5-thiazolethanol (THZ) or 4-amino-5-aminomethyl-2-methylpyrimidine (AmMP), presumably natural thiamin degradation products, in place of thiamin (Droop, 1958; Lewin, 1962). A specific requirement for the thiamin pyrimidine precursor 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) has been described for the protist *Plasmodium falciparum* (Wrenger *et al.*, 2006) and the bacterium *Listeria monocytogenes* (Schauer *et al.*, 2009). Moreover, thiamin is exclusively obtained through salvage of thiamin moieties by the bacterium *Rhizobium leguminosarum* bv. viciae strain 3841 (Karunakaran *et al.*, 2006). Environmental concentrations of these thiamin precursors or degradation products have not been measured, and thiamin metabolism in marine bacteria is a relatively unexplored topic.

This study examines thiamin metabolism in the SAR11 clade of α-proteobacteria (*Pelagibacterales*). These organisms are the most abundant chemoheterotrophic bacterioplankton in the oceans, often comprising 25–50% of the cells in the euphotic zone (Morris *et al.*, 2002; Carlson *et al.*, 2009). Both *in situ* studies and those with axenic cultures show that the *Pelagibacterales* contribute significantly to the cycling of carbon and sulfur in the ocean (reviewed in Tripp, 2013). The first cultivated *Pelagibacterales* bacterium, '*Candidatus* Pelagibacter ubique' strain HTCC1062 (*Ca.* P. ubique), contains one of the smallest genomes found in free-living organisms. The small genome of *Ca.* P. ubique is attributed to streamlining selection (Giovannoni *et al.*, 2005). Gene loss related to streamlining selection has been proposed as an explanation for the unusual combination of amino acids, reduced organosulfur compounds and organic acids required for the growth of *Ca.* P. ubique (Carini *et al.*, 2013; Tripp, 2013). Although the macronutrient requirements of *Ca.* P. ubique have been identified, their requirements for vitamins and other trace molecules have not been investigated.

We used comparative genomics to examine the distribution of genes for thiamin metabolism among the *Pelagibacterales*, and studied the requirement for thiamin or its precursors in *Ca.* P. ubique. Following up on the surprising finding that *Ca.* P. ubique requires the thiamin precursor HMP, we applied high-performance liquid chromatography-coupled tandem mass spectrometry (LC-MS) to show that dissolved HMP is present at picomolar concentrations in the oceans. These findings offer important new insights into thiamin cycling, and identify HMP as a growth factor that is likely to have

an important role in vitamin-mediated interactions in the ocean.

## Materials and methods

*Metabolic reconstruction of thiamin biosynthesis in* Ca. *P. ubique and other* Pelagibacterales

To identify putative protein domains involved in thiamin biosynthesis, amino-acid sequences of known *Escherichia coli* (ThiC, ThiD, ThiE, ThiS, ThiG, ThiL, ThiF, IscS and ThiH), *Bacillus subtilis* (ThiO) and *Saccharomyces cerevisiae* (NMT1) thiamin biosynthesis proteins were used as query sequences in an HMMER search against the Pfam database (v.27.0), using the Pfam website (http://pfam.sanger.ac.uk/search) with default settings. Identified Pfam domains were extracted from the Pfam-A database and prepared as an hmmscan (v.3.1b) compliant database. This database was used to search predicted amino-acid sequences of *Ca.* P. ubique ORFs for putative protein domains involved in thiamin biosynthesis using hmmscan (http://hmmer.janelia.org; v.3.1b) (Supplementary Data set 1). A similar approach was used to identify *Ca.* P. ubique genes involved in thiamin biosynthesis using the Sifting Families (Sfam) Hidden Markov Model (HMM) database (Sharpton *et al.*, 2012) in place of Pfam (Supplementary Data set 2). When an ORF from *Ca.* P. ubique was predicted to match a Pfam and/or Sfam identified from a Thi_ query (*e*-value $\leqslant 1.0 \times 10^{-35}$), it was assumed that the *Ca.* P. ubique gene was a homolog of the query. The best hit for *E. coli* ThiL in the Pfam database (PF00586) is the N-terminal domain of aminoimidazole ribonucleotide synthase-related proteins—a putative ATP-binding domain. Proteins associated with this Pfam model are numerous and functionally diverse. Therefore, ThiL homologs in *Ca.* P. ubique were assigned based on the strength of their best-hit Sfam model alone.

The Hal pipeline (Robbertse *et al.*, 2011) was used to identify genes encoding Thi biosynthesis homologs, in seven additional *Pelagibacterales* genomes (HTCC1002, HTCC9565, HTCC7211, HIMB5, HIMB114, HIMB59 and IMCC9063). Orthologous groups were established using the pipeline Hal, as described in Thrash *et al.* (2014). The Hal pipeline connects the programs BLASTP, MCL, user-specified alignment programs, GBlocks, ProtTest and user-specified phylogenetic programs. Hal uses an all-versus-all BLASTP search and MCL clustering to identify orthologs, as described in detail in Robbertse *et al.* (2011).

*Construction of ThiV phylogenetic trees*

RAxML (Stamatakis, 2006) was used for phylogenetic inference, after alignment with MUSCLE (Edgar, 2004), curation with Gblocks (Castresana, 2000) and amino-acid substitution modeling with ProtTest (Abascal *et al.*, 2005). SAR11_0811 was initially identified as a ThiV homolog by searching the amino-acid sequence against others at MicrobesOnline (http://microbesonline.org/). This search identified SAR11_0811 as a member of the COG591 gene family, which had orthologs in the genomes of eight additional organisms: *Methylobacillus flagellatus* KT, *Marinobacter* sp. ELB17, *Clostridium* sp. OhILAs, *Haloquadratum walsbyi* DSM 16790, *Haloarcula marismortui* ATCC 43049, *Halorhabdus utahensis* DSM 12940, *Haloferax volcanii* DS2 and *Halogeometricum borinquense* PR3, DSM 11551. Eight SAR11_0811 orthologs in other SAR11 genomes (HTCC1002, HTCC9565, HTCC7211, HIMB5, AAA240-E13, AAA288-G21, HIMB114 and IMCC9063) were identified with the Hal pipeline (Robbertse *et al.*, 2011; Thrash *et al.*, 2014). To provide a fuller phylogenetic context for the trees, additional homologs to ThiV amino-acid sequences from the genomes above were searched against the Sfam HMM database (Sharpton, *et al.*, 2012). Further details are provided in Supplementary Documentation.

*Organism source and cultivation details*

*Ca.* P. ubique was revived from 10% glycerol stocks and propagated in AMS1, without added vitamins, amended with oxaloacetate (1 mM), glycine (50 μM), methionine (50 μM) and $FeCl_3$ (1 μM) (Carini *et al.*, 2013). Thiamin or precursors were added as indicated in figure legends and text. All cultures were grown in acid-washed and autoclaved polycarbonate flasks and incubated at 20 °C with shaking at 60 r.p.m. in the dark, unless noted otherwise. Cells for counts were stained with SYBR green I and counted with a Guava Technologies flow cytometer (Millipore, Billerica, MA, USA) at 48–72 h intervals as described elsewhere (Tripp *et al.*, 2008).

Cultures tested for HMP exudation were grown in acid-washed and autoclaved polycarbonate flasks, incubated at 20 °C with shaking at 60 r.p.m. on a 14-h/10-h light (140–180 μmol photons $m^{-2} s^{-1}$)/dark cycle and monitored by flow cytometry as described for *Ca.* P. ubique. For HMP exudation assays, axenic batch cultures of *Synechococcus* sp. WH8102 and *Prochlorococcus* sp. MED4 (CCMP2389) were grown in PCRS-11 Red Sea medium (Rippka *et al.*, 2000). *Dunaliella tertiolecta* (CCMP1320) was grown in AMS1 medium without vitamins (Carini *et al.*, 2013). The OM43 clade isolate, sp. HTCC2181, was grown in natural seawater with no added vitamins as described elsewhere (Giovannoni *et al.*, 2008).

All AMS1 constituents, reagents and vitamins were of the highest available quality (labeled 'ultrapure' when possible). To minimize unintended traces of vitamins from glassware, all nutrient and vitamin stocks were prepared in combusted glassware (450 °C for 4 h) with nanopure water, 0.1 μm filter sterilized and frozen in amber tubes immediately after preparation. HMP was synthesized as described in Reddick *et al.* (2001). AmMP was synthesized as described in Zhao *et al.* (2012).

HMP was purified by chromatography and then recrystallized. It was characterized by $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectroscopy and by mass spectrometry. AMP was purified by crystallization and was characterized by $^{1}H$ and $^{13}C$ nuclear magnetic resonance spectroscopy spectroscopy. No impurities were detected.

*HMP and thiamin concentrations in seawater*

Seawater for vitamin analysis was collected from Hydrostation S (32°10′N, 64°30′W) from casts at 2000 hours (local time) on 19 September 2012, and 0800 hours (local time) on 20 September 2012. At the time of collection, samples were filtered through nanopure water-rinsed 0.2 μm pore-size supor filters into acid-washed amber polypropylene bottles and frozen immediately.

HMP and thiamin were extracted from 300 ml seawater to a reverse-phase C18 silica bead solid phase (Agilent HF-Bondesil, Agilent Technologies, Santa Clara, CA, USA) as described in Sañudo-Wilhelmy *et al.* (2012). For quantification purposes, standard curves were constructed from aged seawater (collected from Hydrostation S in July of 2009) spiked with known amounts of HMP and thiamin (ranging from 0 to 100 pM). These standard curves (Supplementary Figures S1 and S2) were extracted alongside samples using identical procedures.

Extracts were reconstituted in 125 μl high-performance liquid chromatography-grade water. Samples were centrifuged to pellet insoluble matter and the supernatant was transferred to sampling vials. HMP was quantified using an Applied Biosystems MDS Sciex 4000 Q TRAP (Foster City, CA, USA) mass spectrometer coupled to a Shimadzu high-performance liquid chromatography system. An Agilent Zorbax SB-Aq (Agilent Technologies) ($2.1 \times 100\,mm^2$, 3.5 μm) high-performance liquid chromatography column was used for separation over a 10-min gradient flow with mobile phases of pH 4 (formic acid) methanol (MeOH) and pH 4 (formic acid) 5 mM ammonium formate (AmF). The flow rate was $0.4\,ml\,min^{-1}$ and a gradient starting at 98% AmF:2% MeOH for 1 min changing to 75% AmF:25% MeOH over 3 min, 50% AmF:50% MeOH over 0.2 min, and finally to 10% AmF:90% MeOH over 0.8 min. The retention time of HMP was approx. 1.8 min.

For HMP quantification, the mass spectrometer was run in 'Multiple Reaction Monitoring' mode. The HMP parent ion $m/z$ was 140.2, and ion transitions of 81.1 and 54.1 were used for quantification and qualification, respectively. Peaks were analyzed using the Analyst software package v.1.5.2 (AB SCIEX, Concord, ON, Canada). Measured HMP values are the average of technical LC-MS replicates. The greatest standard deviation of replicate measurements was 3.5 pM (coefficient of variation = 10%) in the 120 m 0800 hour sample, and the lowest was 0.22 pM (coefficient of variation = 3.5%) in the 200 m 20:00 hour sample. Thiamin was detected and quantified as described in Sañudo-Wilhelmy *et al.* (2012) The limit of detection is defined as three times the standard deviation of the procedural controls and the limit of quantification as 10 times the standard deviation of the procedural controls. The limit of detection for HMP was 2.4 pM (limit of quantification: 8.0 pM) and for thiamin it was 0.81 pM (limit of quantification: 2.7 pM; from Sañudo-Wilhelmy *et al.* (2012)).

*Cell harvesting of marine microbes for HMP exudation assays and detection of HMP background in AMS1*

During mid-logarithmic growth (approx $1.0 \times 10^7$ cells $ml^{-1}$), 100 ml of culture was gently filtered (to prevent cell lysis) through 0.1 or 0.2 μm pore-size supor filters to remove cells. The filtrate was collected in an acid-washed amber polypropylene bottle and frozen immediately. Uninoculated media (negative control) for each media type (AMS1, PCRS-11 Red Sea medium and natural seawater medium for HTCC2181) was extracted alongside spent medium treatments for comparison. HMP extraction and detection by LC-MS were performed as described for natural seawater samples.

## Results

Thiamin biosynthetic pathways were incomplete in all eight *Pelagibacterales* genomes we studied (Table 1). Despite the apparent inability to synthesize thiamin *de novo*, multiple genes encoding ThPP-dependent enzymes were identified in *Ca.* P. ubique, indicating thiamin is necessary for normal metabolism (Supplementary Figure S3). Four *Pelagibacterales* strains contained the same thiamin biosynthesis and transport genes as *Ca.* P. ubique (Table 1). Two additional *Pelagibacterales* strains, IMCC9063 and HIMB114, have complements of thiamin biosynthesis and transport genes similar to *Ca.* P. ubique, except both are missing *thiL* (Table 1). Additionally, IMCC9063 encodes the AmMP salvage enzyme, *tenA* (Table 1). In *Pelagibacterales* str. HIMB59, *thiC*, *thiD*, *thiG*, *thiE* and *thiE2* and *thiS* are absent. However, a gene encoding a thiamin-specific periplasmic binding protein (*thiB*) (Webb *et al.*, 1998) was identified in HIMB59 (Table 1).

Genes encoding the HMP synthase (*thiC*) are absent from all *Pelagibacterales* genomes (Table 1). ThiC catalyzes the molecular rearrangement of the purine nucleotide biosynthetic intermediate 5-aminoimidazole ribotide to form HMP (Figure 1) (Martinez-Gomez and Downs, 2008) and is essential for *de novo* thiamin biosynthesis in bacteria, archaea and plants. Genes that encode alternate HMP synthesis or salvage proteins were not identified in the *Ca.* P. ubique genome. For example, *Ca.* P. ubique lacks genes encoding for NMT1, which

**Table 1** Comparative genomics of thiamin biosynthesis in the *Pelagibacterales*

| *Strain* | thiC | thiD | thiE_0583[a] | thiE_0360[a] | thiF | thiS | thiG | csdB[b] | thiL | thiB | tenA |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Ca*. P. ubique | Absent | 637671479 | 637671458 | 637671224 | 637671266 | 637671603 | 637671604 | 637671616 | 637671913 | Absent | Absent |
| HTCC1002 | Absent | 639129819 | 639129840 | 639130075 | 639130033 | 639129702 | 639129701 | 639130662, 639129689 | 639130810 | Absent | Absent |
| HTCC7211 | Absent | 2503353714 | 2503353735 | 2503352435 | 2503352394 | 2503352877 | 2503352878 | 2503352890 | 2503353193 | Absent | Absent |
| HTCC9565 | Absent | 2503364149 | 2503364170 | 2503364413 | 2503364372 | 2503364883 | 2503364884 | 2503364896 | 2503365124 | Absent | Absent |
| HIMB5 | Absent | 2504109247 | 2504109269 | 2504109551 | 2504109508 | 2504108506 | 2504108507 | 2504108519, 2504109389 | 2504108893 | Absent | Absent |
| HIMB114 | Absent | 2503356000 | 2503356022 | 2503356319 | 2503356274 | 2503355884 | 2503355883 | 2503355872 | Absent | Absent | Absent |
| IMCC9063 | Absent | 2505688345 | 2505688367 | 2505687345 | 2505687250 | 2505687878 | 2505687879 | 2505688259 | Absent | Absent | 2505687352 |
| HIMB59 | Absent | Absent | Absent | Absent | 2504110146 | Absent | Absent | 2504110964 | 2504110802 | 2504111022 | Absent |

Gene numbers are IMG/ER Gene IDs (https://img.jgi.doe.gov/er).
[a]There are two copies of *thiE* in *Ca*. P. ubique: SAR11_0583 and SAR11_0360.
[b]*csdB* is predicted to encode the cysteine desulfurase activity necessary for thiazole biosynthesis (see Supplementary Methods).

synthesizes HMP from vitamin $B_6$ and histidine in *Saccharomyces cerevisiae* (Figure 1) (Wightman and Meacock, 2003). Genes encoding TenA homologs, which catalyze the hydrolysis of AmMP to yield HMP (Jenkins *et al.*, 2007), were also not present in *Ca*. P. ubique (Figure 1). Some organisms can transport thiamin intact with the thiamin-specific ABC transporter encoded by *thiBPQ*. No homologs of the thiamin-specific binding protein, ThiB, were identified in *Ca*. P. ubique genomes (Figure 1). Further, *Ca*. P. ubique does not encode homologs of the predicted bacterial HMP/AmMP ABC transport complexes ThiXYZ (Jenkins *et al.*, 2007) and YkoEDC, or for the putative HMP/AmMP permeases HmpT and CytX (Rodionov *et al.*, 2002, 2008).

A single predicted ThPP-activated RNA riboswitch was identified in the *Ca*. P. ubique genome (Meyer *et al.*, 2009) in an unusual configuration upstream of a coding sequence annotated as a sodium:solute symporter family protein (encoded by *Ca*. P. ubique ORF SAR11_0811). A similarly configured riboswitch was previously identified in the genome of *Methylobacillus flagellatus*, upstream of a coding sequence for an uncharacterized putative transporter named *thiV* (Rodionov *et al.*, 2002). Maximum-likelihood phylogenetic analysis of the *Pelagibacterales thiV* homologs showed that they form a monophyletic group with the *thiV* sequences from *M. flagellatus* and a diverse group of microbes, including, Haloarchaea, Gram-positive bacteria and β- and γ-proteobacteria (Figure 2a and Supplementary Figure S4). Genes orthologous to *thiV* in all organisms (except for *Marinobacter algicola*) are either (i) in an operon with genes encoding enzymes that enable the salvage of HMP and THZ moieties for thiamin synthesis (*thiD*, *thiM* and *thiE*; Figure 2b); (ii) in an operon with one or two copies of the *tenA* gene (encoding an AmMP salvage enzyme; Figure 2b); or (iii) are preceded by a ThPP-riboswitch motif (Figures 2b and c).

We hypothesized that *Ca*. P. ubique is auxotrophic for HMP because genes coding for known HMP synthesis pathways (*thiC* and *NMT1*) and AmMP salvage mechanisms (*tenA*) were absent (Figure 1 and Table 1). To test this hypothesis, the growth responses of *Ca*. P. ubique to HMP, AmMP and thiamin were investigated across seven orders of magnitude (Figure 3). Cultures grown in medium containing no added HMP, without additional thiamin or precursors, attained maximum cell densities of $3.09 \pm 0.75 \times 10^7$ cells ml$^{-1}$ (mean ± s.d., $n = 3$) (Figure 3). Cell yields responded linearly to HMP additions between 1 and 100 pM (Supplementary Figure S5) and reached maximal cell yields (ca. $3.5 \times 10^8$ cells ml$^{-1}$) at HMP concentrations $\geqslant 1$ nM (Figure 3). The cellular HMP requirement was calculated to be 0.66 zeptomoles (396 molecules) per cell from the slope of the linear regression between 1 and 100 pM (Supplementary Figure S5). Thiamin and AmMP were ineffective at restoring thiamin-limited growth at pico- or nanomolar

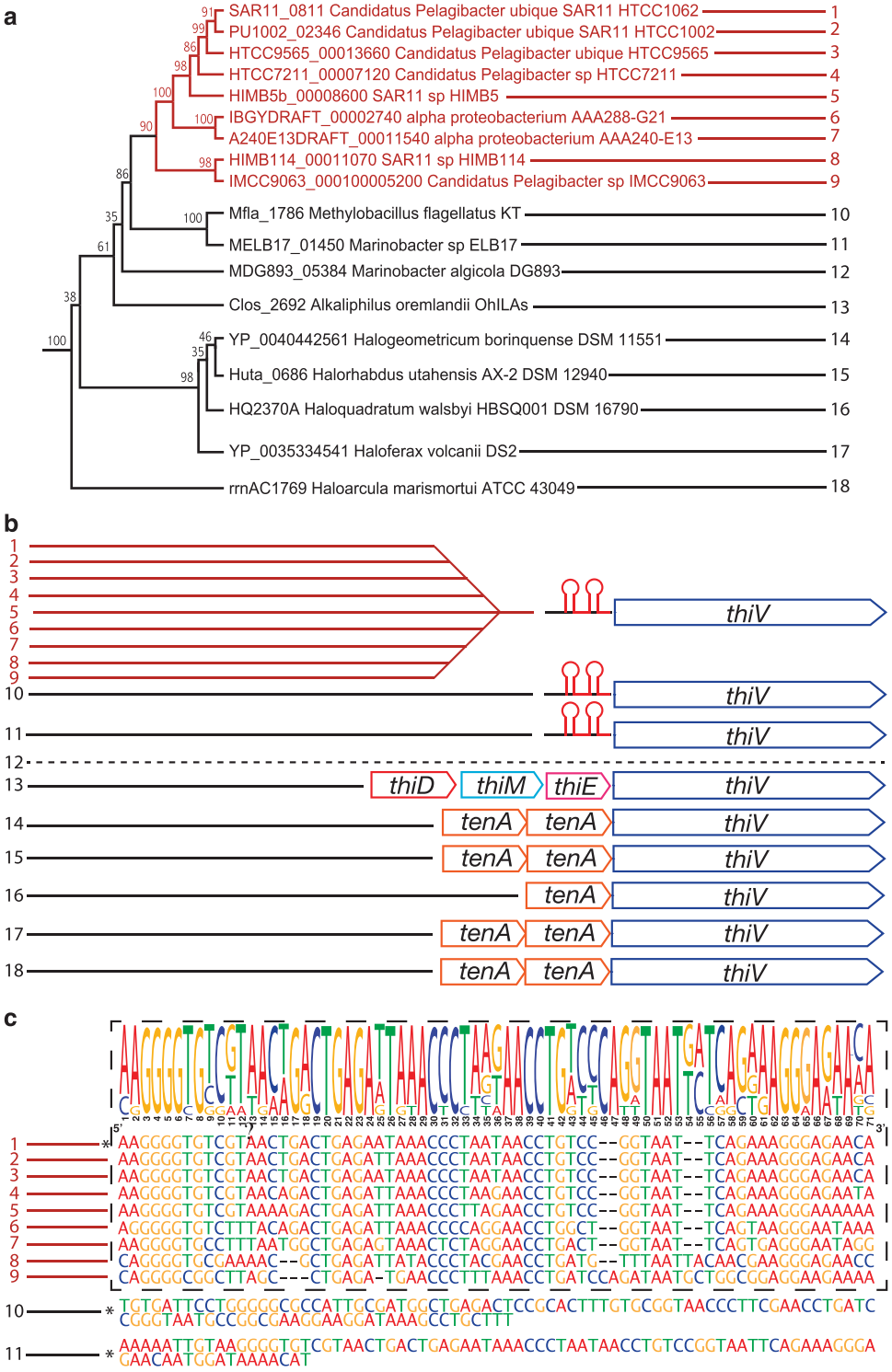


**Figure 2** Gene phylogeny, synteny and conservation of riboswitch structure for the *Pelagibacterales* ThiV family sodium:solute symporter. *Pelagibacterales* genome elements are highlighted in red. (**a**) Maximum-likelihood phylogenetic tree showing a subset of amino-acid sequences extracted from a complete tree (Supplementary Figure S4). (**b**) For the same taxa shown in **a**, the chromosomal colocalization of *thiV* genes with putative ThPP-binding riboswitches (red stem-loop structure) and genes encoding thiamin salvage enzymes (*thiDME* or *tenA*). Dashed line indicates no ThPP-binding riboswitch or associated salvage genes were identified. (**c**) Nucleotide sequences of predicted ThPP-binding riboswitches depicted in **b**. Dashed box encapsulates the riboswitch sequences from nine *Pelagibacterales* genomes and their consensus sequence (illustrated at the top). Sequences that are marked with (*) were predicted to contain ThPP-binding motifs using the rfam (http://rfam.sanger.ac.uk) sequence search tool.

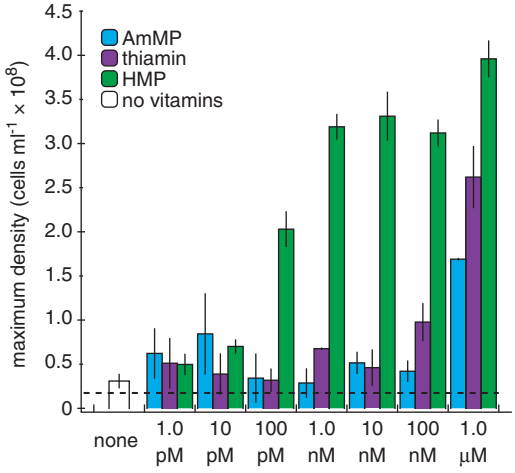


**Figure 3** Maximum cell yields of *Ca.* P. ubique batch cultures in response to AmMP, thiamin and HMP additions. Cells were grown in AMS1 amended with thiamin, HMP or AmMP as indicated. Bar heights are the average densities of biological replicates ± s.d. ($n = 3$). The dashed line represents the calculated maximum density expected ($\sim 1.8 \times 10^7$ cells ml$^{-1}$) from the 'background' level of HMP (see text for details). We attribute the growth with 1 µM thiamin or AmMP to 'contaminating' HMP (see text for details).

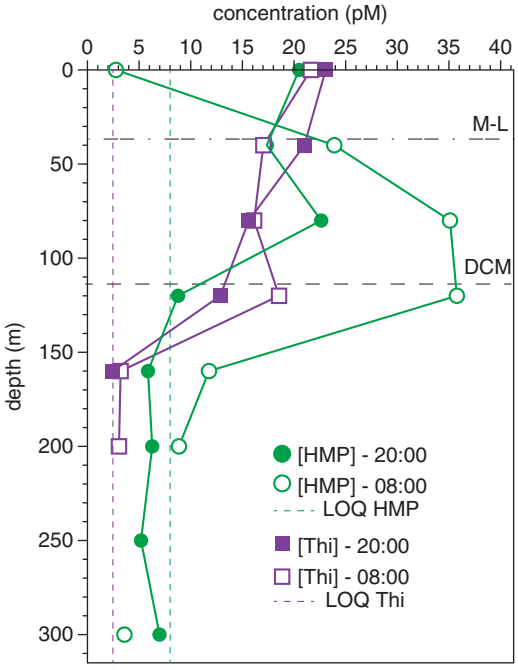


**Figure 4** Depth distribution of dissolved HMP and thiamin in the Sargasso Sea. Times of collection are presented in local time. HMP values are the average of technical replicate analyses for each sample. There was no technical replication for the thiamin measurements owing to insufficient sample. HMP was not detected in the 250 m sample collected at 0800 hours. Thiamin was not detected in samples collected from 200 m at 2000 hours or at 250 and 300 m at either time. DCM, deep chlorophyll maximum; LOQ, limit of quantification; M-L, mixed layer.

concentrations; these compounds restored growth only when supplied at 1.0 µM (Figure 3). The average growth rate of *Ca.* P. ubique was 0.29 ± 0.03 per day (mean ± s.d., $n = 123$) and did not vary with vitamin or precursor treatments (for example, see Supplementary Figure S6).

To rule out NMT1 activity as a potential source of HMP, thiamin was replaced with histidine and vitamin $B_6$ (NMT1's substrates; Ishida *et al.*, 2008). Consistent with the prediction that *Ca.* P. ubique lacks the ability to synthesize HMP through NMT1 activity, histidine + vitamin $B_6$ did not alleviate thiamin-limited growth (Supplementary Figure S7). Thiamin-limited growth was not relieved by pantothenate or THZ addition (Supplementary Figure S7) as has been reported previously for other organisms (Droop, 1958; Downs, 1992).

To date, measurements of HMP or AmMP concentrations in the environment have not been reported. To determine if HMP is present in an environment where *Pelagibacterales* bacteria are also found, thiamin and HMP were extracted from Sargasso Sea seawater collected at two different times of the day (2000 and 0800 hours local time, approximately 1 h after sunset and sunrise, respectively) and quantitatively measured by LC-MS. HMP ranged from undetectable (detection limit: 2.4 pM) to 35.7 pM (Figure 4). The maximum concentration of HMP was observed in samples collected at 0800 hours near the deep chlorophyll maximum (Figure 4). HMP concentrations at 2000 hours were substantially higher at 0 m depth, but lower at depths of 40, 80, 120, 160 and 200 m, compared with samples collected at 0800 hours (Figure 4). HMP was not detected in the 250 m sample collected at 0800 hours. Thiamin was measured in the same samples and ranged from undetectable (detection limit: 0.81 pM; Sañudo-Wilhelmy *et al.*, 2012) to 23 pM, and was present in samples from 0 to 160 m, but not detected in samples from 250 to 300 m (Figure 4).

To determine whether marine microbes exude HMP, we measured HMP concentrations in growth media before and after cell growth in strains known to have a complete complement of thiamin biosynthetic genes (Table 2). The marine cyanobacterium *Synechococcus* sp. WH8102 and the marine chlorophyte *D. tertiolecta* exuded nanomolar amounts of HMP during growth (Table 2). Moderate amounts of excess HMP were also detected in spent media from cyanobacterium *Prochlorococcus* MED4 and the OM43 clade of marine β-proteobacteria isolate, strain HTCC2181 (Giovannoni *et al.*, 2008). Two *Pelagibacterales* cultures were also tested: *Ca.* P. ubique and *Pelagibacterales* sp. strain HTCC7211. In both cases, HMP was not detected after cell growth (Table 2).

## Discussion

Thiamin has long been recognized as an important vitamin for microalgal growth (reviewed in Croft

**Table 2** HMP concentrations in uninoculated and partially spent media

| *Organism* | *HMP (pM)* | |
|---|---|---|
| | *Uninoculated* | *Partially spent* |
| *Synechococcus* sp. WH8102 | N/D | 2909.6 |
| *Dunaliella tertiolecta* | 11.6 | 1584.3 |
| *Prochlorococcus* sp. MED4 | N/D | 32.8 |
| OM43 isolate HTCC2181 | 12.9 | 33.0 |
| *Ca.* P. ubique strain HTCC1062 | 11.6 | N/D |
| *Pelagibacterales* sp. strain HTCC7211 | 11.6 | N/D |

Abbreviations: HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; N/D, not detected.
Limit of detection = 2.4 pM.

*et al.*, 2006). The physiological requirement for thiamin led to the hypothesis that environmental concentrations of thiamin may exert control over some phytoplankton populations (Natarajan, 1968; Panzeca, *et al.*, 2006). Environmental distributions of thiamin, as determined by bioassay, were variable, and in some cases, coupled to productivity (Natarajan and Dugdale, 1966; Natarajan, 1968, 1970). Studies of thiamin auxotrophy in the laboratory showed that thiamin moieties or degradation products were able to satisfy the thiamin requirement of some microalgae (Droop, 1958; Lewin, 1962). However, research pursuing the ecological importance of these findings tapered off. The experimental results presented here reintroduce the idea that thiamin pyrimidines are important growth determinants in marine ecosystems. We show that the thiamin pyrimidine precursor, HMP, is required for growth of the marine chemoheterotrophic bacterium *Ca.* P. ubique (Figure 3), a representative isolate of one of the most abundant groups of organisms on the planet. Surprisingly, neither thiamin itself nor AmMP satisfied this requirement (Figure 3). Comparative genomics extended the significance of this requirement to multiple members of the *Pelagibacterales* clade (Table 1). The importance of these findings were further supported by the detection of dissolved HMP in the Sargasso Sea (Figure 4), one of the most oligotrophic ocean systems on Earth, at concentrations often exceeding those of thiamin. This discovery shows that fundamental information needed to understand thiamin biogeochemistry in marine ecosystems is incomplete—specifically that environmental measurements of thiamin alone may only partially explain interactions related to the thiamin requirements of planktonic cells.

The inability of *Ca.* P. ubique to use thiamin or its degradation product AmMP was surprising given that many algal species are able to use these compounds (Droop, 1958; Lewin, 1962). The *Ca.* P. ubique genome encodes no thiamin transporter (Figure 1 and Table 1), consistent with the observation that exogenous thiamin does not support growth (Figure 3). Likewise, we propose that the absence of the *tenA* gene (Figure 1 and Table 1), necessary for the conversion of AmMP to HMP, explains why AmMP does not substitute for HMP in thiamin biosynthesis. However, genome analysis of *Pelagibacterales* strain HIMB59 shows that this strain lacks genes required for *de novo* synthesis of thiamin (*thiC*, *thiD*, *thiG*, *thiE* and *thiS*), as well as the AmMP salvage enzyme (*tenA*; Table 1) and *thiV*; therefore, we postulate that this strain requires exogenous thiamin. Supporting this idea, *thiB*, encoding the periplasmic subunit of a thiamin-specific thiamin ABC transporter, was identified in HIMB59 (Table 1).

The new data reported here indicate that thiamin cycling in the oceans may follow complex patterns and involve multiple processes and intermediates. Whereas we show that marine microbes can release HMP into the surrounding environment (Table 2), some phytoplankton exude thiamin (Carlucci and Bowes, 1970a, b). Although thiamin is labile in seawater (Gold, 1968; Gold *et al.*, 1966), its decomposition products in seawater have not been fully characterized and the effect of various environmental factors on degradation are poorly understood. For example, thiamin is a light-sensitive molecule that is readily cleaved by UV-B radiation to AmMP and other products (Okumura, 1961; Machlin, 1984). Although no measurements of AmMP concentrations in the environment have been reported, the physiological responses of phytoplankton to AmMP (Droop, 1958; Lewin, 1962) and the presence of *tenA* genes in some bacterial genomes that lack the *thiC* gene (Supplementary Table S1), including *Pelagibacterales* sp. strain IMCC9063 (Table 1), suggest that environmental AmMP is present, and might also be an important growth determinant in marine ecosystems.

Light-mediated decay of thiamin may be an important factor in thiamin geochemistry and influence HMP production patterns in marine surface waters. The depth profiles showing that the dissolved HMP maximum coincides with the deep chlorophyll maximum (Figure 4) suggest that marine phytoplankton may be important HMP producers. Intriguingly, previous studies reported diel periodicity in the transcription and translation of *thiC* (the HMP synthase) in laboratory cultures of *Prochlorococcus* MED4 (Waldbauer *et al.*, 2012). Similarly, the abundance of environmental transcripts mapping to *thiC* of *Synechococcus* sp. followed a diel pattern (Ottesen *et al.*, 2013). In both reports, maximum *thiC* transcript levels were observed in the mid-afternoon, shortly after the periods of highest light intensity. We speculate that the large differences in dissolved HMP concentrations from profiles collected at different times (Figure 4) may be an indication that HMP exudation by *thiC*-containing cyanobacteria also follows a diel pattern. Although measurements of dissolved vitamins (and precursors) reflect equilibrium concentrations,

not fluxes, reports of rapid rates of $^{3}$H-thiamin uptake by plankton communities (Koch *et al*., 2012) suggest that rapid water column vitamin depletion due to biological scavenging is feasible. The notable production of HMP by *Synechococcus* sp. WH8102 and modest exudation by *Prochlorococcus* MED4 batch cultures (Table 2) is consistent with the idea that cyanobacteria are important HMP producers; however, diel patterns of HMP production were not tested in our experiments.

The absence of *thiC*, and thus the requirement for exogenous thiamin pyrimidines, is not unique to the *Pelagibacterales*, but is broadly and unevenly distributed among diverse microbial taxa inhabiting marine waters. Incomplete thiamin biosynthetic gene complements were previously reported in the genomes of the uncultivated SAR86 clade of marine γ-proteobacteria (Dupont *et al*., 2012) and in some phytoplankton (reviewed in Bertrand and Allen, 2012; Helliwell *et al*., 2013). Genes for ThiC are also absent from the genomes of many other ecologically important marine bacteria and archaea (Supplementary Table S1). The observation that canonical thiamin biosynthetic pathways are incomplete in sequenced organisms was further mirrored in metagenomic data sets. Comparisons of the abundances of *thiC*, *thiD* and *thiG* across a metagenomic depth profile from the Sargasso Sea found that *thiC* genes were depleted relative to *thiD* and *thiG* genes at 0, 40 and 80 m, but near the deep chlorophyll maximum, copies of *thiC* exceeded those of *thiD* (Supplementary Figure S8). The relative deficiency of *thiC* to other essential thiamin biosynthesis genes in shallow waters is consistent with the idea that HMP salvage is important for thiamin synthesis at those depths.

We postulate that ThiV sodium:solute symporters constitute a new family of thiamin pyrimidine transport proteins. Previously Worden *et al*. hypothesized that ThiV and its homologs (identified as 'SSSF-P') might transport thiamin precursors in some eukaryotic phytoplankton and SAR11 that lacked canonical thiamin biosynthesis genes (Worden *et al*., 2009). Noting conservation of the relationship between TPP and ThiV across taxa, they concluded ThiV and its homologs might represent 'ancient thiamine-pathway components', but their function remained uncertain. A phylogeny of bacterial and archaeal ThiV orthologs supports this interpretation by showing that *thiV* genes colocalize with genes encoding for thiamin pyrimidine salvage enzymes (*tenA* in archaeal genomes and with *thiD*, *thiM* and *thiE* in the *Alkaliphilus oremlandii* genome) (Figure 2), implying that ThiV orthologs transport thiamin pyrimidines (HMP or AmMP). We speculate that *Ca*. P. ubique regulates the acquisition of HMP from the environment by controlling the expression of ThiV with a ThPP-binding riboswitch, in a manner akin to the ThPP-binding riboswitch regulation of *de novo* HMP synthesis (via ThiC) in other organisms (Winkler *et al*., 2002). When thiamin is bound to ThPP riboswitches, transcription and translation of the downstream coding sequence is repressed, and thus the detection of ThiV and other ThPP-regulated gene products in metaproteomes may be useful indicators of thiamin deprivation in the environment. For example, peptides mapping to *Pelagibacter* ThiV orthologs were detected in environmental metaproteomes from the Sargasso Sea (Sowell *et al*., 2009), but not the Southern Ocean (Williams *et al*., 2012), perhaps indicating differences in the thiamin status of the two biomes.

The dependence of *Ca*. P. ubique, and likely other *Pelagibacterales*, on HMP implies that these cells gain an advantage by outsourcing HMP production to other plankton, in essence relying on HMP as a publically available commodity. This perspective is consistent with genome streamlining theory, and previous reports of unusual nutrient requirements associated with genome reduction in *Pelagibacterales* (Tripp *et al*., 2008; Carini *et al*., 2013). Streamlining theory predicts that atypical nutrient requirements can arise in microorganisms that have large effective population sizes in response to selection favoring small cell size and the efficient use of limiting nutrient resources (Giovannoni *et al*., 2005). The 'Black Queen Hypothesis' explored the coevolutionary implications of genome streamlining theory, examining the broader context of adaptive gene loss in a framework that considered competition for public goods (Morris *et al*., 2012). In this context, because the *Pelagibacterales* depend on environmental HMP, there is potential for *Pelagibacter* growth limitation by HMP, intimately tying the success of these organisms to HMP producers.

Because *Ca*. P. ubique cells are among the smallest known, and replicate efficiently at very low nutrient concentrations, elucidating the trace nutrient requirements of these cells is technically challenging. Even in a defined minimal medium, when precautions were taken to minimize trace vitamin background, *Ca*. P. ubique reached $2–3 \times 10^{7}$ cells ml$^{-1}$ in the absence of added vitamins or precursors (Figure 3 and Supplementary Figures S6 and S7). These yields are within a factor of two of theoretical yields ($1.8 \times 10^{7}$ cells ml$^{-1}$) based on the cellular HMP requirement (Supplementary Figure S5) and the amount of 'background' HMP measured in the medium (12 pM). This 'background' HMP disappeared in the presence of *Ca*. P. ubique, implying consumption of the nutrient (Table 2). Previously, background levels of vitamins in heterotrophic growth medium were proposed to underlie scant growth of vitamin auxotrophs in the absence of added vitamins (Norman *et al*., 1981; Wu *et al*., 2005), and the difficulty associated with thiamin removal from growth medium has been noted (Button, 1968). The number of HMP molecules required per *Ca*. P. ubique cell is on the order of 400 molecules per cell (Supplementary Figure S5).

Assuming each HMP molecule is used to make one thiamin molecule, and an estimate of 6 fg carbon per cell (unpublished data), the thiamin/carbon ratio of *Ca*. P. ubique was calculated to be 25 ng thiamin per mg carbon—similar to the values measured for marine phytoplankton (5–100 ng thiamin per mg carbon; Carlucci and Bowes, 1972; Brown *et al.*, 1999). Thus, the cell titers we observed in the absence of added HMP are consistent with the explanation that even pure reagents (e.g., 98–99%) and water from reverse osmosis purifiers can contain very low concentrations of vitamins and vitamin precursors—enough to support the growth of cells that require miniscule amounts of vitamins.

Contaminating HMP was detected in the thiamin stock solution that was added to thiamin-amended treatments. The level of HMP 'contamination' in the concentrated thiamin stock was measured (via LC-MS) to be $\sim$2.6 nM HMP per 1 μM thiamin ($=0.0012$ g HMP per g thiamin) (Supplementary Figure S9). The unintended addition of approximately 2.6 nM HMP as a contaminant of the thiamin stock is the probable explanation for the growth restoration by thiamin at culture concentrations of 1 μM (Figure 3). The source of contaminating HMP appears to be the result of the commercial thiamin manufacturing process. Contaminating amounts of HMP in the AmMP stock could not be determined because HMP and AmMP have similar liquid chromatography retention times, and thus the application of large amounts of AmMP to the chromatography column obscured the detection of possible traces of HMP. We propose that HMP contamination in the AmMP preparation is also a plausible explanation for the slightly elevated yields at high AmMP concentrations.

This investigation illustrates the value of combining metabolic reconstruction from genomes with experimentation in the laboratory and field measurements of specific compounds to explore biogeochemical cycles. The demonstration that HMP exclusively satisfies the thiamin requirement of a highly abundant marine organism (Figure 3), is found in the ocean (Figure 4), and is exuded by some marine organisms (Table 2), identifies this compound as an important, previously unknown growth factor in marine systems. It is particularly surprising that thiamin and AmMP were not used by *Ca*. P. ubique, implying that HMP-producing organisms potentially could exert control over *Pelagibacterales* populations. Extending these findings outside of the *Pelagibacterales*, multiple genomes of cosmopolitan marine bacteria display incomplete thiamin synthesis pathways (Supplementary Table S1), suggesting that thiamin moiety scavenging may be a common strategy in marine waters. The specific mechanism of HMP exudation by marine phytoplankton is unknown. It is possible that in high light environments, intracellular thiamin is relatively unstable, preventing repression of the ThPP-regulated HMP synthase gene (*thiC*), and resulting in HMP overproduction. However, HMP might also partition to the membrane and from there to the extracellular environment because it is relatively hydrophobic, or its exudation could be driven by coevolutionary interactions. As yet, there is no evidence that favors one of these alternatives over another. A more complete understanding of HMP production patterns, as they pertain to vitamin cycling, will likely be important for understanding turnover and connectedness in plankton communities (Fuhrman *et al.*, 2006; Steele *et al.*, 2011).

## Conflict of Interest

The authors declare no conflict of interest.

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