Title:

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

Running title: SAR11 requires HMP

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Abstract

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 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 sub-picomolar 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 $cell^{-1}$ (*ca*. 400 HMP molecules $cell^{-1}$). Measurements of dissolved HMP in the Sargasso Sea surface layer, showed 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.

Keywords: vitamins, thiamine, B1, phytoplankton, micronutrient, auxotrophy
Subject Category: Microbial ecology and functional diversity of natural habitats
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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 TCA 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 & 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 (HMP-PP) and 4-methyl-5-(2-phosphoethyl)-thiazole (THZ-P) (Fig. 1). Phosphorylation of ThP yields the active thiamin coenzyme, thiamin diphosphate (ThPP) (Fig. 1) (reviewed in Jurgenson et al., 2009).

Renewed interest in vitamin distributions in marine ecosystems has been driven by the development of 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 & 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 co-limitation (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 (Helliwell et al., 2013; Bertrand & Allen, 2012), suggesting 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 Helliwell et al., 2013; Croft et al., 2006). 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 str. 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 un-explored 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' str. 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 (Tripp, 2013; Carini et al., 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 pM concentrations in the oceans. These findings offer important new insight into thiamin cycling, and identify HMP as a growth factor that is likely to play an important role in vitamin-mediated interactions in the ocean.

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 *E. 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 a HMMER search against the Pfam database (v27.0), 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 (v3.1b) 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; v3.1b) (Supplementary Dataset 1). A similar approach was used to identify *Ca.* P. ubique genes involved in thiamin biosynthesis using the Sifting Families HMM database (Sfam) (Sharpton et al., 2012) in place of Pfam (Supplementary Dataset 2). When an ORF from *Ca.* P. ubique was predicted to match a Pfam and/or Sfam identified from a Thi_ query (*e*-value $\leq 1.0\times10^{-35}$), 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

118 diverse. Therefore, ThiL homologs in *Ca.* P. ubique were assigned on the strength of their
119 best-hit Sfam model alone.

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

128 *Construction of ThiV phylogenetic trees:* RAxML (Stamatakis, 2006) was used
129 for phylogenetic inference, after alignment with MUSCLE (Edgar, 2004), curation with
130 Gblocks (Castresana, 2000), and amino acid substitution modeling with ProtTest
131 (Abascal et al., 2005). SAR11_0811 was initially identified as a ThiV homolog by
132 searching the amino acid sequence against others at MicrobesOnline
133 (http://microbesonline.org/). This search identified SAR11_0811 as a member of the
134 COG591 gene family, which had orthologs in the genomes of eight additional organisms:
135 *Methylobacillus flagellatus* KT, *Marinobacter* sp. ELB17, *Clostridium* sp. OhILAs,
136 *Haloquadratum walsbyi* DSM 16790, *Haloarcula marismortui* ATCC 43049,
137 *Halorhabdus utahensis* DSM 12940, *Haloferax volcanii* DS2 and *Halogeometricum*
138 *borinquense* PR3, DSM 11551. Eight SAR11_0811 orthologs in other SAR11 genomes
139 (HTCC1002, HTCC9565, HTCC7211, HIMB5, AAA240-E13, AAA288-G21,
140 HIMB114, and IMCC9063) were identified with the Hal pipeline (Robbertse et al., 2011;

141 Thrash et al., 2014). To provide a fuller phylogenetic context for the trees, additional
142 homologs to ThiV amino acid sequences from the genomes above were searched against
143 the SFam Hidden Markov Model (HMM) database (Sharpton, et al., 2012). Further
144 details are provided in supplementary documentation.

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

153 Cultures tested for HMP exudation were grown in acid-washed and autoclaved
154 polycarbonate flasks, incubated at 20°C with shaking at 60 RPM on a 14-h/10-h light
155 (140-180 μmol photons m^{-2} s^{-1})/dark cycle and monitored by flow cytometry as described
156 for *Ca*. P. ubique. For HMP exudation assays, axenic batch cultures of *Synechococcus sp*.
157 WH8102 and *Prochlorococcus sp*. MED4 (CCMP2389) were grown in PCRS-11 Red
158 Sea medium (Rippka et al., 2000). *Dunaliella tertiolecta* (CCMP1320) was grown in
159 AMS1 medium without vitamins (Carini et al., 2013). The OM43-clade isolate, sp.
160 HTCC2181, was grown in natural seawater with no added vitamins as described
161 elsewhere (Giovannoni et al., 2008).

162 All AMS1 constituents, reagents and vitamins were of the highest available
163 quality (labeled 'ultrapure' when possible). To minimize unintended traces of vitamins

164 from glassware, all nutrient and vitamin stocks were prepared in combusted glassware
165 (450°C for 4 h) with nanopure water, 0.1 μm-filter-sterilized and frozen in amber tubes
166 immediately after preparation. HMP was synthesized as described in Reddick et al.,
167 (2001). AmMP was synthesized as described in Zhao et al., (2012). HMP was purified by
168 chromatography and then recrystallized. It was characterized by ^{1}H and ^{13}C NMR
169 spectroscopy and by mass spectrometry. AMP was purified by crystallization and was
170 characterized by ^{1}H NMR and ^{13}C NMR spectroscopy. No impurities were detected.
171 *HMP and thiamin concentrations in seawater:* Seawater for vitamin analysis was
172 collected from Hydrostation S (32°10'N, 64°30'W) from casts at 20:00 (local time) on 19
173 September 2012, and 08:00 (local time) on 20 September 2012. At the time of collection,
174 samples were filtered through nanopure water-rinsed 0.2 μm pore-size supor filters into
175 acid-washed amber polypropylene bottles and frozen immediately.
176 HMP and thiamin were extracted from 300 mL seawater to a reverse-phase C18
177 silica bead solid phase (Agilent HF-Bondesil) as described in Sañudo-Wilhelmy et al.,
178 (2012). For quantification purposes, standard curves were constructed from aged
179 seawater (collected from Hydrostation S in July of 2009) spiked with known amounts of
180 HMP and thiamin (ranging from 0 pM to 100 pM). These standard curves
181 (Supplementary Figures S1 and S2) were extracted alongside samples using identical
182 procedures.
183 Extracts were reconstituted in 125 μL HPLC-grade water. Samples were
184 centrifuged to pellet insoluble matter and the supernatant was transferred to sampling
185 vials. HMP was quantified using an Applied Biosystems MDS Sciex 4000 Q TRAP mass
186 spectrometer coupled to a Shimadzu HPLC system. An Agilent Zorbax SB-Aq (2.1 × 100

mm, 3.5-micron) HPLC column was used for separation over a 10-minute 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 minute changing to 75% AmF: 25% MeOH over 3 minutes, 50% AmF: 50% MeOH over 0.2 minutes, and finally to 10% AmF:90% MeOH over 0.8 minutes. The retention time of HMP was appx. 1.8 minutes.

For HMP quantification, the mass spectrometer was run in 'Multiple Reaction Monitoring' (MRM) 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 (CV = 10%) in the 120 m 08:00 sample, and the lowest was 0.22 pM (CV=3.5%) in the 200 m 20:00 sample. Thiamin was detected and quantified as described in Sañudo -Wilhelmy et al., (2012). The limit of detection (LOD) is defined as 3 times the standard deviation of the procedural controls and the limit of quantification (LOQ) as 10 times the standard deviation of the procedural controls. The LOD for HMP was 2.4 pM (LOQ: 8.0 pM) and, for thiamin, 0.81 pM (LOQ: 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 (appx 1.0×10^7 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 Fig. 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* & *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 (Fig. 1) (Martinez-Gomez & 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 synthesizes HMP from vitamin B_6 and histidine in *Saccharomyces cerevisiae* (Fig. 1) (Wightman & 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 (Fig. 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 (Fig. 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 of *M. flagellatus* and a diverse group of microbes, including, Haloarchaea, Gram-positive bacteria and β- and γ-proteobacteria (Fig. 2A and Supplementary Fig. 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*; Fig. 2B); ii) in an operon with one or two copies of the

256 *tenA* gene (encoding an AmMP salvage enzyme; Fig. 2B); or iii) are preceded by a ThPP-
257 riboswitch motif (Fig. 2B, C).

258 We hypothesized that *Ca*. P. ubique is auxotrophic for HMP because genes
259 coding for known HMP synthesis pathways (*thiC* and *NMT1*) and AmMP salvage
260 mechanisms (*tenA*), were absent (Fig. 1; Table 1). To test this hypothesis, the growth
261 responses of *Ca*. P. ubique to HMP, AmMP, and thiamin, were investigated across seven
262 orders of magnitude (Fig. 3). Cultures grown in medium containing no added HMP,
263 without additional thiamin or precursors, attained maximum cell densities of
264 $3.09 \pm 0.75 \times 10^7$ cells ml^{-1} (mean ± s.d., n=3) (Fig. 3). Cell yields responded linearly to
265 HMP additions between 1 and 100 pM (Supplementary Fig. S5) and reached maximal
266 cell yields (ca. 3.5×10^8 cells ml^{-1}) at HMP concentrations ≥1 nM (Fig. 3). The cellular
267 HMP requirement was calculated to be 0.66 zeptomoles (396 molecules) $cell^{-1}$ from the
268 slope of the linear regression between 1 pM and 100 pM (Supplementary Fig. S5).
269 Thiamin and AmMP were ineffective at restoring thiamin-limited growth at pico- or
270 nanomolar concentrations; these compounds restored growth only when supplied at 1.0
271 μM (Fig. 3). The average growth rate of *Ca*. P. ubique was 0.29 ± 0.03 d^{-1} (mean ± s.d.,
272 n=123) and did not vary with vitamin or precursor treatments (for example, see
273 Supplementary Fig. S6).

274 To rule out NMT1 activity as a potential source of HMP, thiamin was replaced
275 with histidine and vitamin B_6 (NMT1's substrates (Ishida et al., 2008)). Consistent with
276 the prediction that *Ca*. P. ubique lacks the ability to synthesize HMP through NMT1
277 activity, histidine + vitamin B_6 did not alleviate thiamin-limited growth (Supplementary
278 Fig. S7). Thiamin-limited growth was not relieved by pantothenate or THZ addition

279 (Supplementary Fig. S7) as has been reported previously for other organisms (Downs,
280 1992; Droop, 1958).

281 To date, measurements of HMP or AmMP concentrations in the environment
282 have not been reported. To determine if HMP is present in an environment where
283 *Pelagibacterales* bacteria are also found, thiamin and HMP were extracted from Sargasso
284 Sea seawater collected at two different times of day (20:00 and 08:00 local time,
285 approximately 1 hour after sunset and sunrise, respectively) and quantitatively measured
286 by LC-MS. HMP ranged from undetectable (detection limit: 2.4 pM) to 35.7 pM (Fig. 4).
287 The maximum concentration of HMP was observed in samples collected at 08:00 near
288 the deep chlorophyll maximum (Fig. 4). HMP concentrations at 20:00 were substantially
289 higher at 0 meters depth, but lower at depths of 40, 80, 120, 160 and 200 m, compared to
290 samples collected at 08:00 (Fig. 4). HMP was not detected in the 250 m sample collected
291 at 08:00. Thiamin was measured in the same samples and ranged from undetectable
292 (detection limit: 0.81 pM (Sañudo-Wilhelmy et al., 2012)) to 23 pM, and was present in
293 samples from 0 to 160 m, but not detected in samples from 250 and 300 m (Fig. 4).

294 To determine whether marine microbes exude HMP, we measured HMP
295 concentrations in growth media before and after cell growth in strains known to have a
296 complete complement of thiamin biosynthetic genes (Table 2). The marine
297 cyanobacterium *Synechococcus sp*. WH8102 and the marine chlorophyte *Dunaliella*
298 *tertiolecta* exuded nanomolar amounts of HMP during growth (Table 2). Moderate
299 amounts of excess HMP were also detected in spent media from cyanobacterium
300 *Prochlorococcus* MED4 and the OM43-clade of marine β-proteobacteria isolate, str.
301 HTCC2181 (Giovannoni, et al., 2008). Two *Pelagibacterales* cultures were also tested:

302 *Ca*. P. ubique and *Pelagibacterales sp*. str. HTCC7211. In both cases, HMP was not
303 detected after cell growth (Table 2).
304
305 **Discussion**
306 Thiamin has long been recognized as an important vitamin for microalgal growth
307 (reviewed in Croft et al., 2006). The physiological requirement for thiamin led to the
308 hypothesis that environmental concentrations of thiamin may exert control over some
309 phytoplankton populations (Natarajan, 1968; Panzeca, et al., 2006). Environmental
310 distributions of thiamin, as determined by bioassay, were variable, and in some cases,
311 coupled to productivity (Natarajan, 1968; 1970; Natarajan & Dugdale, 1966). Studies of
312 thiamin auxotrophy in the laboratory showed that thiamin moieties or degradation
313 products were able to satisfy the thiamin requirement of some microalgae (Lewin, 1962;
314 Droop, 1958). However, research pursuing the ecological importance of these findings
315 tapered off. The experimental results presented here reintroduce the idea that thiamin
316 pyrimidines are important growth determinants in marine ecosystems. We show that the
317 thiamin pyrimidine precursor, HMP, is required for growth of the marine
318 chemoheterotrophic bacterium *Ca*. P. ubique (Fig. 3), a representative isolate of one of
319 the most abundant groups of organisms on the planet. Surprisingly, neither thiamin itself,
320 nor AmMP satisfied this requirement (Fig. 3). Comparative genomics extended the
321 significance of this requirement to multiple members the *Pelagibacterales* clade (Table
322 1). The importance of these findings were further supported by the detection of dissolved
323 HMP in the Sargasso Sea (Fig. 4), one of the most oligotrophic ocean systems on earth, at
324 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 utilize thiamin or its degradation product AmMP was surprising given that many algal species are able to utilize these compounds (Lewin, 1962; Droop, 1958). The *Ca*. P. ubique genome encodes no thiamin transporter (Fig. 1 & Table 1); consistent with the observation that exogenous thiamin does not support growth (Fig. 3). Likewise, we propose that the absence of the *tenA* gene (Fig. 1 & 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* str. HIMB59 indicates 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 & Bowes, 1970a; 1970b). 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 (Machlin, 1984;

348 Okumura, 1961). Although no measurements of AmMP concentrations in the
349 environment have been reported, the physiological responses of phytoplankton to AmMP
350 (Lewin, 1962; Droop, 1958) and the presence of *tenA* genes in some bacterial genomes
351 that lack the *thiC* gene (Supplementary Table S1), including *Pelagibacterales sp.* str.
352 IMCC9063 (Table 1), suggest that environmental AmMP is present, and might also be an
353 important growth determinant in marine ecosystems.

354 Light-mediated decay of thiamin may be an important factor in thiamin
355 geochemistry and influence HMP production patterns in marine surface waters. The
356 depth profiles showing that the dissolved HMP maximum coincides with the deep
357 chlorophyll maximum (Fig. 4) suggest that marine phytoplankton may be important HMP
358 producers. Intriguingly, previous studies reported diel periodicity in the transcription and
359 translation of *thiC* (the HMP synthase) in laboratory cultures of *Prochlorococcus* MED4
360 (Waldbauer et al., 2012). Similarly, the abundance of environmental transcripts mapping
361 to *thiC* of *Synechococcus sp*. followed a diel pattern (Ottesen et al., 2013). In both
362 reports, maximum *thiC* transcript levels were observed in the mid afternoon, shortly after
363 the periods of highest light intensity. We speculate that the large differences in dissolved
364 HMP concentrations from profiles collected at different times (Fig. 4) may be an
365 indication that HMP exudation by *thiC*-containing cyanobacteria also follows a diel
366 pattern. Although measurements of dissolved vitamins (and precursors) reflect
367 equilibrium concentrations, not fluxes, reports of rapid rates of ^{3}H-thiamin uptake by
368 plankton communities (Koch et al., 2012) suggest that rapid water column vitamin
369 depletion due to biological scavenging is feasible. The notable production of HMP by
370 *Synechococcus sp*. WH8102 and modest exudation by *Prochlorococcus* MED4 batch

371 cultures (Table 2) is consistent with the idea that cyanobacteria are important HMP
372 producers, however diel patterns of HMP production were not tested in our experiments.

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

388 We postulate that ThiV sodium:solute symporters constitute a new family of
389 thiamin pyrimidine transport proteins. Previously it was hypothesized that ThPP-
390 regulated sodium:solute symporters, like ThiV, might transport thiamin moieties in
391 eukaryotes (Worden et al., 2009). A phylogeny of bacterial and archaeal ThiV orthologs
392 supports this interpretation by showing that *thiV* genes co-localize with genes encoding
393 for thiamin pyrimidine salvage enzymes (*tenA* in archaeal genomes and with *thiD*, *thiM*

394 and *thiE* in the *Alkaliphilus oremlandii* genome) (Fig. 2), implying that ThiV orthologs
395 transport thiamin pyrimidines (HMP or AmMP). We speculate that *Ca.* P. ubique
396 regulates the acquisition of HMP from the environment by controlling the expression of
397 ThiV with a ThPP-binding riboswitch, in a manner akin to the ThPP-riboswitch
398 regulation of *de novo* HMP synthesis (via ThiC) in other organisms (Winkler et al.,
399 2002). When thiamin is bound to ThPP-riboswitches, transcription and translation of the
400 downstream coding sequence is repressed, thus the detection of ThiV and other ThPP-
401 regulated gene products in metaproteomes may be useful indicators of thiamin
402 deprivation in the environment. For example, peptides mapping to *Pelagibacter* ThiV
403 orthologs were detected in environmental metaproteomes from the Sargasso Sea (Sowell
404 et al., 2009), but not the Southern Ocean (Williams et al., 2012), perhaps indicating
405 differences in the thiamin status of the two biomes.

406 The dependence of *Ca*. P. ubique, and likely other *Pelagibacterales*, on HMP
407 implies that these cells gain an advantage by outsourcing HMP production to other
408 plankton, in essence relying on HMP as a publically available commodity. This
409 perspective is consistent with genome streamlining theory, and previous reports of
410 unusual nutrient requirements associated with genome reduction in *Pelagibacterales*
411 (Tripp et al., 2008; Carini et al., 2013). Streamlining theory predicts that atypical nutrient
412 requirements can arise in microorganisms that have large effective population sizes in
413 response to selection favoring small cell size and the efficient use of limiting nutrient
414 resources (Giovannoni et al., 2005). The 'Black Queen Hypothesis' explored the co-
415 evolutionary implications of genome streamlining theory, examining the broader context
416 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 (Fig. 3 and Supplementary Figures S6 & S7). These yields are within a factor of two of theoretical yields (1.8×10^7 cells ml^{-1}) based on the cellular HMP requirement (Supplementary Fig. 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 (Wu et al., 2005; Norman et al., 1981), 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 $cell^{-1}$ (Supplementary Fig. S5). Assuming each HMP molecule is used to make one thiamin molecule, and an estimate of 6 fg carbon $cell^{-1}$ (unpublished data), the thiamin/carbon ratio of *Ca*. P. ubique was calculated to be 25 ng thiamin/mg carbon - similar to the values measured for marine phytoplankton (5-100 ng thiamin/mg carbon (Carlucci & 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 small 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 ~2.6 nmoles HMP per 1 μmole thiamin (=0.0012 g HMP per g thiamin) (Supplementary Fig. 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 (Fig. 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, 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 (Fig. 3), is found in the ocean (Fig. 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

463 that HMP-producing organisms potentially could exert control over *Pelagibacterales*
464 populations. Extending these findings outside of the *Pelagibacterales*, multiple genomes
465 of cosmopolitan marine bacteria display incomplete thiamin synthesis pathways
466 (Supplementary Table S1), suggesting thiamin moiety scavenging may be a common
467 strategy in marine waters. The specific mechanism of HMP exudation by marine
468 phytoplankton is unknown. It is possible that in high light environments, intracellular
469 thiamin is relatively unstable, preventing repression of the ThPP-regulated HMP synthase
470 gene (*thiC*), and resulting in HMP overproduction. But, HMP might also partition to the
471 membrane and from there to the extracellular environment because it is relatively
472 hydrophobic, or its exudation could be driven by co-evolutionary interactions. As yet,
473 there is no evidence that favors one of these alternatives over another. A more complete
474 understanding of HMP production patterns, as they pertain to vitamin cycling, will likely
475 be important for understanding turnover and connectedness in plankton communities
476 (Steele et al., 2011; Fuhrman et al., 2006) .
477
478 Supplementary information is available at ISMEJ's website.
479
480
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674

675 **Figure Legends:**

676 Figure 1: Simplified illustration of thiamin metabolism in *Ca*. P. ubique. Black colored

677 lines and enzyme abbreviations represent reactions and enzymes encoded by the *Ca*. P.

678 ubique genome. Red colored lines and enzyme abbreviations represent reactions and

679 enzymes that are absent from the *Ca*. P. ubique genome. Abbreviations: AIR -

680 aminoimidazole ribotide; his – histidine; HMP(-P) - 4-amino-5-hydroxymethyl-2-

681 methylpyrimidine (-phosphate); HMP-PP - 4-amino-5-hydroxymethyl-2-

682 methylpyrimidine diphosphate; THZ-P - 4-methyl-5-(2-phosphoethyl)-thiazole; AmMP -

683 4-amino-5-aminomethyl-2-methylpyrimidine; ThP – thiamin monophosphate; ThPP –

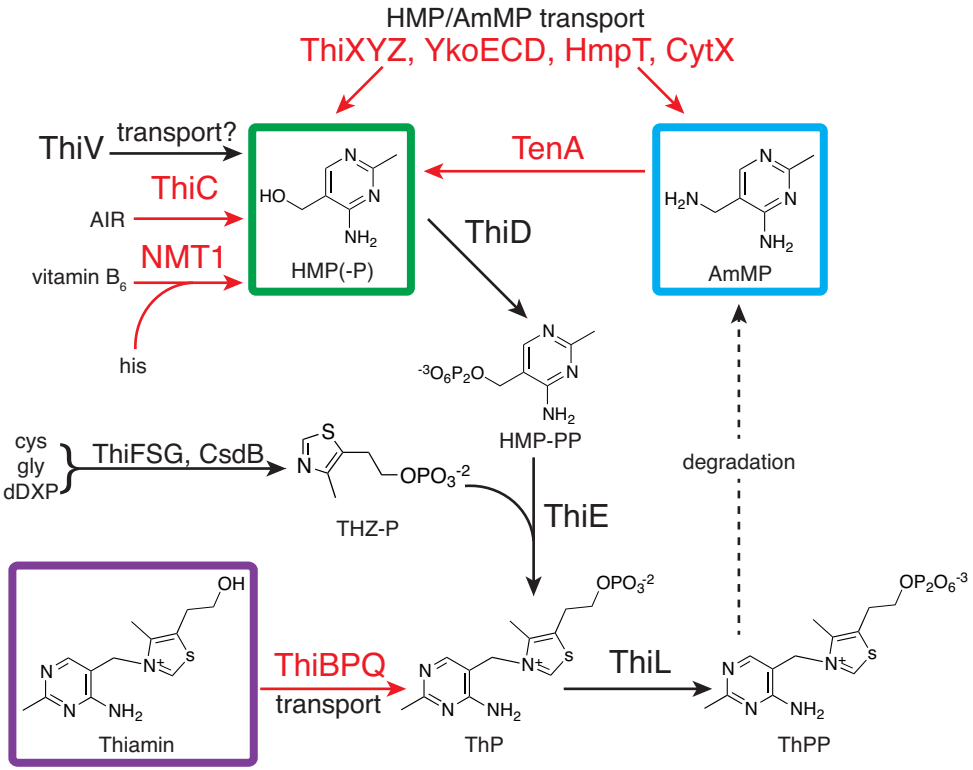
684 thiamin diphosphate; dDXP - 1-deoxy-D-xylulose 5-phosphate; gly – glycine; cys -
685 cysteine.

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

698

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

Figure 4: Depth distribution of dissolved 4-amino-5-hydroxymethyl-2-methylpyrimidine (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 due to insufficient sample. HMP was not detected in the 250 m sample collected at 08:00. Thiamin was not detected in samples collected from 200 m at 20:00 or at 250 m and 300 m at either time. LOQ: limit of quantification. M-L: Mixed Layer. DCM: deep chlorophyll maximum.


HMP/AmMP transport
ThiXYZ, YkoECD, HmpT, CytX
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transport?
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TenA
ThiD
AmMP
HMP-PP
cys
gly
dDXP
ThiFSG, CsdB
THZ-P
ThiE
degradation
Thiamin
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transport
ThP
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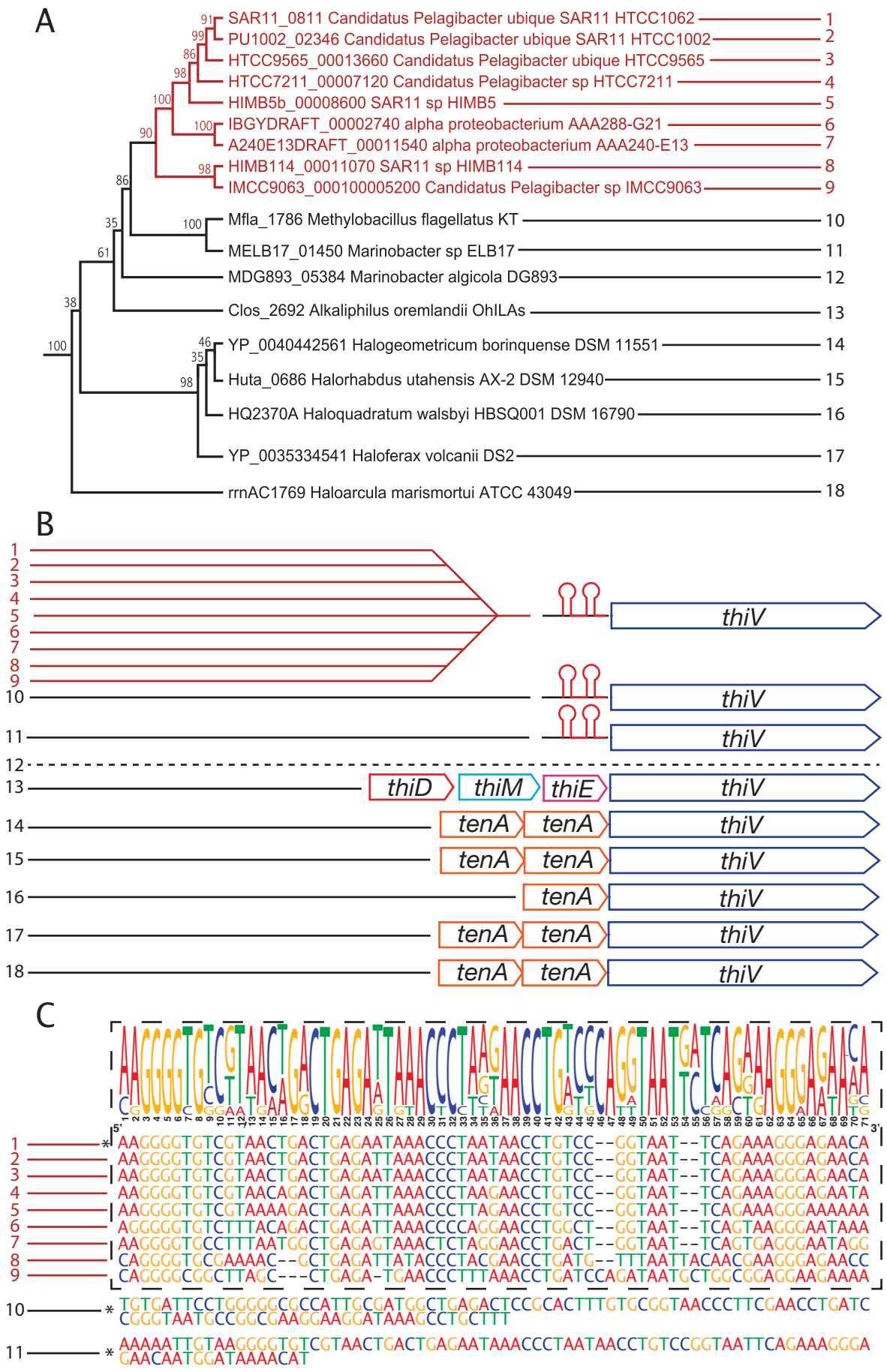
A
SAR11_0811 Candidatus Pelagibacter ubique SAR11 HTCC1062
PU1002_02346 Candidatus Pelagibacter ubique SAR11 HTCC1002
HTCC9565_00013660 Candidatus Pelagibacter ubique HTCC9565
HTCC7211_00007120 Candidatus Pelagibacter sp HTCC7211
HIMB5b_00008600 SAR11 sp HIMB5
IBGYDRAFT_00002740 alpha proteobacterium AAA288-G21
A240E13DRAFT_00011540 alpha proteobacterium AAA240-E13
HIMB114_00011070 SAR11 sp HIMB114
IMCC9063_000100005200 Candidatus Pelagibacter sp IMCC9063
Mfla_1786 Methylobacillus flagellatus KT
MELB17_01450 Marinobacter sp ELB17
MDG893_05384 Marinobacter algicola DG893
Clos_2692 Alkaliphilus oremlandii OhILAs
YP_0040442561 Halogeometricum borinquense DSM 11551
Huta_0686 Halorhabdus utahensis AX-2 DSM 12940
HQ2370A Haloquadratum walsbyi HBSQ001 DSM 16790
YP_0035334541 Haloferax volcanii DS2
rrnAC1769 Haloarcula marismortui ATCC 43049
B
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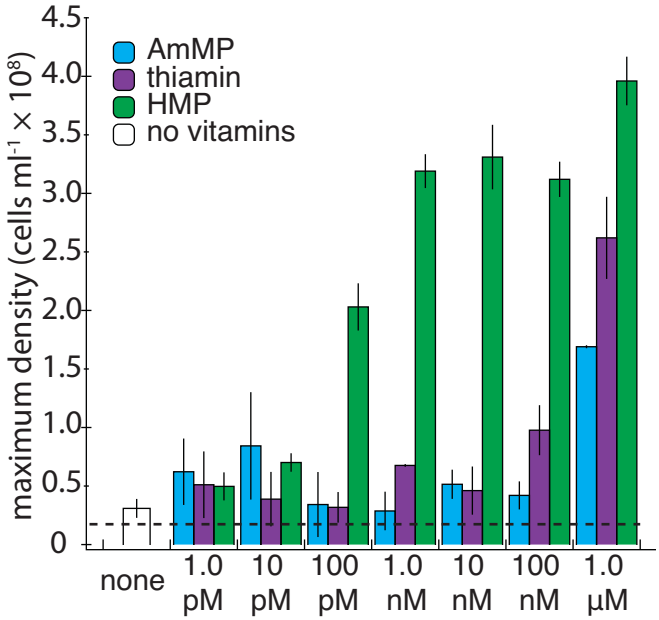
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maximum density (cells ml-1 × 10^8)
AmMP
thiamin
HMP
no vitamins
none
1.0 pM
10 pM
100 pM
1.0 nM
10 nM
100 nM
1.0 µM

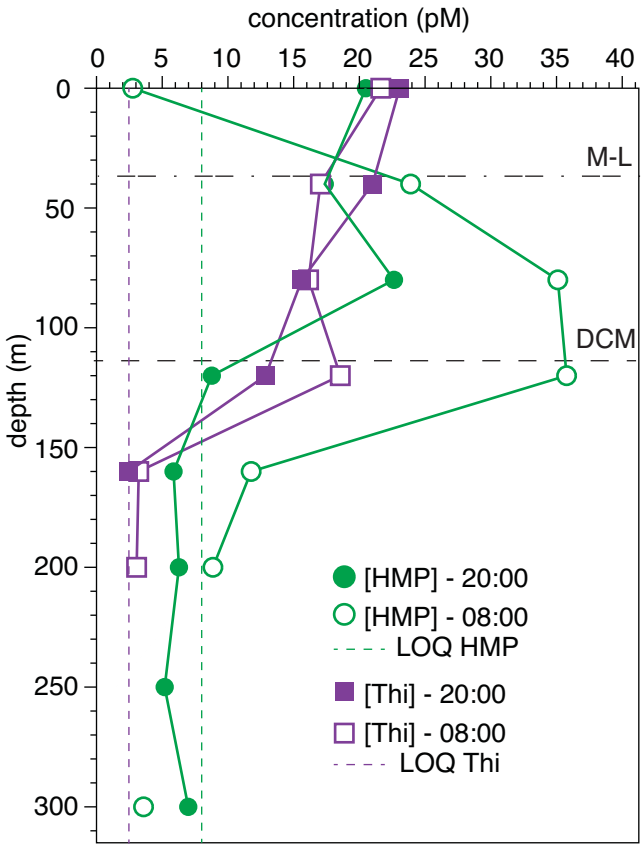
concentration (pM)
0
5
10
15
20
25
30
35
40
depth (m)
0
50
100
150
200
250
300
M-L
DCM
[HMP] - 20:00
[HMP] - 08:00
LOQ HMP
[Thi] - 20:00
[Thi] - 08:00
LOQ Thi

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

Strain	***thiC***	***thiD***	***thiE_0583*** †	***thiE_0360*** †	***thiF***	***thiS***	***thiG***
***Ca.* P. ubique**	absent	637671479	637671458	637671224	637671266	637671603	637671604
HTCC1002	absent	639129819	639129840	639130075	639130033	639129702	639129701
HTCC7211	absent	2503353714	2503353735	2503352435	2503352394	2503352877	2503352878
HTCC9565	absent	2503364149	2503364170	2503364413	2503364372	2503364883	2503364884
HIMB5	absent	2504109247	2504109269	2504109551	2504109508	2504108506	2504108507
HIMB114	absent	2503356000	2503356022	2503356319	2503356274	2503355884	2503355883
IMCC9063	absent	2505688345	2505688367	2505687345	2505687250	2505687878	2505687879
HIMB59	absent	absent	absent	absent	2504110146	absent	absent

Gene numbers are IMG/ER Gene ID's (https://img.jgi.doe.gov/er)

†There are two copies of *thiE* in *Ca.* P. ubique: *SAR11_0583* and *SAR11_0360.*

[1]*csdB* is predicted to encode the cysteine desulfurase activity necessary for thiazole biosynthesis (see supplementary methods)

csdB [1]	***thiL***	***thiB***	***tenA***
637671616	637671913	absent	absent
639130662, 639129689	639130810	absent	absent
2503352890	2503353193	absent	absent
2503364896	2503365124	absent	absent
2504108519, 2504109389	2504108893	absent	absent
2503355872	absent	absent	absent
2505688259	absent	absent	2505687352
2504110964	2504110802	2504111022	absent

Table 2: HMP concentrations in uninoculated and partially spent media

	HMP (pM)	
Organism	uninoculated	partially spent
Synechococcus sp. WH8102	N/D	2,909.6
Dunaliella tertiolecta	11.6	1,584.3
Prochlorococcus sp. MED4	N/D	32.8
OM43 isolate HTCC2181	12.9	33.0
Ca. P. ubique str. HTCC1062	11.6	N/D
Pelagibacterales sp. str. HTCC7211	11.6	N/D

N/D: not detected. Limit of detection = 2.4 pM