Cyanobacteria and Eukaryotic Algae Use Different Chemical Variants of Vitamin B$_{12}$

Graphical Abstract

Highlights

- Dominant marine cyanobacteria synthesize only pseudocobalamin
- Pseudocobalamin is orders of magnitude less bioavailable to eukaryotic algae
- Certain algae can remodel pseudocobalamin to a bioavailable form
- This implies a complex B$_{12}$ cycle between microbes in the photic zone

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In Brief

Helliwell et al. demonstrate that the two major groups of photosynthetic microbes in the photic zone, cyanobacteria and microalgae, use different forms of vitamin B$_{12}$. These findings challenge the oversimplified assumption of a linear flux of B$_{12}$ from producers to consumers, in favor of a more complex network of B$_{12}$ production, uptake, and cycling.
Cyanobacteria and Eukaryotic Algae Use Different Chemical Variants of Vitamin B₁₂

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SUMMARY

Eukaryotic microalgae and prokaryotic cyanobacteria are the major components of the phytoplankton. Determining factors that govern growth of these primary producers, and how they interact, is therefore essential to understanding aquatic ecosystem productivity. Over half of microalgal species representing marine and freshwater habitats require for growth the corrinoid cofactor B₁₂, which is synthesized de novo by certain prokaryotes, including the majority of cyanobacteria. There are several chemical variants of B₁₂, which are not necessarily functionally interchangeable. Cobalamin, the form bioavailable to humans, has as its lower axial ligand 5,6-dimethylbenzimidazole (DMB). Here, we show that the abundant marine cyanobacterium Synechococcus synthesizes only pseudocobalamin, in which the lower axial ligand is adenine. Moreover, bioinformatic searches of over 100 sequenced cyanobacterial genomes for B₁₂ biosynthesis genes, including those involved in nucleotide loop assembly, suggest this is the form synthesized by cyanobacteria more broadly. We further demonstrate that pseudocobalamin is several orders of magnitude less bioavailable than cobalamin to several B₁₂-dependent microalgae representing diverse lineages. This indicates that the two major phytoplankton groups use a different B₁₂ currency. However, in an intriguing twist, some microalgal species can use pseudocobalamin if DMB is provided, suggesting that they are able to remodel the cofactor, whereas Synechococcus cannot. This species-specific attribute implicates algal remodelers as novel and keystone players of the B₁₂ cycle, transforming our perception of the dynamics and complexity of the flux of this nutrient in aquatic ecosystems.

INTRODUCTION

Eukaryotic microalgae are photosynthetic microbes estimated to be responsible for up to 50% of global carbon fixation [1]. Elucidation of factors that control algal community structure and dynamics is thus fundamental to understanding the global cycling of carbon. Nutrients such as iron, nitrogen, and phosphorus clearly play an important role [2], but many microalgae also require the vitamins B₁ (thiamine), B₇ (biotin), or B₁₂ for growth [3]. B₁₂ is required as a cofactor for methionine synthase (MET; EC 2.1.1.13) activity, a key enzyme of cellular one-carbon (C₁) metabolism important for production of the universal methyl donor S-adenosylmethionine (SAM), and for folate cycling necessary for DNA synthesis [4]. Those algae that do not need a supply of B₁₂ cannot synthesize the vitamin; rather, they possess an alternative form of methionine synthase (METE; EC 2.1.1.14) that can catalyze the same reaction in a B₁₂-independent fashion [5, 6].

Measurement of B₁₂ levels in the water column have indicated concentrations of ~10 pM in freshwater ecosystems [7] and are often below the threshold of detection in certain marine habitats, including large areas of the northeast Pacific margin [8]. The scarcity of this micronutrient is therefore thought to limit phytoplankton abundance [8], so competition for B₁₂ among those organisms that require/use it is likely. Indeed, field-enrichment experiments found that, whereas N addition stimulated all microbial growth, there was a specific growth enhancement of phytoplankton >5 µm (i.e., the larger eukaryote fraction) with B₁₂ supplementation [9]. However, several recent studies have demonstrated that heterotrophic bacteria can satisfy microalgal requirements for B₁₂ via mutualistic interactions (e.g., [5, 10]).

Provision from prokaryotes is particularly pertinent because the biosynthetic pathway of this cofactor is confined to certain archaea and bacteria. B₁₂ is an umbrella term that refers to cobalt-containing corrinoids (ring-contracted tetrapyrroles), which have upper and lower axial ligands to the cobalt ion (Figure 1A). The nature of these ligands varies, leading to diversity in the structural forms of B₁₂. Methylcobalamin, where the upper axial ligand is a methyl group, is involved in methyl-transfer
reactions, whereas adenosylcobalamin (coenzyme B₁₂) is used for radical-based rearrangements and reductions [11]. The identity of the base found as the lower axial ligand, bound via a nucleotide loop, may vary too. In cobalamin, the base is 5,6-dimethylbenzimidazole (DMB). Many bacteria, including methanogens and anaerobes such as *Clostridium* species, synthesize a B₁₂ vitamer known as pseudocobalamin [12, 13], which differs from cobalamin in that DMB is replaced by adenine. Intrinsic factor, the mammalian B₁₂-binding protein important in uptake from the gut, has a lower affinity for pseudocobalamin than cobalamin [14]. Pseudocobalamin is therefore considered not “bioavailable” to humans, and the efficacy of vitamin supplements produced from cyanobacteria such as *Spirulina* and *Aphanizomenon*, which also contain pseudocobalamin, has been questioned [15, 16].

Cyanobacteria are the numerically dominant photosynthetic microbes in the marine environment [17]. Two cyanobacterial strains, *Crocosphaera watsonii* WH8501 and *Synechococcus* sp. WH7803, were reported to release B₁₂ into the media at rates exceeding those estimated for the heterotrophic bacterium *Halomonas*, suggesting that cyanobacteria might be the major source of B₁₂ for marine algae [18]. However, indications from the early literature suggest consideration of algal specificity toward different B₁₂-like factors may be pertinent [19]. Here, we investigate corrinoids in several strains of *Synechococcus*, an abundant and ubiquitous marine cyanobacterium [17, 20]; the nature of their axial ligands; and their ability to support growth of eukaryotic primary producers.

**RESULTS**

*Synechococcus* Species Make Only Pseudocobalamin

The biosynthesis of the corrinoid ring of B₁₂ from the common tetapyrrole progenitor uroporphyrinogen III requires at least 20 enzymatic steps, and several routes are known [21]. In a preliminary investigation, San˜udo-Wilhelmy et al. [22] searched for the presence of B₁₂-biosynthesis genes in /C24/40 marine cyanobacteria with sequenced genomes. They found that all but one species had putative homologs for at least 11 of these genes and so concluded that they were capable of making B₁₂. However, they did not investigate the genes involved in synthesis of the lower axial ligand and so could not conclude whether the cyanobacteria studied produced cobalamin or pseudocobalamin. To refine the analysis of B₁₂-biosynthesis genes, we searched 123 sequenced cyanobacterial genomes for all 20 genes of the corrinoid pathway. All but six species contained at least 15/20 of these genes and were predicted to be B₁₂ producers (Data S1; Supplemental Experimental Procedures). Additionally, we searched for genes involved in DMB biosynthesis, for which two routes are currently known [23, 24]. The BluB enzyme, first characterized in *Sinorhizobium meliloti* (Rhizobia) makes DMB from riboflavin under aerobic
conditions [23]. Using this sequence as a query, no hits were found in 118 cyanobacterial genomes including C. watsonii WH8501 and Synechococcus sp. WH7803. For five species (including three from the Fischerella genus), hits for BluB were obtained: two were annotated as cob(iii)lyric acid a,c-diamide reductase (CobR), which is an enzyme of an earlier stage of B12 biosynthesis, whereas the others were unknown. In contrast, BluB homologs were found in 80% of sequenced rhizobia (227/284 genomes; Data S1C) and 60% of Rhodobacterales species (77/128; Data S1D) including Mesorhizobium loti, Sinorhizobium melloti, Rhizobium leguminosarum, and the marine bacterium Dinoroseobacter shibae, all of which can support algal B12-auxotrophic growth [10, 25]. More recently, a second route for DMB biosynthesis was identified in the obligate anaerobic bacterium Eubacterium limosum [24], and enzymes encoded by the bzaABCDDE operon were shown to direct DMB production via an oxygen-sensitive reaction from the purine precursor 5-aminoimidazole ribotide (AIR) [24]. We found that none of the cyanobacterial genomes encoded the full bzaABCDDE operon (Data S1B). Moreover, CobT, which is required for DMB activation, is absent from all but two cyanobacterial genomes. Taken together, these searches suggest that the vast majority of cyanobacteria cannot make DMB.

To validate the observations from the bioinformatics analysis, we wanted to assess directly what corrinoids are synthesized by cyanobacteria so investigated the B12 content of strains of marine Synechococcus, because this is an ancient and ecologically abundant lineage [20] with a mean global abundance of 7.0 ± 0.3 × 10^26 cells y^-1, high-biomass-specific CO2 fixation rates [17, 26] and axenic strains are available. Corrinoids can be extracted from cells as their cyano-derivatives and then analyzed by high-performance liquid chromatography (HPLC)-mass spectrometry (LC-MS). First, using purified cyanocobalamin (obtained commercially) and cyanopseudocobalamin, prepared from Propionibacterium acidi-propionici [12], we were able to distinguish the two variants of B12 by their different retention times on the LC (Figure 1B) and different mass (Figures S1A and S1B). Derivatized cell lysate obtained from axenic cultures of the heterotrophic marine bacterium D. shibae DFL12T contained only cyanocobalamin. We next tested five members of the Synechococcus lineage representing different clades and habitats (highlighted in red in Figure S2): coastal strain CC9311 (sub-cluster [SC] 5.1A; clade I); oligotroph WH8102 (SC5.1A; clade III); WH7803 and WH7805 (SC5.1B; clades V and VI, respectively) which are widely distributed in various oceanic waters; and the estuarine strain WH5701 (SC5.2). A single peak was observable in these samples at a retention time consistent with the pseudocobalamin standard (Figure 1B), and its identity was confirmed by MS (Figures S1D–S1H). To facilitate subsequent physiological work, we also tested two model freshwater cyanobacterial species Synechocystis sp. PCC6803 and Synechococcus elongatus PCC7942, because these species grow quickly and easily in the laboratory. Again, cell lysates from these strains contained only pseudocobalamin (Figures 1B, S1I, and S1J). Together, these data demonstrate that the cyanobacterial species sampled here make only pseudocobalamin in axenic laboratory culture conditions.

However, some B12-synthesizing bacteria can modify endogenous B12 forms with an alternative base [27]. For instance, although Salmonella enterica cannot make DMB, it can import it and then make cobalamin instead of pseudocobalamin [27]. To investigate whether Synechococcus can perform this so-called “guided biosynthesis,” we grew strains WH8102 and WH7803 (representing SC5.1A and 5.1B) in the presence of pseudocobalamin and 1 μM DMB, but only pseudocobalamin was detected (Figures 1B, S1K, and S1L). We conclude therefore that Synechococcus cannot replace the adenine base with DMB to make cobalamin.

**Pseudocobalamin Is Orders of Magnitude Less Bioavailable to Eukaryotic Algae**

We next tested whether cyanobacterially derived B12 could be utilized by eukaryotic algae. Cell-free extracts of S. elongatus PCC7942 (pseudocobalamin producer) were unable to rescue growth of the B12-dependent freshwater alga Lobomonas rostrata, whereas there is clear growth with the addition of extracts of three rhizobial bacteria (Figure S3), which all encode BluB [28]. It is conceivable that the growth is due to other compounds in the crude lysate, so this initial experiment was extended using the purified compounds, cyanocobalamin and cyanopseudocobalamin. Equivalent concentrations of each B12 variant were added to axenic cultures of B12-dependent microalgae from different algal lineages: marine species Ostreococcus tauri (Chlorophyta, Mamiellophyceae); Amphidinium carterae (Alveolata, Dinoflagellate); Pavlova lutheri (Haptophyta, Prymnesiophyceae); Thalassiosira pseudonana (Heterokontophyta, Coscinodiscophyceae); Aureococcus anophagefferens (Heterokontophyta, Pelagophyceae); and the freshwater species Euglena gracilis (Excavata, Euglenoza) and L. rostrata (Chlorophyta, Volvocales). We also tested a B12-dependent metE mutant of Chlamydomonas reinhardtii (Chlorophyta, Volvocales) [29]. When pseudocobalamin was supplied at a concentration of 0.07 or 0.7 nM, we observed little or no growth in any of the marine species, nor with the C. reinhardtii metE mutant or L. rostrata. This is in contrast to cobalamin, which supported growth of all algal cultures at equivalent concentrations (Figure 2; Student’s t test; p < 0.05; n = 3). For O. tauri, A. carterae, and T. pseudonana, and to a lesser extent the C. reinhardtii metE mutant, provision of pseudocobalamin at 7 nM (10 μg/l) supported growth to a similar extent as cobalamin (Figures 2A, 2B, 2D, and 2H), although this amount is significantly higher than found in natural ecosystems (with reported concentrations ranging from below the detection threshold to 0.03 nM across large areas of the northeast Pacific margin, for instance) [7, 8]. One way to compare the efficacy of the different B12 variants is to carry out dose-response experiments, which enable determination of an EC50 (that is the effective concentration required to support half-maximal biomass accumulation) and also provide an indication of the minimum amount needed to observe any growth, and so we carried these out with the C. reinhardtii metE mutant. Figure S4 shows that the EC50 was ~0.07 nM for cobalamin, compared to ~7 nM (~100-fold higher) for pseudocobalamin. In addition, it is clear that even the highest concentration of pseudocobalamin used (40 nM) is not saturating, whereas 0.2 nM cobalamin supports maximum growth. For E. gracilis, some growth was observed even at the lowest pseudocobalamin concentration, but it was still significantly lower than with cobalamin (Figure 2G). Thus, pseudocobalamin is orders of magnitude less effective as a B12 source than cobalamin.
bioavailable to eukaryotic algae. It is notable to mention that *E. gracilis* has also been demonstrated to encode a B₁₂-dependent (type II) ribonucleotide reductase [30], which could account for the growth response to pseudocobalamin observed in this alga.

We reasoned that the reduced ability of pseudocobalamin to support growth of algal B₁₂-auxotrophs may be either because the molecule cannot be used as a cofactor or because it does not get transported into algal cells. To investigate the latter possibility, we took advantage of the presence of B₁₂-responsive genes previously identified in the marine diatom *Phaeodactylum tricornutum* and *C. reinhardtii* [31, 32]. These algae do not need B₁₂ for growth but will uptake and use it if it is available [5, 6]. Several genes in these algae are responsive to B₁₂: METE (in *P. tricornutum* and *C. reinhardtii*) [31, 32]; CBA1, encoding a novel cobalamin acquisition protein (in *P. tricornutum* only) [31]; and S-adenosylhomocysteine hydrolase, SAHH (in *C. reinhardtii* only) [32]. Using qRT-PCR, we analyzed their expression in cells grown in the presence of 0.7 nM cobalamin or pseudocobalamin. For *P. tricornutum*, both forms of B₁₂ resulted in downregulation of METE, but the effect was less pronounced with pseudocobalamin compared to that with cobalamin (Student’s t test; *p* < 0.001; *n* = 3; Figure 3A). As previously demonstrated, cobalamin suppressed CBA1 [31], but this gene was significantly upregulated by pseudocobalamin (Student’s t test; *p* < 0.05; *n* = 3; Figure 3A). In *C. reinhardtii*, both METE (Student’s t test; *p* < 0.05; *n* = 3) and SAHH (Student’s t test; *p* < 0.01; *n* = 3) were downregulated relative to the no supplementation control (Figure 3B) with both forms of B₁₂. Subsequent western blot analysis using polyclonal antibodies against *C. reinhardtii* METE protein [32] demonstrated a modest reduction of METE abundance when cells were grown with pseudocobalamin, although not to the same extent as cobalamin (Figure S5). Nonetheless, the effect of pseudocobalamin on the expression of these four B₁₂-responsive genes indicates that the molecule can enter both *C. reinhardtii* and *P. tricornutum* cells.

**Certain Algae Are Capable of Remodeling Pseudocobalamin**

By analogy with the guided biosynthesis described earlier, some bacteria that cannot synthesize B₁₂ de novo can modify imported forms with an alternative base, via “corrinoid remodeling” [33, 34]. To investigate this possibility in algae, we grew B₁₂-requiring species in the presence of pseudocobalamin and a range of DMB concentrations. For most, growth was not restored by DMB supplementation (Figures 4A, 4B, and 4D–4G). However, for *P. lutheri* and the *C. reinhardtii* metE mutant, addition of DMB alongside pseudocobalamin rescued growth to the same extent as cobalamin (Figures 4C and 4H). A dose-response experiment with *P. lutheri* established an EC₅₀ value of ~18 pM for cobalamin (Figure 5A). A similar experiment with
a fixed concentration (0.7 nM) of pseudocobalamin but varying the amount of DMB revealed a similar EC$_{50}$ (~23 pM; Figure 5B). Interestingly, an equivalent dose-response curve (and EC$_{50}$ value: ~26 pM) was observed when cells were grown in medium made using natural filtered seawater rather than artificial sea salts. Thus, the level of DMB in the natural filtered seawater is not sufficient to allow remodeling; otherwise, the dose-response curve would be shifted to the left. That comparable levels of B$_{12}$ and DMB (at a fixed level of pseudocobalamin) are able to rescue B$_{12}$-dependent growth implies that $P$. lutheri is remodeling pseudocobalamin with DMB to generate cobalamin. Dosage experiments with the C. reinhardtii B$_{12}$-dependent metE mutant also identified similar EC$_{50}$ values of ~28 pM and ~70 pM for cobalamin and DMB, respectively (Figures 5C and 5D). In this case, the EC$_{50}$ value for DMB was slightly higher than cobalamin. We also tested whether C. reinhardtii is capable of de novo lower-loop synthesis and grew the C. reinhardtii B$_{12}$-dependent metE mutant with DMB, alongside (dicyano)cobinamide, a B$_{12}$ precursor that lacks the DMB ribonucleotide tail, but no restoration of growth was observed (Figure 6A).

Our data indicate that, of eight diverse algal species studied, six do not appear to be able to use exogenous DMB. Nevertheless, the observation that growth of the C. reinhardtii B$_{12}$-dependent metE mutant (alongside that of $P$. lutheri) with pseudocobalamin is restored by DMB provision suggests that these algae are able to chemically modify pseudocobalamin to a form that can support B$_{12}$-auxotrophic growth. To test more directly whether pseudocobalamin is being remodeled, we grew samples of C. reinhardtii in the presence of (1) cobalamin, (2) pseudocobalamin (1 nM), and (3) pseudocobalamin (1 nM) + DMB (1 µM) and prepared cell lysate for LC-MS analysis. However, we could not detect any form of B$_{12}$ from lysed cells. We infer from this that intracellular B$_{12}$ levels are extremely low, i.e., the quantity from ~1 x 10$^{10}$ cells is below the threshold detection of the LC-MS (which in our system is ~1 x 10$^{12}$ molecules). Without a clear idea of what order of magnitude more biomass would be required and constrained by the limitations of scale, we turned to alternative means of characterizing remodeling activity and investigated the effect of DMB + pseudocobalamin on gene expression in C. reinhardtii. Previously, we had generated several transgenic lines of C. reinhardtii expressing the B$_{12}$-responsive element of the METE gene fused to the BLE gene, which confers resistance to the antibiotic Zeocin [32]. This reporter gene construct enables rapid and easy measurement of B$_{12}$-responsive gene expression, whereby growth with cobalamin represses expression of BLE so that cells die in the presence of Zeocin (Figure 6B). In contrast, pseudocobalamin alone had little effect, but the inclusion of DMB impaired growth to the same extent as cobalamin, demonstrating that C. reinhardtii converts DMB and pseudocobalamin into a form that is able to repress the METE promoter.

The pathway for pseudocobalamin remodeling has been investigated previously in the purple bacterium Rhodobacter sphaeroides, and cobinamide amidohydrolase (CbIZ) and cobinamide-phosphate synthase (CbIB) have been implicated in this process [34]. We could not identify cbiZ or cbiB in any of the algal genomes we analyzed. We therefore searched for proteins shown to be involved in lower-loop assembly and activation [34] in S. enterica (CobT, CobS, and CobO) [27], where mutants of CobT are unable to incorporate exogenous DMB (Figure 6C). We identified genes encoding all three of these proteins in C. reinhardtii, which exhibits the remodeling phenotype (Table S1; Figure 5). In contrast, BLAST searches of the genomes of O. tauri and T. pseudonana, species that do not appear to remodel, were negative for CobT and CobS. Although a hit for CobC was identified in T. pseudonana, it should be noted that CobC catalyzes a dephosphorylation step [21, 27], and therefore BLAST searches may retrieve genes encoding unrelated phosphatases. Interestingly, we identified hits for CobT and CobC, but not CobS, in A. anophagefferens, which can use pseudocobalamin with DMB, but only with very high levels of the latter (10 µM; Figure 4E). Transcript sequences for P. lutheri are available via the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP), a database of 396 unique strains representing ecologically significant and taxonomically diverse marine microbial eukaryotes [35]. This alga, which can use pseudocobalamin alongside DMB, also expresses COBT, COBS, and COBC (Data S2A). Thus, the presence of these novel proteins correlates with the ability to remodel pseudocobalamin, implicating them in B$_{12}$ metabolism. We also identified another 46 candidate remodelers in the MMETSP (Data S2A), including several that encoded METE, and so are like C. reinhardtii in being independent of a source of B$_{12}$ for growth. In total, the potential remodelers represented ~11% of unique strains and included representatives of the higher class levels Alveolata, Stramenopila, Hacrobia, and Viridiplantae (Data S2B). Incidentally, none of the sequenced Synechococcus genomes encode CobT (Data S1), which might explain why Synechococcus strains cannot remodel pseudocobalamin to cobalamin in the presence of DMB (Figure 1B).
Eukaryotic microalgae and cyanobacteria are the major components of the phytoplankton in marine and freshwater systems. Because they both inhabit the photic zone, they will compete for resources including light and limiting nutrients such as nitrogen and Fe. We have demonstrated that, in contrast to heterotrophic bacteria such as *D. shibae* and certain rhizobial bacteria [28] that make cobalamin, members of the ubiquitous marine *Synechococcus* genus synthesize only pseudocobalamin, in which the lower base is adenine instead of DMB (Figure 1). Moreover, a survey of diverse cyanobacterial genomes, encompassing marine and freshwater species, showed the vast majority do not encode *bluB* or the *bzaABCDE* operon (Data S1) [23, 24]. This strongly suggests that pseudocobalamin is the major form of B$_{12}$ synthesized by most if not all cyanobacteria.

We found that pseudocobalamin is considerably less bioavailable than cobalamin to several B$_{12}$-dependent algae (Figure 2). This reduced bioavailability suggests these organisms are compromised in their ability to acquire or use pseudocobalamin as a cofactor. Human intrinsic factor, part of the B$_{12}$ uptake system in the gut, exhibits a 500-fold-lower binding affinity for pseudocobalamin [14], thus reducing the bioavailability of the compound to humans. In algae, currently only one protein has been implicated in B$_{12}$ uptake [31] (CBA1), although the precise molecular mechanism and role of CBA1 in B$_{12}$ binding are not fully understood. Nevertheless, the ability of pseudocobalamin to affect the expression of algal B$_{12}$-responsive genes (Figure 3) and protein levels (Figure S5) suggests this compound can enter algal cells, albeit that it has the opposite effect on CBA1 to cobalamin, suggesting that the cells are experiencing cobalamin deficiency. Transport of pseudocobalamin into the cell is also indicated by our observed remodeling of pseudocobalamin in *C. reinhardtii* and *P. lutheri* following DMB addition (Figures 4 and 5). The identification of genes encoding enzymes of lower ligand activation (COBT) and nucleotide-loop assembly (COBS) [27] in these algae (Table S1) provides a likely mechanism for corrinoid remodeling. We found no evidence of secretory peptide signals in *C. reinhardtii* COBT or COBS using the green algal subcellular localization tool “PredAlgo” [36], implying that remodeling takes place within the cell and providing further support for the ability of pseudocobalamin to be taken up. Whether these genes have been acquired through lateral gene transfer from a bacterial source, which is thought to be the case for *E. coli* [37], remains unknown. However, of the non-algal sequences retrieved via a BLAST search of the
NCBI non-redundant database with the C. reinhardtii COBT/COBS, top hits were derived from the amphipod-associated protist species Aphanomyces astaci (4e−C057) and Sphaeroforma arctica (7e−C077), respectively. A broader phylogenetic analysis of COB genes will be integral to further understanding of what, at a first glance, appears to be an intriguing evolutionary history. Because algae rely on B12 for METH [5], the function of pseudocobalamin as a cofactor for this enzyme is also an important question. Structural data available for the B12-binding pocket and the active site of METH [38, 39] implicate several amino acids in B12 binding, with the DMB “tail” buried within a cleft of the active site [40]. Because pseudocobalamin contains an alternative lower base to B12, it seems plausible that algal METH proteins may have reduced binding affinity for pseudocobalamin.

The combination of DMB with pseudocobalamin improves the bioavailability to certain algae. We infer from this that these remodeling algae are able to generate cobalamin from pseudocobalamin + DMB, although we were unable to measure detectable levels of any form of B12 in C. reinhardtii cells grown under these conditions. It is possible therefore that another form of the vitamin is being generated, though we deem this unlikely. In any case, our results highlight the importance of considering environmental concentrations of DMB. A bioassay to measure free DMB concentrations was recently reported [41].

### Figure 5. Certain Algae Can Remodel the Lower Axial Ligand of Pseudocobalamin with Exogenously Supplied DMB

(A and B) Relative growth yield of (A) P. lutheri cells supplemented with different concentrations of cobalamin after 19 days (values of OD730 were normalized as a proportion of growth at 1,000 pM B12) or (B) P. lutheri cells supplemented with different concentrations of DMB in the presence of 0.7 nM pseudocobalamin (after 19 days) in artificial seawater or natural filtered seawater (values of OD730 normalized to growth at 1,000 pM DMB). (C and D) Equivalent experiments with C. reinhardtii, evolved B12-dependent metE mutant [29] are displayed in (C) and (D) after 96 hr growth (mean ± SEM; n = 3).

### Figure 6. Further Characterization of the Remodeling Phenotype

(A) Relative growth yield (OD730) of C. reinhardtii B12-dependent mutant grown with DMB alongside (dicyano)cobinamide, a B12 precursor that lacks the DMB ribonucleotide tail. Cells were grown in liquid medium (Table S2) with 0.7 nM cobalamin (open bars), 0.7 nM pseudocobalamin (gray bars), or 0.7 nM (dicyano)cobinamide (black bars) in the presence of different DMB concentrations. (B) Relative growth yield (OD730) of C. reinhardtii reporter line containing a Zeocin resistance gene controlled by the METE promoter [32] after 13 days in the presence (white bar) or absence (black bar) of DMB (1 μM) and 20 μg/ml Zeocin without or with 0.7 nM cobalamin or pseudocobalamin. Values of OD730 were normalized as a proportion of growth with no B12 (mean ± SEM; n = 3). (C) The pathway for the activation of DMB and nucleotide loop assembly in S. enterica (adapted from [27]). CobT catalyzes the attachment of a phosphoribosyl moiety derived from nicotinate mononucleotide to form α-ribazole phosphate. CobS and CobC catalyze the attachment of the activated base to the cobamide precursor (GDP-cobinamide).
natural environmental samples (Eucalyptus groove soil/creek water) determined concentrations in the picomolar and sub-picomolar range. Our observation that P. lutheri (whose growth can be supported by pseudocobalamin + DMB) could not grow in natural seawater from the English Channel supplemented with pseudocobalamin (Figure 5B) suggests DMB levels were not sufficient in these coastal waters to support remodeling. Nevertheless, further work is required to quantify DMB in marine (and freshwater) environments more broadly. In a similar vein, a recent field study determined concentrations in the picomolar and sub-picomolar range. Our observation that P. lutheri capable of remodelling can access this essential cofactor if DMB is also present.

Representsives of all major algal lineages require B12, yet its biosynthesis is limited to a subset of prokaryotes. As such, the flux of B12 between microbes is integral to the growth of auxotrophic species. Our results imply heterotrophic bacteria are likely to be a more important source of B12 for eukaryotic algae than cyanobacteria. An increasing body of evidence provided by this study and others suggests the relationship between scavengers and remodelers. That different B12 forms are not functionally equivalent between organisms means that biochemical transformations between vitamer classes are essential for this micronutrient to reach different members of the community. This complicates our current view of B12 cycling in aquatic environments (Figure 7). Whether cyanobacteria synthesize a currency of B12 that is inaccessible to competing eukaryotic microbes as a strategy to exclude competitors remains unknown. Nevertheless, the observation that certain algae possess a counter-mechanism to convert pseudocobalamin to a bioavailable form suggests the selective pressure to devise and refine strategies of B12 acquisition/utilization in order to enhance accessibility to this limiting micronutrient is strong. In any case, the importance of B12 and its derivatives in structuring microbial communities in aquatic ecosystems may have been previously underestimated.
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