Title: Cross-exchange of B-vitamins underpins a mutualistic interaction between *Ostreococcus tauri* and *Dinoroseobacter shibae*

Authors: Matthew B. Cooper[1,3*], Elena Kazamia[1,4*], Katherine E. Helliwell[1,5*], Ulrich Johan Kudahl[1], Andrew Sayer[1], Glen L. Wheeler[2] and Alison G. Smith[1]

Affiliations: *[1]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, United Kingdom.*

[2]Marine Biological Association of UK, The Laboratory, Citadel Hill, Plymouth, PL1 2PB, UK.

[3]Current address: Faculty of Biology, Medicine and Health, The University of Manchester, Michael Smith Building, Oxford Road, Manchester, M13 9PT, UK.

[4]Current address: Institut de Biologie de l'École Normale Supérieure, 46 Rue d'Ulm, Paris, 75005, France.

[5]Current address: Marine Biological Association of UK, The Laboratory, Citadel Hill, Plymouth, PL1 2PB, UK.

*These authors contributed equally to the work.

Running title (50 characters): B-vitamin exchange between algae and bacteria

Subject category: Microbe-microbe and microbe-host interactions

Abstract

Ostreococcus tauri, a picoeukaryotic alga that contributes significantly to primary production in oligotrophic waters, has a highly streamlined genome, lacking the genetic capacity to grow without the vitamins thiamine (B_1) and cobalamin (B_{12}). Here we demonstrate that the B_{12} and B_1 auxotrophy of *O. tauri* can be alleviated by co-culturing with a heterotrophic bacterial partner *Dinoroseobacter shibae*, a member of the *Rhodobacteraceae* family of alpha-proteobacteria, genera of which are frequently found associated with marine algae. *D. shibae* lacks the complete pathway to synthesise three other B vitamins: niacin (B_3), biotin (B_7), and *p*-aminobenzoic acid (a precursor for folate, B_9), and the alga is in turn able to satisfy the reciprocal vitamin requirements of its bacterial partner in a stable long-term co-culture. Bioinformatics searches of 197 representative marine bacteria with sequenced genomes identified just 9 species that had a similar combination of traits (ability to make vitamin B_{12}, but missing one or more genes for niacin and biotin biosynthesis enzymes), all of which were from the *Rhodobacteraceae*. Further analysis of 70 species from this family revealed the majority encoded the B_{12} pathway, but only half were able to make niacin, and fewer than 13% biotin. These characteristics may have either contributed to or resulted from the tendency of members of this lineage to adopt lifestyles in close association with algae. This study provides a nuanced view of bacterial-phytoplankton interactions, emphasising the complexity of the sources, sinks and dynamic cycling between marine microbes of these important organic micronutrients.

Key words: *Ostreococcus tauri, Dinoroseobacter shibae,* B-vitamins, auxotrophy, symbiosis, functional genome analysis, microbial communities

Introduction

Photosynthetic picoeukaryotic (PPE) algae are the main primary producers in many marine and freshwater aquatic ecosystems, playing a significant role in biogeochemical processes and food-web dynamics (1, 2, 3). Prasinophytes, a paraphyletic group of green algae, are a major group of PPE (4). Members of the *Ostreococcus* genus in this class have cells typically <1 μm in diameter, making them the smallest free-living eukaryotes described to date. *Ostreococcus* are found globally in a range of conditions and habitats including the nutrient poor oligotrophic oceans, some of the largest and most challenging biomes on Earth (5). This underscores their important ecological role and raises interesting questions about how the metabolism of these species equips them for survival.

Analysis of several *Ostreococcus* species reveals highly reduced genomes, although they retain the majority of metabolic attributes essential for autotrophic growth, including a complete set of genes for transport and assimilation of ammonium, nitrate and urea (6). However, like many other algae their growth is dependent on the presence of certain organic micronutrients in the environment, specifically thiamine (vitamin B_1) (7) and cobalamin (vitamin B_{12}) (8), both of which are required as cofactors for enzymes involved in central metabolism. Genomic evidence revealed that *O. tauri* has only a partial biosynthetic pathway for thiamine, suggesting it may have lost its ability to synthesise this cofactor *de novo* (9, 10). The B_{12}-biosynthetic pathway, comprising over 20 enzyme-catalysed steps, is found only in certain prokaryotes (11). The requirement for this vitamin by B_{12}-dependent algae is as a cofactor for the essential B_{12}-dependent enzyme, methionine synthase (METH), rather than the inability to synthesise the vitamin. Those species that can grow independently of an external supply of B_{12} encode an alternative methionine synthase enzyme (METE) that does not use B_{12} as a cofactor (8, 12). *O. tauri* encodes *METH* but not *METE*, and thus requires vitamin B_{12} for growth (8).

Algae may acquire vitamins in their natural environment via different routes. Seasonal upwelling of deep nutrient-rich water may provide a source for some coastal strains (13). However, some vitamins are photolabile and would not persist for long in the water column. Moreover, using a sensitive analytical method Sanudo-Wilhelmy *et al* (2012) found that ambient concentrations along the coast of California were significantly lower than needed for the growth of vitamin-dependent

algae such as *O. tauri*, and were below the detection threshold in some regions (14). Addition of vitamin B_{12} (together with Fe and N) enhanced chlorophyll *a* levels threefold (beyond stimulating effects of adding just Fe + N) in the eastern boundary of the South Atlantic Gyre (15). This highlights the crucial role of this compound in controlling phytoplankton growth in natural marine microbial communities, and demonstrates that the vitamin auxotrophy of *O. tauri* is representative of the natural world, rather than a particularity of this species.

Although small populations may persist under limiting micronutrient conditions, many PPEs including *Ostreococcus* periodically form characteristic blooms, implying that levels of vitamins must increase. Prokaryotes are the ultimate and only source of vitamin B_{12} (11). However, structural diversity in the forms of B_{12} produced by different prokaryote taxa has important implications in terms of their bioavailability to eukaryote auxotrophs. In particular, evidence indicates that B_{12}-synthesising cyanobacteria produce a variant, pseudocobalamin, that is considerably less bioavailable to eukaryotic algae (16). This suggests cobalamin-producing heterotrophic bacteria and archaea are likely to produce the majority of the cofactor that is bio-available for algae (12, 17). Further, certain bacterial phyla are more likely to be found associated with algae. In an analysis of over 40 different species of both macro and microalgae, six phyla (Bacteroidetes, Proteobacteria, Firmicutes, Actinobacteria, Verrucomicrobia and Planctomycetes) accounted for the majority of bacteria (18), and both alpha- and gamma-Proteobacteria are almost invariably found in studies of algal-associated microbiomes (19). In particular, species from the family *Rhodobacteraceae* (20) have been shown to deliver vitamins and other metabolites to marine algae. For example, *Dinoroseobacter shibae,* a cosmopolitan obligately aerobic bacterium, originally isolated as a symbiont to cultured marine dinoflagellates (21), could supply thiamine and B_{12} to its hosts in exchange for a source of fixed carbon (22). Similarly, *Ruegeria pomeroyi* supported the B_{12} requirements of the diatom *Thalassiosira pseudonana* in exchange for organosulphur compounds (23). Analysis of environmental isolates of another diatom, *Pseudo-nitzschia multiseries,* showed association with a number of bacteria one of which, a *Sulfitobacter* species, promoted algal cell division by providing indole acetic acid (24).

The hypothesis of mutualistic exchange of nutrients between microorganisms is challenging to test in the field. Most rely on correlations between co-existing species (25, 26), because in the absence

of tight physical associations, which are not necessary for mutualism, nutrient flux is difficult to deduce even using the most advanced methodologies (27). In light of this, simplified laboratory-based systems are invaluable for the study of nutrient cycling between microbes (28), and may provide important clues for our understanding of biogeochemical cycling of nutrients in natural systems. In this study, we established a stable co-culture between *O. tauri* and *D. shibae,* organisms that are reported to co-exist in marine microbial assemblages, such as in the North Pacific subtropical gyre (29). Using this system in the laboratory, we examined the nature of the metabolic exchange, probing the dynamics and stability of the interaction over successive generations. Our findings demonstrate that there is a complex two-way exchange of B vitamins that underpins this stable mutualistic relationship.

Materials & methods

Algal and bacterial strains and culture conditions

Ostreococcus tauri (OTH95) was a gift from Herve Moreau at the Oceanological Observatory of Banyuls-ser-Mer, France. This culture is a non-axenic environmental isolate from the Thau lagoon of the Mediterranean sea, just off the south of France (6). The strain was made axenic by fluorescence activated cell sorting (FACS), which provided starter cultures growing from single cells (Figure S2). The FACS protocol was specifically adapted and optimised to allow sorting, in collaboration with Nigel Miller (Department of Pathology, University of Cambridge, UK) for a MoFlo MLS high-speed cell sorter (Becton Dickinson). Chlorophyll fluorescence was measured through a 670/40 nm band pass filter (FL3) after excitation using a 488-nm argon laser (dot plots shown in Figure S2). Cells positive for chlorophyll fluorescence were sorted into single wells of a 24-well plate containing 2 ml of L1 medium, monitored for growth, and treated with ampicillin (1 mg/ml), neomycin (0.25 mg/ml), kanamycin (1 mg/ml), and streptomycin (50 µg/ml) for 14 days. Cultures were then tested for the presence of bacteria by replica plating onto MB agar. For this, 10 µl of liquid medium from stationary phase algal cultures was streaked onto MB plates, and incubated at room temperature for 14 days,

which yielded no bacterial colonies. Its purity was further verified by staining with the nucleic acid-specific stain 4′,6- diamidino-2-phenylindole (DAPI). Cells stained with (1 ng/ml, 5 min at 20 °C), placed in a microscopy dish and viewed under epifluorescent illumination (excitation 330–380 nm, emission above 420 nm). Bacteria were clearly visible in non-treated control cultures but not in the FAC-sorted, antibiotic treated *O. tauri* cultures. Cultures, which tested negative for bacteria in this way, were used for subsequent experiments.

Dinoroseobacter shibae DF-12 was a gift from Michael Cunliffe (Marine Biological Association, UK). It was maintained on 1.5% marine broth (MB) plates (Difco™) at 24°C. Its purity was verified by PCR amplification of the V3-V4 variable region of the 16S rRNA gene using a universal primer set (31), as described in (12, 32). The sequenced PCR product matched the correct bacterial ribotype by searching against the NCBI 16S rRNA genomic database (http://blast.ncbi.nlm.nih.gov/).

All axenic and co-cultures containing *O. tauri* were grown at 18°C with a 16:8 hour light:dark cycle (irradiance of 150 µmol photons.m^{-2}.s^{-1}) in unmodified L1 medium (30) unless otherwise stated. For experiments testing exchange of B vitamins, monocultures of *D. shibae* and *O. tauri* were pre-washed 3 times in L1 medium and then starved of vitamins for 7 days prior to the start of the experiment. B-vitamins were added to cultures at the following concentrations: 0.40 nM cobalamin, 2.05 nM biotin, 296 nM thiamine, 812 nM niacin and 291 nM *p*-aminobenzoic acid. These are concentrations known to be sufficient for unhindered algal cell growth in L1 medium. However, it is likely that these amounts are in excess of requirements, and the effect on cellular metabolism of providing a different ratio of these metabolites has not been demonstrated in dedicated experimental analyses.

Measuring cell densities of O. tauri and D. shibae

Optical densities of *O. tauri* cultures were measured using excitation at 750 nm (UV1, Thermo Spectronic, UK). For cultures with OD values between 0.1 and 0.45, the measured scatter was proportional to cell concentrations (Figure S1). For lower cell concentrations, cell density was determined using fluorescence activated cell sorting (FACS) with a FacsScan500 flow cytometer

(Becton-Dickinson, San Jose, California). *O. tauri* cell cultures at stationary phase were diluted 2-40 times using L1 medium and spiked with CountBright™ absolute counting beads (Life Technologies). Cells were discriminated by forward scatter and red (chlorophyll) fluorescence and CountBright™ beads discriminated by forward scatter and orange fluorescence. Samples were analysed until 100-300 beads events had been noted, with three technical replicates per biological sample. Cyflogic software (version 1.2.1, developed by CyFlo Ltd) was used to determine cell density from the collected data.

For bacteria, colony-forming units were used as a proxy for population density, determined using a variation of the replica plating method as described by (33). Harvested bacterial cells were diluted serially with L1 medium, and 3×10 µl of the diluted solution placed as drops on a single agar plate. The plate was then angled so the liquid formed 3 vertical lanes, and left to dry. After 10 days at room temperature colonies were counted from each individual lane, and counts from one plate averaged together as technical replicates per biological sample.

Bioinformatics Analysis

Sequence similarity searches were performed to assess the presence of vitamin biosynthesis genes in different organisms. Enzymes required for the biosynthesis of each vitamin were identified from a literature search and reference pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (34). Validated reference protein sequences for enzymes of vitamin biosynthesis were identified from the Universal Protein Resource (UniProt) archive. BLASTP (35) was used to search for each of these enzymes in the *O. tauri* (GenBank ID: GCA_000214015.1) and *D. shibae* (GenBank ID: GCA_000018145.1) genome assembly hosted at the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/), using the online BLAST server at the European Bioinformatics Institute (EMBL-EBI; https://www.ebi.ac.uk/Tools/sss/ncbiblast/). Default parameters including a word size of 3 and the BLOSUM62 scoring matrix were used for all searches. Low complexity regions were also filtered out. Only hits with an expectation coefficient of less than $1 \times e^{-15}$ were taken through for extended analysis. The protein sequences of the putative biosynthesis enzymes were aligned using the ClustalW algorithm (36) with the query protein sequences using the

AlignX module of the vector NTI software (Invitrogen) to confirm sequence similarities. Conserved functional domains of putative proteins were also confirmed using the NCBI's conserved domain searchable database. These database and literature searches were also used to ascertain conserved active sites of enzymes if available. For whole genome searches to identify vitamin pathways in sequenced bacterial genomes, the automated approach as described in (16) was carried out, using the same query sequences.

Results

Complementary B vitamin synthesis capabilities of O. tauri and D. shibae revealed by in silico pathway analyses

Figure 1a shows the growth requirements of *O. tauri* and *D. shibae* in minimal medium with different supplements. *O. tauri* requires both vitamins B_{12} and B_1 (7, 8), as cofactors for cobalamin-dependent methionine synthase (B_{12}) and several enzymes of intermediary carbon metabolism including transketolase (B_1). *D. shibae* DFL-12 on the other hand encodes the genes for the biosynthesis of B_{12} and B_1 (22), and can grow without either supplement. However, *D. shibae* is known to need exogenous sources of three other B vitamins: biotin (vitamin B_7), niacin (B_3) and *p*-aminobenzoic acid a precursor for folate (B_9) biosynthesis, whereas *O. tauri* does not require these molecules for growth. All three of these compounds have vital roles in central metabolism: biotin is a co-factor for enzymes necessary for essential carboxylation, transcarboxylation and decarboxylation reactions, folate is required for enzymes of 1-carbon metabolism, and niacin is a precursor to the hydrogen carriers NAD^+ & $NADP^+$, redox carriers used ubiquitously within the cell (reviewed in 37). Despite these fundamental roles, the exact genetic basis underlying the auxotrophy of *D. shibae* for these vitamins is currently unknown. We therefore carried out a detailed assessment of the genomes of the two organisms to investigate this, and whether they may be able to support one another's growth in co-culture.

The possible biosynthetic pathways of biotin, niacin and pABA are shown in Figure 1b. In bacteria, biotin is synthesised from its precursor molecule pimeloyl-coA by four enzymes: 8-amino-7-oxononanoate synthase (KAPAS, BioF) (38), 7,8-diamino-pelargonic acid aminotransferase (DAPAS, BioA) (39), dethiobiotin synthase (BioD) (40), and finally biotin synthase (BioB) (41), which converts dethiobiotin into biotin. In plants and some algae, the reactions of BioA and BioD can be performed by a bi-functional enzyme, known as BIO1 (42). A BLASTP search for biotin synthesis enzymes in *O. tauri* identified a significant hit for this bi-functional enzyme, alongside the

biosynthesis proteins BIOF and BIOB (green dots, Figure 1a; Table S1) supporting the physiological data. In contrast, the genome for *D. shibae* contains putative genes for only two of the four required enzymes: BioF and BioA (two putative hits were found for BioA) (blue dots, Figure 1b; Table S2). No orthologues of genes for BioD or BioB were identified, explaining the inability of *D. shibae* to grow without an exogenous source of biotin.

There are two biosynthetic routes for synthesis of niacin: i) from anthranilate produced from the degradation of tryptophan (as in animals), or ii) *de novo* synthesis from aspartate (characteristic of plants). Bacteria have been shown to use both pathways (43). *O. tauri* does not encode biosynthesis genes from the tryptophan degradation pathway, but has all the genes required to produce nicotinamide mononucleotide through the aspartate pathway, using L-aspartate oxidase (NADB), quinolinate synthase (NADA) and quinolinic acid phosphoribosyl transferase (NADC) (Figure 1b and supporting material in Table S3). Whilst *D. shibae* has a significant hit for proteins related to aspartate dehydrogenase (nadX, which is functionally equivalent to NADB (44)) and kynureninase (KYNU) (Table S4), we found no convincing orthologues for any other enzymes necessary for niacin biosynthesis. The lack of biosynthesis genes for the full aspartate or tryptophan pathway in *D. shibae* corroborates previous physiological evidence that *D. shibae* cannot make niacin itself (22), and thus requires an exogenous supply of this essential molecule (Figure 1b).

The compound *p*-aminobenzoic acid, an intermediate in the synthesis of folate and its bioactive forms, is produced from chorismate (45). In bacteria, two enzymes are required for this process. The first, aminodeoxychorismate synthase (ADCS) is made up of two protein sub-units, PabA and PabB (46). Sub-unit II of the ADCS complex (PabA) acts as a glutamine amidotransferase, transferring the amino group from glutamine to sub-unit I (PabB), which is then used to aminate the chorismate molecule (46). *Arabidopsis thaliana,* some yeasts, and certain algae have been shown to encode a bi-functional enzyme (PAB-AB) that performs both of these steps (47). In both bacteria and plants, the final enzyme in the pathway is 4-amino-4-deoxychorismate lyase (ADCL, PabC) that catalyses the reversible conversion of aminodeoxychorismate into *p*-aminobenzoic acid and pyruvate (48). *O. tauri* has significant hits for PAB-AB and PABC (Figure 1b and supporting material in Table S5), suggesting it is able to synthesise *p*-aminobenzoic acid. Incidentally, separate PABA and PABB

244 homologues were also identified. *D. shibae* also encodes *pabA* and *pabB* genes (Table S6).
245 Additionally, a possible *pabC* gene was identified, but this was annotated as a D-alanine
246 aminotransferase (D-AAT). ADCL and D-AAT are both class IV pyridoxal 5'-phosphate (PLP)
247 dependent enzymes and share similar domain architecture. Using the NCBI conserved domain search
248 function (49), we looked at the affiliation of our hit to reference ADCL and D-AAT sequences (Figure
249 S3). The *D. shibae* gene (YP_001534484.1, highlighted in red) clusters together with other D-AAT
250 enzymes (green) including that from another member of the *Rhodobacteraceae* family, *Rhodobacter*
251 *sphaeroides,* rather than with ADCL-like enzymes (blue). These results indicate this gene is thus more
252 likely to encode a D-AAT. The incomplete biosynthesis pathway of *D. shibae* for *p*-aminobenzoic
253 acid thus corroborates the observation that this bacterium requires an exogenous source of this
254 molecule for growth.

255 In the present study we did not investigate the secretory pathways, which are associated with
256 the uptake and potential release of these vitamins into the environment. For algal species, vitamin
257 transporters have not been identified conclusively so bioinformatics characterisation is not possible.
258 This is with the exception of CBA1, a protein important for cobalamin uptake (50). However, this
259 does not appear to be encoded by *O. tauri*. Our work therefore only addresses the question of whether
260 the species are capable of metabolic synthesis and modes of exchange – whether passive (such as for
261 example by viral lysis) or active (through transport proteins), remains unknown.

262 In summary, using curated sequences as queries on the *O. tauri* genome, complete
263 biosynthetic pathways for *p*-aminobenzoic acid, niacin and biotin were identified, demonstrating
264 potential biosynthetic capacity for each of these metabolites. In contrast, *D. shibae* lacks a complete
265 biosynthetic pathway for all three of these vitamins.

266

267 *Reciprocal complementation O. tauri/D.shibae B vitamin requirements by mutualism*

268 To explore further the complementary vitamin synthesis capabilities of *O. tauri* and *D. shibae*, we
269 investigated their ability to grow together in the absence of exogenous B-vitamins. This required an
270 axenic culture of both organisms. All cultures of *O. tauri* available from culture collections are
271 maintained as uni-algal, with one or more contaminating bacteria present. Treatment of *O. tauri* OTH

95 with a cocktail of antibiotics over several subcultures reduced the load considerably, but one bacterium, *Zeaxanthibacterium* sp. (*Flavobacteriaceae*), was always present. Although supplementation with B_1 & B_{12} was still required, indicating that it was not providing the vitamins to *O. tauri*, we nonetheless wanted to separate the algal and bacterial cells to avoid confounding factors. Since these are essentially the same dimensions (~1 μm in diameter), we explored the possibility of using fluorescence activated cell sorting (FACS). Initially, cells were gated on their size and shape, and then gated further based on their pulse width/pulse area, as described in Methods. This process could distinguish between single cells and doublets or triplets, indicative of bacteria attached to algae. Once settings were established that allowed only single cells to flow through the channel, chlorophyll fluorescence was used to separate algal cells from bacteria (Figure S2). Cells were treated again with antibiotics for 14 days, following which no bacteria were detected by light microscopy or growth on marine broth plates.

Given the non-overlapping B-vitamin dependencies of *O. tauri* and *D. shibae*, we then investigated whether a stable co-culture of the two organisms could be established in medium containing none of the five B vitamins, to instigate the transfer of complementary micronutrients. Cultures were maintained for three sub-cultures (each of 21-25 day duration) to ensure that any rescue effect was long-term and stable. At stationary phase, total cell numbers for both *O. tauri* and *D. shibae* were taken and compared to control mono-cultures grown in parallel (Figure 2). Maximal growth was evident when *O. tauri* and *D. shibae* were grown in monoculture with all the necessary B vitamins. However, a significant increase in *O. tauri* cell density was observed when the alga was grown in co-culture with *D. shibae* (Figure 2a, white bars) in medium lacking thiamine, B_{12} or all B-vitamins compared with the respective axenic monocultures (grey bars) (Student's t-test $p<0.05$), although the rescue effect varied in magnitude. We found that whilst *D. shibae* was able to satisfy the requirements of *O. tauri* for B_{12}, the rescue effect was weaker when thiamine was omitted, with the maximum carrying capacity achieved only ~30% of that when the vitamin was included in the growth medium. In turn, co-culturing with *O. tauri* satisfied the requirements of *D. shibae* for each of niacin, biotin and *p*-aminobenzoic acid (Figure 2b), with statistically significant differences observed compared to growth in monoculture under the equivalent deficiency profiles (Student's t-test $P<0.05$

for each comparison). Interestingly, for axenic cultures of *D. shibae* (Figure 2b, grey bars), omission of niacin had the greatest inhibitory effect on the final carrying capacity, and was equivalent to omitting all B-vitamins from the medium.

O. tauri can satisfy D. shibae requirements for niacin and p-aminobenzoic acid in stable long-term co-culture

The previous experiment indicated that growth yields of both *O. tauri* (OTH95) and *D. shibae* DFL-12 were lower in co-culture compared to mono-cultures. The observation that carrying capacities of organisms engaged in mutualisms are different to mono-culture is common, and has been termed regulation (51). This is particularly well documented for plants in association with rhizobial symbionts, where the balance of host to mutualist is carefully maintained, and allows a carefully maintained exchange of resources (e.g. 52). For algal and bacterial systems in mutualism regulation has been observed (53) but often the signalling mechanism remains unclear (reviewed in 54).

To determine whether *O. tauri* and *D. shibae* actively regulate each other's growth, we inoculated the two organisms at different relative proportions into minimal medium without niacin, *p*-aminobenzoic acid or B_{12}; both thiamine and biotin were included to enable reasonable growth rates and minimise the time needed for each experiment (omission of these vitamins had the greatest inhibitory effect on *O. tauri* and *D. shibae* cell density in co-culture, respectively (Figure 2)). For those cultures with higher initial algal numbers, the starting inoculation density for the algae was set to ~2×10^6 cells per ml, with bacterial numbers adjusted accordingly to obtain the different ratios. When bacteria were initially in excess, the starting inoculation density of bacterial cells was ~1×10^6 cells per ml. Growth of the cultures were monitored over time by cell counting as described in the Methods. We found that regardless of starting numbers of either algal or bacterial cells, after 21-25 days in co-culture a ~1:1 ratio of the two organisms was reached (Figure 3a). *D. shibae* cells have a faster growth rate than *O. tauri*, and so the 1:1 equilibrium was reached within 8 days when starting cultures had a higher ratio of algae to bacteria, whereas 25 days were required for *O. tauri* cells to reach parity from an initial inoculum with more bacterial cells.

Previously, we had shown that a similar co-culture could be established between the B_{12}-dependent freshwater green alga *Lobomonas rostrata* with the soil bacterium *Mesorhizobium loti* (53). An exogenous supply of either vitamin B_{12} or a fixed carbon source disrupted the nature of the co-culture. We therefore attempted to disrupt the *O. tauri*/*D. shibae* interaction through the addition of different B vitamins and a carbon source that favour bacterial growth. When the suite of vitamins required by the bacteria were provided exogenously into the algal-bacterial co-culture together with a source of fixed carbon, the ratio of bacteria:algae altered significantly in favour of *D. shibae*, releasing the co-cultures from the established 1:1 ratio (Figure 3b). Addition of just thiamine and B_{12} to the co-cultures also encouraged the growth of bacteria slightly relative to controls, even though these conditions satisfy the vitamin requirements of *O. tauri* and would be expected to favour the growth of the alga. This suggests that *O. tauri* is unable to regulate the growth of *D. shibae* when not engaged in mutualism with the bacteria. Nevertheless, when conditions for mutualism were maintained in minimal medium over successive subcultures (i.e. without supplementation with fixed carbon or B vitamins), *O. tauri* and *D. shibae* continued to grow in a stable self-sustaining mutualistic relationship over four consecutive sub-cultures regardless of starting inoculum, resulting in a ratio of around 1:1 for the two organisms (Figure 4).

Niacin and biotin auxotrophy is enriched in B_{12}-biosynthesising bacteria belonging to the Rhodobacteraceae

The co-culture between *O. tauri* (OTH95) and *D. shibae* DFL-12 demonstrates a novel role for the vitamin cofactors niacin, biotin and *p*-aminobenzoic acid in nutrient exchange. To assess the broader relevance of this observation, we investigated whether the potential to form mutualistic interactions with eukaryotic marine algae based on reciprocal B vitamin exchanges is a likely common characteristic of algae-associated bacteria. A survey of reports in the literature of bacteria found associated with marine algae from 7 different locations identified a total of 28 genera (Table S8). Strikingly, in each location species of the *Rhodobacteraceae* family (order Rhodobacterales) were found (asterisked in Table S8). We chose 70 species from this family that had sequenced genomes, and analysed the gene complement for the presence of the 63 genes involved in the biosynthesis of

355 cobalamin, niacin and biotin, using our previously established bioinformatics approach that allows
356 rapid searching of complete bacterial genomes for large numbers of genes (16). We were not able to
357 use this method to search for genes encoding pABA biosynthesis enzymes because the close
358 similarity between sequences for other enzymes requires manual inspection of the hits to verify their
359 identity. The results revealed that that the majority (87%) of the species possess the genes required for
360 vitamin B_{12} synthesis, whereas those for niacin were found in half, and only 9 (13%) appeared to
361 encode the entire biotin biosynthetic pathway (Supplementary dataset S1). In total, 29 species (41%)
362 shared the genotype of *D. shibae*, namely the ability to synthesis vitamin B_{12} but not biotin or niacin.
363 To assess whether the *Rhodobacteraceae* exhibit a disproportionately high frequency of this
364 trait compared to other bacteria, we extended the search more generally, using the GOLD database
365 (55), which characterises species from known locations. A total of 197 verified marine eubacteria
366 from 39 different orders, including 19 members of the *Rhodobacteraceae* family, were searched for
367 the presence or absence of these 63 genes (Supplementary dataset S2). Only 9 species capable of
368 producing B_{12} and at the same time lacking the capacity to synthesise both biotin and niacin were
369 identified, all of which were *Rhodobacteraceae*. For the remaining 178 species from non-
370 *Rhodobacteraceae* families, 149 (84%) encoded the complete niacin pathway, and 123 (69%) the
371 entire biotin pathway, much higher percentages than for the *Rhodobacteraceae* (37% and 16%). Thus,
372 using two independent datasets combined with information from previous studies, we find that, in
373 contrast to marine bacteria generally, bacteria of the *Rhodobacteraceae* family, which includes *D.*
374 *shibae,* are characterised by the ability to synthesise vitamin B_{12}, but not B_3 or B_7 (Figure 5).
375 Members of this family are also commonly found together with eukaryotic marine algae (19, 54). We
376 therefore find it plausible that they could supply algae with their required cobalamin, whilst receiving
377 niacin and biotin in return.
378
379 **Discussion**
380
381 The notion that photosynthetic microorganisms are at the bottom of the food web in aquatic
382 communities, supplying metabolites and energy to heterotrophic bacteria and macro-organisms in

progressive trophic levels is out-dated (56, 57). Instead, studies on aquatic microbial interactions are unveiling a suite of different lifestyles and cross-feeding interactions at the heart of these complex communities. Vitamins have emerged as important players in associations between aquatic microbes (e.g. 23, 24, 56, 57), since by definition they are required obligately by auxotrophs for survival, so the demands for them must be met by other species capable of their biosynthesis, even if not directly (58). The findings presented here are the first physiological demonstration of a bilateral B-vitamin exchange between aquatic microorganisms. Perhaps particularly important is our finding that *O. tauri* was able to provide *D. shibae* with niacin, the vitamin for which *D. shibae* exhibited the highest dependency. Very few studies have attempted to determine the concentration of niacin in oceanic environments: at the time of writing the only available paper is from half a century ago (59) and states niacin was undetectable in the Alaskan surface waters sampled. Our *in silico* survey found 20% of marine bacteria sampled do not have the genetic capacity to synthesise it *de novo* (Supplementary Dataset S2), but niacin auxotrophy is significantly enriched in the *Rhodobacteracaeae* (35/70 species sampled), whilst in contrast these are more likely to synthesise vitamin B_{12} than marine bacteria generally. Two *Roseobacter* species, *R. litoralis* and *R. denitrificans,* have previously been shown experimentally to require an exogenous source of niacin and biotin for growth (22). These bacteria were originally isolated from the surface of green seaweeds, and were not found in open waters. More recently, another member of the *Rhodobacteraceae* (20), a *Sulfitobacter* species named SA11, was identified as a key symbiont with the toxic bloom forming alga *Pseudo-nitzschia* (24). Previous studies have also identified niacin auxotrophy in host-associated bacteria, including in pathogenic and enteric bacteria (60). For example, *Shigella flexneri*, the pathogen responsible for shigellosis in primates, is a niacin auxotroph, having lost the *nadA* and *nadB* genes responsible for synthesis of quinolinate from aspartate, a precursor to niacin (61). The central role played by the bioactive forms of niacin as redox carriers and signalling molecules (62) makes it a crucial micronutrient for cell growth and survival in all organisms. The inability of some microbes to synthesise it is therefore perhaps surprising, especially considering that it requires just 3 enzymatic steps from central metabolites (37). It is tempting to conclude that niacin auxotrophy in enteric bacteria, commensalists

410 and pathogens, as well as in those found associated with algae, may be a common type of genetic
411 streamlining in bacteria that live closely with other organisms.

412 The rescue effect of the mutualistic symbiosis that we describe here between *O. tauri* and *D.*
413 *shibae* is stable over multiple generations for both individual B-vitamins and combinations thereof
414 (Figures 2 and 4). *D. shibae* was found to support the bulk of the B_{12} requirement for growth of *O.*
415 *tauri* in co-culture but only a small proportion of its thiamine requirement (Figure 2a). This matches
416 the known Ks values of thiamine for several algal species, which have been reported to be up to four
417 orders of magnitude higher than those for B_{12} (63). Our findings indicate that thiamine is the most
418 important regulator of *O. tauri* growth. For *D. shibae,* biotin limitation had the greatest effect on
419 bacterial cell density in co-culture. We also investigated whether the interaction between the algae and
420 bacteria exhibited regulation of population size, a phenomenon observed both in terrestrial systems
421 and aquatic symbioses (e.g. 54, 64). Our findings suggest that regulation between *D. shibae* and *O*
422 tauri is likely, as the ratio of bacterial to algal cells reproducibly stabilises at ~ 1:1 during the
423 exponential phase of growth, regardless of initial starting cell concentrations (Figure 3a). This is a
424 higher proportion of bacterial cells than the 1:30 measured in another co-culture, between *L. rostrata*
425 and *M. loti* (53), and may reflect the fact that *L. rostrata* is much larger cell than *M. loti*, whereas *O.*
426 *tauri* and *D. shibae* are essentially the same size. However, as for the *L. rostrata/M. loti* system the
427 1:1 equilibrium in cell numbers was only maintained when the medium was minimal, without any B
428 vitamins or an exogenous source of fixed carbon. Addition of these micronutrients favoured the
429 growth of both algae and bacteria, disrupting their dependency on one another and destabilising the
430 1:1 ratio observed (Figure 3b). This indicates vitamin (and carbon) availability are partially
431 responsible for the observed regulatory effect. Thiamine and biotin may be particularly important in
432 this regard, given that omission of these vitamins had the greatest inhibitory effect on *O. tauri* and *D.*
433 *shibae* cell density in co-culture, respectively (Figure 2). However, since bacterial and algal cell
434 density in co-culture is reduced even in the presence of all B vitamins (Figure 2) other factors must be
435 important too. For instance, it remains possible that the equilibrium observed is reached due to
436 competition of the cells for other nutrients in the medium. This can be excluded by further studies
437 under chemostat or semi-continuous batch culturing, which replenish the demand for macronutrients.

438 However, factors beyond nutrient limitation may control algal-bacterial cell densities too. Other work
439 (65) observed "Jekyll and Hyde" dynamics regulated algal cell density in co-cultures of the marine
440 alga *Emiliania hyxleyi* and bacterium *Phaeobacter gallaeciensis*. In this instance, population
441 regulation occurs via production of the algicidal compound p-coumaric acid. Further experiments into
442 possible signalling between the cells and quorum sensing is required to confirm the presence of
443 regulation in this interaction.

444 Recent evidence has highlighted further complex exchange between algae and bacteria. Several
445 marine bacteria have been demonstrated to synthesise auxins such as indole acetic acid (IAA), which
446 have growth-promoting roles for microalgae (24, 65). Moreover, algal-derived tryptophan may
447 provide the precursor for IAA synthesis. Together with our results, these studies identify a remarkably
448 complex and important aspect of marine microbial ecology that we are only beginning to understand.
449 By providing the genetic basis underlying the physiological observations, a portion of the "meta-
450 metabolome" of two organisms in symbiosis (representing the combined metabolic capabilities of the
451 interacting species) can be visualised with respect to B-vitamins, highlighting the nodes of exchange
452 (Figure 6). Without considering co-limitation of macronutrients as a factor, when both these
453 organisms are present in oligotrophic waters containing little or no B-vitamins free in solution they
454 could support each other's growth. Our experiments add to accumulating evidence of generalised
455 "niche-fitting" (66) between microbial species *Chlamydomonas reinhardtii* and the budding yeast
456 *Saccharomyces cerevisiae* were shown to form complex physiological structures in the laboratory,
457 providing each other with limiting nutrients (67). Similarly, here, *O. tauri* could provide and receive
458 B-vitamins from *D. shibae*. It should be mentioned that the formation of non-specific mutualisms
459 between organisms in co-limiting conditions is not a given; for example, complementary auxotrophs
460 of *S. cerevisiae* deficient in various aspects of amino acid and nucleotide metabolism regularly fail to
461 rescue each other upon co-culturing (68) even though they are the same species. Consequently, our
462 study makes an important contribution to the study of microbial interactions by providing an example
463 where metabolic complementarity is achieved between partners from different branches of life. The
464 fact that this is mediated by exchange of B vitamins highlights the significance of these compounds,
465 and expands the repertoire of nutrients involved in interactions in natural ecosystems.

Evidence is accumulating to demonstrate a range of complex ecological interactions within microbial communities, so it is important to consider how such interactions may arise in an environment such as the ocean, where species assemblages are likely to be transient due to the fluid and dynamic medium. It remains an open question whether these symbiotic interactions are the product of evolutionary history, which presumes a number of generations of co-occurrence with metabolic exchange as the outcome rather than the driver for co-existence, or the opposite (69). Two hypotheses for the evolution of mutualism within microbes have been proposed, which explore these contrasting scenarios: the Black Queen Hypothesis (70) and the Forager-to-Farmer model (57). The identification and characterization of microbial interactions at the genetic and physiological levels, as described here, will provide data to enable the hypotheses to be tested and refined.

Acknowledgements

We thank Herve Moreau at Banyuls-ser-Mer Oceanological Observatory in France for the gift of *O. tauri* OTH 95. We are grateful to Nigel Miller (Department of Pathology, University of Cambridge) for help with FACS. We acknowledge funding from BBSRC grant (BB/I013164/1) and EU FP7 DEMA (Project no. 309086). MBC was in receipt of a CASE studentship with the UK Biotechnology and Biological Sciences Research Council (BBSRC) and Plymouth Marine Laboratory Applications Ltd, UJK was supported by EU FP7 Marie Curie ITN Photo.Comm (Project No 317184), and AS by the Cambridge BBSRC Doctoral Training Partnership.

Conflict of Interest Statement

The authors declare no conflict of interest

References

1. Li WKW. (1992). Primary production of prochlorophytes, cyanobacteria, and eukaryotic ultraphytoplankton: Measurements from flow cytometric sorting. *Limnol Oceanogr* **39**: 169–175.
2. Countway PD, Caron DA. (2006). Abundance and Distribution of *Ostreococcus* sp. in the San Pedro Channel, California, as Revealed by Quantitative PCR. *Appl Environ Microbiol* **72**(4): 2496-2506.
3. Worden AZ, Nolan JK, Palenik B. (2004). Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component. *Limnol Oceanogr* **49**: 168–179.
4. Massana R. (2011). Eukaryotic picoplankton in surface oceans. *Annu Rev Microbiol* **65**: 91–110.
5. Demir-Hilton E, Sudek S, Cuvelier ML, Gentemann CL, Zehr JP, Worden AZ. (2011). Global distribution patterns of distinct clades of the photosynthetic picoeukaryote Ostreococcus. *ISME J* **5**: 1095–107.
6. Derelle E, Ferraz C, Rombauts S, Rouzé P, Worden AZ, Robbens S, *et al* (2006). Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. *Proc Natl Acad Sci USA* **103**: 11647–11652.
7. Paerl RW, Bertrand EM, Allen AE, Palenik B, Azam F. (2015). Vitamin B_1 ecophysiology of marine picoeukaryotic algae: Strain-specific differences and a new role for bacteria in vitamin cycling. *Limnol Oceanogr* **60**(1): 215–228.
8. Helliwell KE, Wheeler GL, Leptos KC, Goldstein RE, Smith AG. (2011). Insights into the evolution of vitamin B_{12} auxotrophy from sequenced algal genomes. *Mol Biol Evol* **28**: 2921–2933.
9. Bertrand EM, Allen AE. (2012). Influence of vitamin B auxotrophy on nitrogen metabolism in eukaryotic phytoplankton. *Front Microbiol* **3**: 375.
10. McRose D, Guo J, Monier A, Sudek S, Wilken S, Yan S, *et al*. (2014). Alternatives to vitamin B1 uptake revealed with discovery of riboswitches in multiple marine eukaryotic lineages. *ISME J* **8**: 2517–2529.

11. Warren MJ, Raux E, Schubert HL, Escalante-Semerena JC. (2002). The biosynthesis of adenosylcobalamin (vitamin B_{12}). *Nat Prod Rep* **19**: 390–412.
12. Croft MT, Lawrence AD, Raux-deery E, Warren MJ, Smith AG. (2005). Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. *Nature* **438**: 90–93.
13. Karl DM. (2002). Nutrient dynamics in the deep blue sea. *Trends in Microbiol*. **10**(9): 410-419.
14. Sañudo-Wilhelmy S, Cutter LS, Durazo R, Smail E, Gómez-Consarnau L, Webb E, *et al* (2012). Multiple B-vitamin depletion in large areas of the coastal ocean. *Proc Natl Acad Sci USA* **109**: 14041–14045.
15. Browning TJ, Achterberg EP, Rapp I, Engel A, Bertrand EM, Tagliabue A, *et al.* (2017). Nutrient co-limitation at the boundary of an oceanic gyre. *Nature* **551**: 242.
16. Helliwell KE, Lawrence AD, Holzer A, Kudahl UJ, Sasso S, Kräutler B, *et al*. (2016). Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B_{12}. *Curr Biol* **26**: 999–1008
17. Doxey AC, Kurtz DA, Lynch MD, Sauder LA, Neufeld JD. (2015). Aquatic metagenomes implicate Thaumarchaeota in global cobalamin production. *ISME J* **9**: 461–471.
18. Goecke F, Labes A, Wiese J, Imhoff JF. (2013). Phylogenetic analysis and antibiotic activity of bacteria isolated from the surface of two co-occurring macroalgae from the Baltic Sea. *Eur J Phycol* **48**: 47–60.
19. Cooper MB, Smith AG. (2015). Exploring mutualistic interactions between microalgae and bacteria in the omics age. *Curr Opin Plant Biol* **26**: 147–153.
20. Simon M, Scheuner C, Meier-Kolthoff JP, Brinkhoff T, Wagner-Döbler I, Ulbrich M, *et al.* (2017). Phylogenomics of Rhodobacteraceae reveals evolutionary adaptation to marine and non-marine habitats. *ISME J* **11**: 1483–1499
21. Biebl H, Allgaier M, Tindall BJ, Koblizek M, Lünsdorf H, Pukall R, *et al*. (2005). *Dinoroseobacter shibae* gen. nov., sp. nov., a new aerobic phototrophic bacterium isolated from dinoflagellates. *Int J Syst Evol Microbiol* **55**: 1089–1096.

22. Wagner-Döbler I, Ballhausen B, Berger M, Brinkhoff T, Buchholz I, Bunk B, *et al* (2010). The complete genome sequence of the algal symbiont *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea. *ISME J* **4**: 61–77.

23. Durham BP, Sharma S, Luo H, Smith CB, Amin SA, Bender SJ, *et al*. (2015). Cryptic carbon and sulfur cycling between surface ocean plankton. *Proc Natl Acad Sci USA* **112**: 453–457.

24. Amin SA, Hmelo LR, van Tol HM, Durham BP, Carlson LT, Heal KR, *et al.* (2015). Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature* **522**: 98–101.

25. Bertrand EM, McCrow JP, Moustafa A, Zheng H, McQuaid JB, Delmont TO, *et al.* (2015). Phytoplankton-bacterial interactions mediate micronutrient colimitation at the coastal Antarctic sea ice edge. *Proc Natl Acad Sci USA* **112**: 9938–9943.

26. Lima-Mendez G, Faust K, Henry N, Decelle J, Colin S, Carcillo F, *et al*. (2015). Determinants of community structure in the global plankton interactome. *Science* **348**(6237): 1262073–1262073.

27. Giovannoni, SJ. (2012). Vitamins in the sea. *Proc Natl Acad Sci USA* **109**: 13888-13889.

28. Helliwell KE, Pandhal J, Cooper MB, Longworth J, Kudahl UJ, Russo DA, et al. (2018). Quantitative proteomics of a B12 -dependent alga grown in coculture with bacteria reveals metabolic tradeoffs required for mutualism. New Phytol 217: 599–612.

29. Poretsky RS, Hewson I , Sun S, Allen AE, Zehr JP, Moran, MA. (2009), Comparative day/night metatranscriptomic analysis of microbial communities in the North Pacific subtropical gyre. *Environmental Microbiology*, **11**: 1358-1375.

30. Guillard RRL, Hargreaves PE. (1993). *Stichochrysis immobilis* is a diatom, not a crysophyte. *Phycologia* **32**: 234–236.

31. Lane DJ. (1991). 16S/23S rRNA sequencing. In Nucleic Acid Techniques in Bacterial Systematic, E. Stackebrandt, and M. Goodfellow, eds. (New York, USA: John Wiley and Sons), pp. 116–175.

32. Pace NR, A Molecular View of Microbial Diversity and the Biosphere. Science **276:** 743-740

33. Jett BD, Hatter KL, Huycke MM, Gilmore MS. (1997). Simplified agar plate method for quantifying viable bacteria. *Biotechniques* **23**: 648–650.

570 34. Kanehisa M, Goto S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids*
571 *Res* **28**: 27–30.
572 35. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search
573 tool. *J Mol Biol* **215**: 403–410.
574 36. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, *et al*. (2007).
575 Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948.
576 37. Gerdes S, Lerma-Ortiz C, Frelin O, Seaver SMD, Henry CS, de Crecy-Lagard V, *et al*. (2012).
577 Plant B vitamin pathways and their compartmentation: a guide for the perplexed. *J Exp Bot* **63**:
578 5379–5395.
579 38. Webster SP, Alexeev D, Campopiano DJ, Watt RM, Alexeeva M, Sawyer L, *et al*. (2000).
580 Mechanism of 8-amino-7-oxononanoate synthase: spectroscopic, kinetic, and crystallographic
581 studies. *Biochemistry* **39**: 516–28.
582 39. Stoner GL, Eisenberg MA. (1975). Purification and properties of 7, 8-diaminopelargonic acid
583 aminotransferase. *J Biol Chem* **250**: 4029–36.
584 40. Krell K, and Eisenberg MA, (1970). The purification and properties of dethiobiotin synthetase. *J*
585 *Biol Chem*. **245**(24): 6558-66
586 41. Berkovitch F, Nicolet Y, Wan JT, Jarrett JT, Drennan CL. (2004). Crystal structure of biotin
587 synthase, an S-adenosylmethionine-dependent radical enzyme. *Science* **303**: 76–9
588 42. Muralla R, Chen E, Sweeney C, Gray JA, Dickerman A, Nikolau BJ, *et al.* (2007). A Bifunctional
589 Locus (BIO3-BIO1) Required for Biotin Biosynthesis in Arabidopsis. *Plant Physiol* **146**: 60–73.
590 43. Roje S. (2007). Vitamin B biosynthesis in plants. *Phytochemistry* **68**: 1904–1921.
591 44. Yang Z, Savchenko A, Yakunin A, Zhang R, Edwards A, Arrowsmith C, *et al*. (2003). Aspartate
592 Dehydrogenase, a Novel Enzyme Identified from Structural and Functional Studies of TM1643. *J*
593 *Biol Chem* **278**: 8804–8808.
594 45. Green JM, Matthews RG. (2007). Folate Biosynthesis, Reduction, and Polyglutamylation and the
595 Interconversion of Folate Derivatives. *EcoSal Plus* **2**. doi:10.1128/ecosalplus.3.6.3.6
596 46. Viswanathan VK, Green JM, Nichols BP. (1995). Kinetic characterization of 4-amino 4-
597 deoxychorismate synthase from *Escherichia coli*. *J Bacteriol* **177**: 5918–23.

47. Camara D, Richefeu-Contesto C, Gambonnet B, Dumas R, Rébeillé F. (2011). The synthesis of pABA: Coupling between the glutamine amidotransferase and aminodeoxychorismate synthase domains of the bifunctional aminodeoxychorismate synthase from *Arabidopsis thaliana*. *Arch Biochem Biophys* **505**: 83–90.
48. Nakai T, Mizutani H, Miyahara I, Hirotsu K, Takeda S, Jhee KH, *et al.* (2000). Three-dimensional structure of 4-amino-4-deoxychorismate lyase from Escherichia coli. *J Biochem* **128**: 29–38.
49. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, *et al*. (2011). CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* **39**: D225–D229.
50. Bertrand EM, Allen AE, Dupont CL, Norden-Krichmar TM, Bai J, Valas RE, Saito MA. (2012), Cobalamin acquisition protein from diatoms, *PNAS*. **109**(26): E1762-E1771
51. Rodriguez RJ, Freeman CD, McArthur DE, Kim YO, Redman RS. (2009). Symbiotic regulation of plant growth, development and reproduction. *Commun Integrat Biol* **2**(2): 1-3.
52. Horvath B, Bachem CWB, Schell J, Kondorosi A. (1987), Host-specific regulation of nodulation genes in Rhizobium is mediated by a plant-signal, interacting with the nodD gene product. *The EMBO Journal*, **6**(4):841-848
53. Kazamia E, Czesnick H, Nguyen TTV, Croft MT, Sherwood E, Sasso S, *et al* (2012). Mutualistic interactions between vitamin B_{12}-dependent algae and heterotrophic bacteria exhibit regulation. *Environ Microbiol* **14**: 1466–1476.
54. Amin SA, Parker MS, Armbrust EV. (2012). Interactions between diatoms and bacteria. *Microbiol Mol Biol Rev* **76**: 667–84
55. Mukherjee S, Stamatis D, Bertsch J, Ovchinnikova G, Verezemska O, Isbandi M, *et al.* (2017). Genomes OnLine Database (GOLD) v.6: data updates and feature enhancements. *Nucleic Acids Res* **45**: D446–D456.
56. Flynn KJ, Stoecker DK, Mitra A, Raven JA, Glibert PM, Hansen PJ, *et al*. (2013). Misuse of the phytoplankton–zooplankton dichotomy: the need to assign organisms as mixotrophs within plankton functional types. *J Plankton Res* **35**: 3–11.

626 57. Kazamia E, Helliwell KE, Purton S, Smith AG. (2016). How mutualisms arise in phytoplankton
627 communities: building eco-evolutionary principles for aquatic microbes Fussmann G (ed). *Ecol*
628 *Lett* **19**: 810–822.
629 58. Helliwell KE. (2017). The roles of B vitamins in phytoplankton nutrition: new perspectives and
630 prospects. *New Phytol* **216**: 62–68.
631 59. Natarajan KV. (1968). Distribution of thiamine, biotin, and niacin in the sea. *Appl Microbiol* **16**:
632 366–369.
633 60. Ford JE, Perry KD, Briggs CA. (1958). Nutrition of lactic acid bacteria isolated from the rumen. *J*
634 *Gen Microbiol* **18**: 273–284.
635 61. Prunier AL, Schuch R, Fernández RE, Mumy KL, Kohler H, McCormick BA, *et al* (2007). nadA
636 and nadB of *Shigella flexneri* 5a are antivirulence loci responsible for the synthesis of quinolinate,
637 a small molecule inhibitor of *Shigella* pathogenicity. *Microbiology* **153**: 2363–2372.
638 62. Opitz CA, Heiland I. (2015). Dynamics of NAD-metabolism: everything but constant. *Biochem*
639 *Soc Trans* **43**: 1127–1132
640 63. Tang YZ, Koch F, Gobler CJ. (2010) Most harmful algal bloom species are vitamin B-1 and B-12
641 auxotrophs. *Proc Natl Acad Sci USA* **107**: 20756-20761
642 64. Parniske M. (2000), Intracellular accommodation of microbes by plants: a common
643 developmental program for symbiosis and disease? *Current Opinion in Plant Biology*, **3**(4): 320-
644 328
645 65. Seyedsayamdost MR, Case RJ, Kolter R, Clardy J. (2011). The Jekyll-and-Hyde chemistry of
646 Phaeobacter gallaeciensis. *Nat Chem* **3**: 331–335
647 66. Agosta SJ, Klemens JA. (2008). Ecological fitting by phenotypically flexible genotypes:
648 implications for species associations, community assembly and evolution. *Ecol Lett* **11**: 1123–
649 1134
650 67. Hom EFY, Murray AW. (2014). Plant-fungal ecology. Niche engineering demonstrates a latent
651 capacity for fungal-algal mutualism. *Science* **345**: 94–98.

68. Campbell K, Vowinckel J, Mülleder M, Malmsheimer S, Lawrence N, Calvani E, *et al*. (2015). Self-establishing communities enable cooperative metabolite exchange in a eukaryote. *Elife* **4**: e09943.
69. Helliwell KE, Wheeler GL, Smith AG. (2013) Widespread decay of vitamin-related pathways: coincidence or consequence? *Trends in Genetics* **29**: 469-478.
70. Morris JJ, Lenski RE, Zinser ER. (2012) The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *mBio* **3**(2)

Figure legends

Figure 1. Determining the genetic basis for vitamin dependency. A. Growth of axenic *O. tauri* and *D. shibae* (with 1% glucose) with and without various B-vitamins at stationary phase after two sub-cultures. **B.** Schematic of biosynthetic pathways for *p*-aminobenzoic acid, niacin and biotin. Results of similarity sequence searches of *D. shibae* and *O. tauri* genomes for genes encoding biosynthetic enzymes for these vitamins are indicated. *O. tauri* encodes the gene set necessary for synthesis for *p*-aminobenzoic acid, niacin and biotin (Supplementary Information), whereas *D. shibae* lacks a complete biosynthetic pathway for all three of these vitamins

Figure 2. Growth of *O. tauri* and *D. shibae* under different B-vitamin profiles.

A. Growth assay to confirm B-vitamin auxotrophy in axenic *O. tauri* and *D. shibae*. Bacterial cultures were supplemented with 1% glucose. The photograph was taken once the cultures reached stationary phase. **B.** Growth of *O. tauri* in mono-culture (grey) or in co-culture with *D. shibae* (white) under different B-vitamin profiles. Cell density was determined at stationary phase. C). Growth of *D. shibae* in mono-culture (grey) or in co-culture with *O. tauri* (white) under different B-vitamin profiles. Error bars show standard deviation for three biological replicates.

Figure 3. Growth dynamics of *O. tauri* in co-culture with *D. shibae*

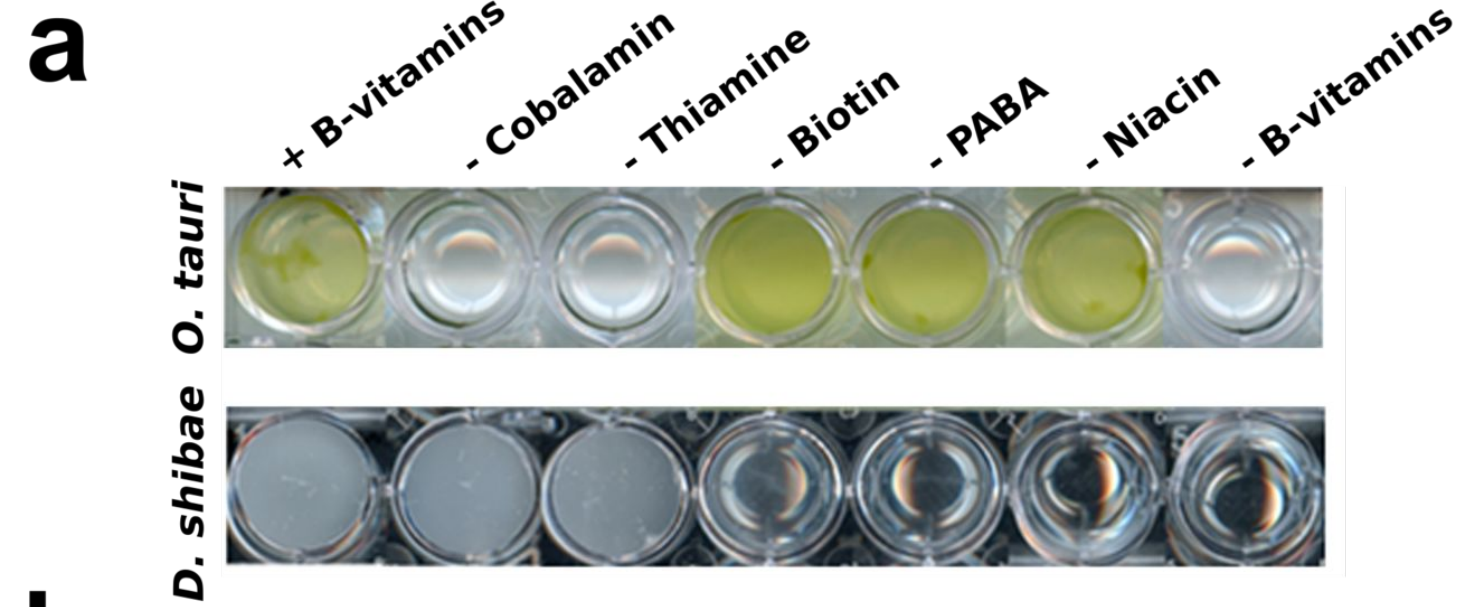
A. The ratio of *D. shibae* to *O. tauri* cells during growth in co-cultures initiated at different starting ratios. Cell densities of algae and bacteria were monitored until *O. tauri* reached stationary phase at

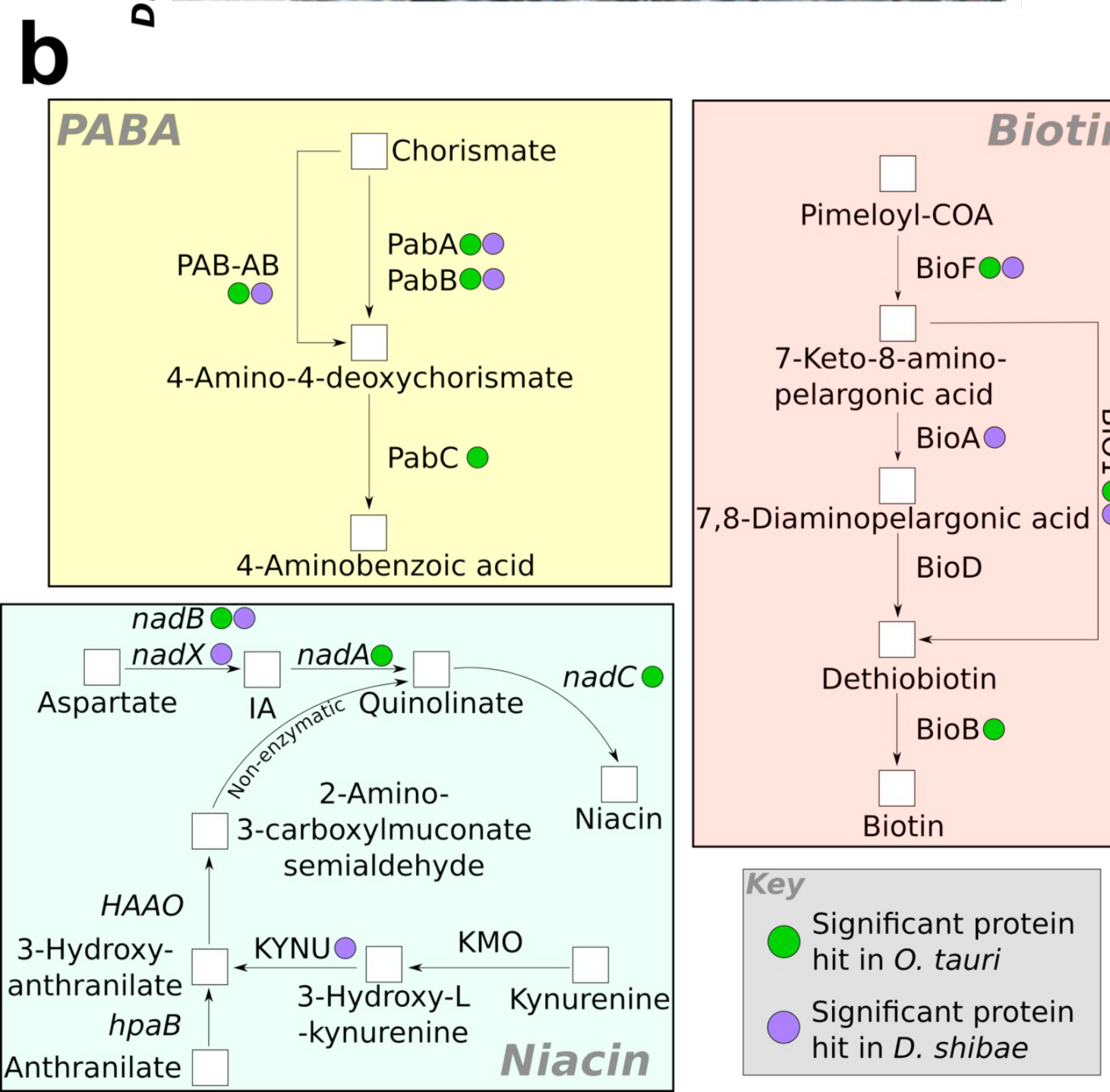
25 days. **B.** The effect of adding-back nutrients on the established ratio. Ratio of *O. tauri* to *D. shibae* cells over the course of one culture when a stable co-culture is inoculated into medium favouring the bacteria (supplemented with 1% glucose and vitamins *p*-aminobenzoic acid (*p*ABA), B_3 and B_7), medium favouring the alga (with B_1 and B_{12}) or no nutrient addback.

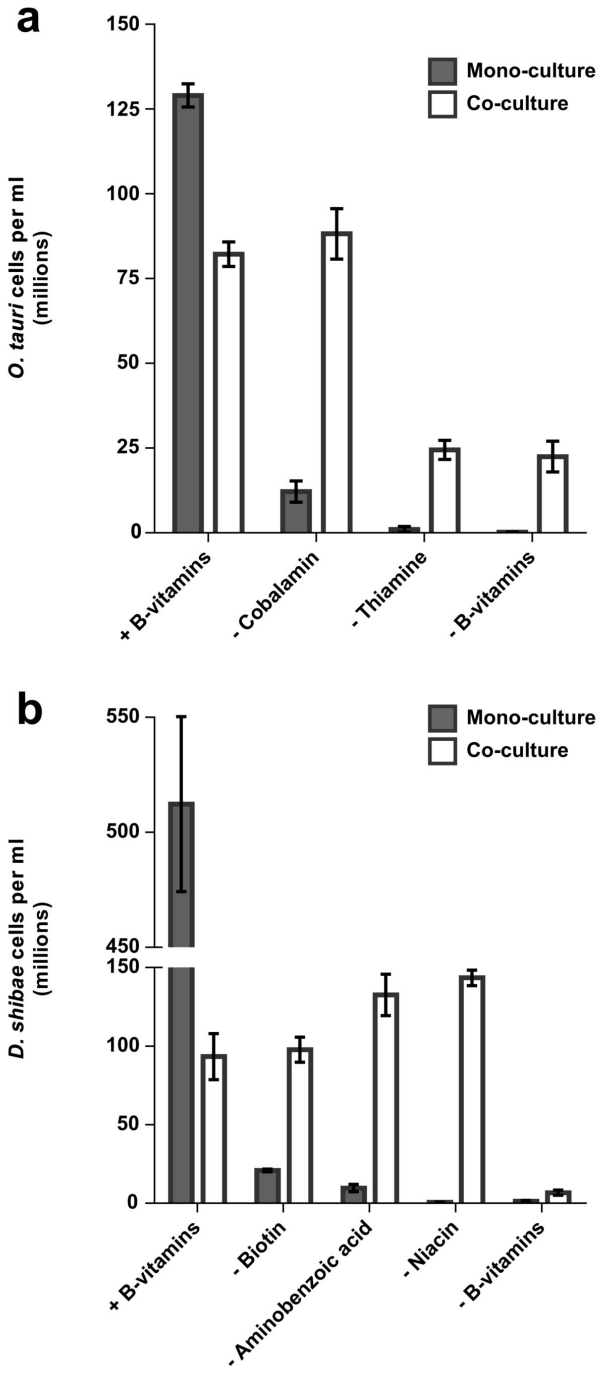
Figure 4. Rescue effect of *O. tauri* and *D. shibae* for required B vitamins is stable and long-term. **A.** Log cell density of *O. tauri* cells at stationary phase over 4 sub-cultures that were inoculated at differing initial ratios to *D. shibae*. **B.** The average ratio of *D. shibae: O. tauri* at each different starting bacterial: algal ratio at stationary phase. Error bars indicate standard error for mean of ratios at stationary phase across all sub-cultures (n=4).

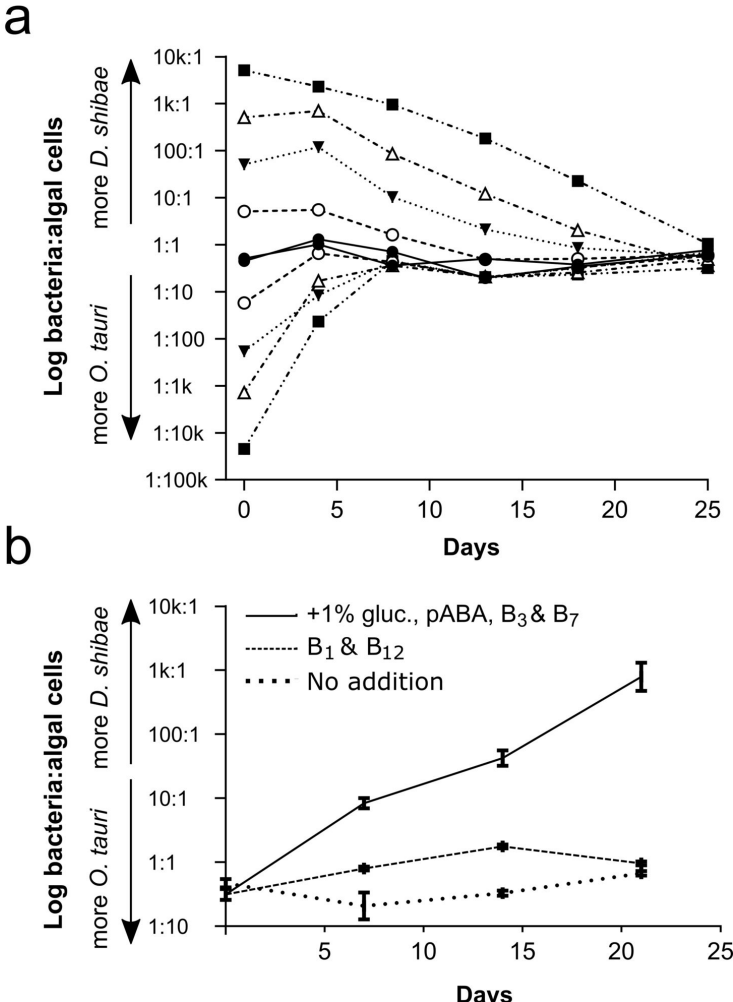
Figure 5. Distribution of pathways for synthesis of vitamins B3, B7 and B12 in selected bacterial species. A range of bacterial species with sequenced genomes were subjected to a bioinformatics pipeline to predict their vitamin biosynthesis capabilities (16). A set of 197 verified marine bacteria was obtained from the GOLD database (51), and a selection of 70 members of the *Rhodobacteraceae* family were sampled from NCBI. Their taxonomic positions were determined using ETE 3 (66), and species that were unclassified at the family level were discarded from the final analysis. The data were summarised at the family level for each of the different datasets – marine non-*Rhodobacteraceae* n=178, marine *Rhodobacteraceae* n=19, *Rhodobacteraceae* n=70.

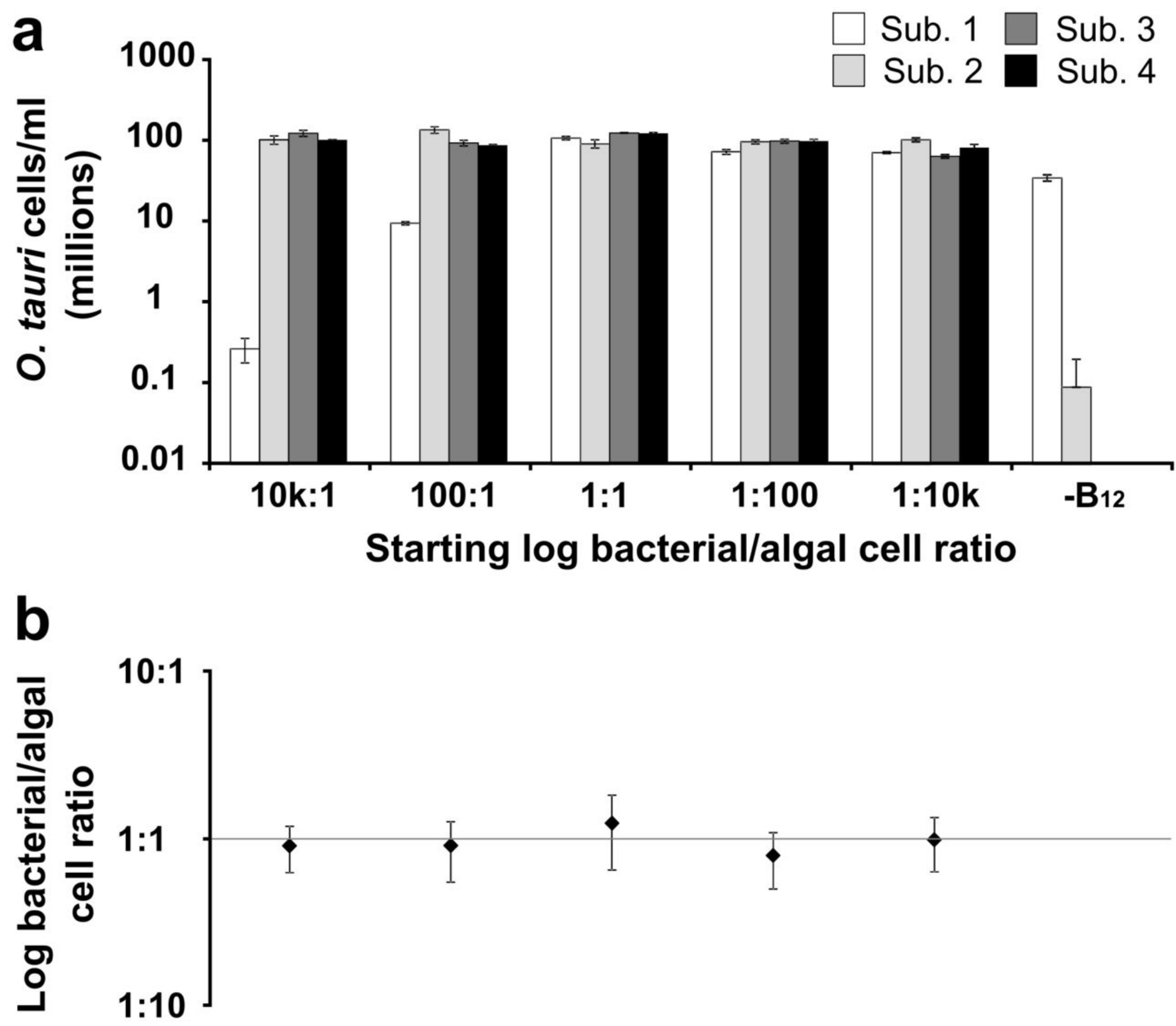
Figure 6. The meta-metabolome of the *O. tauri*-*D. shibae* partnership.
Red arrows indicate reactions dependent on vitamins provided by *D. shibae* in the model system, blue arrows indicate reactions dependent on vitamins produced by *O. tauri*. The provision of niacin, is arguably the most important aspect of this mutualism as many reactions in the *D. shibae* cell are dependent on NAD & NADP, the bioactive forms of niacin.

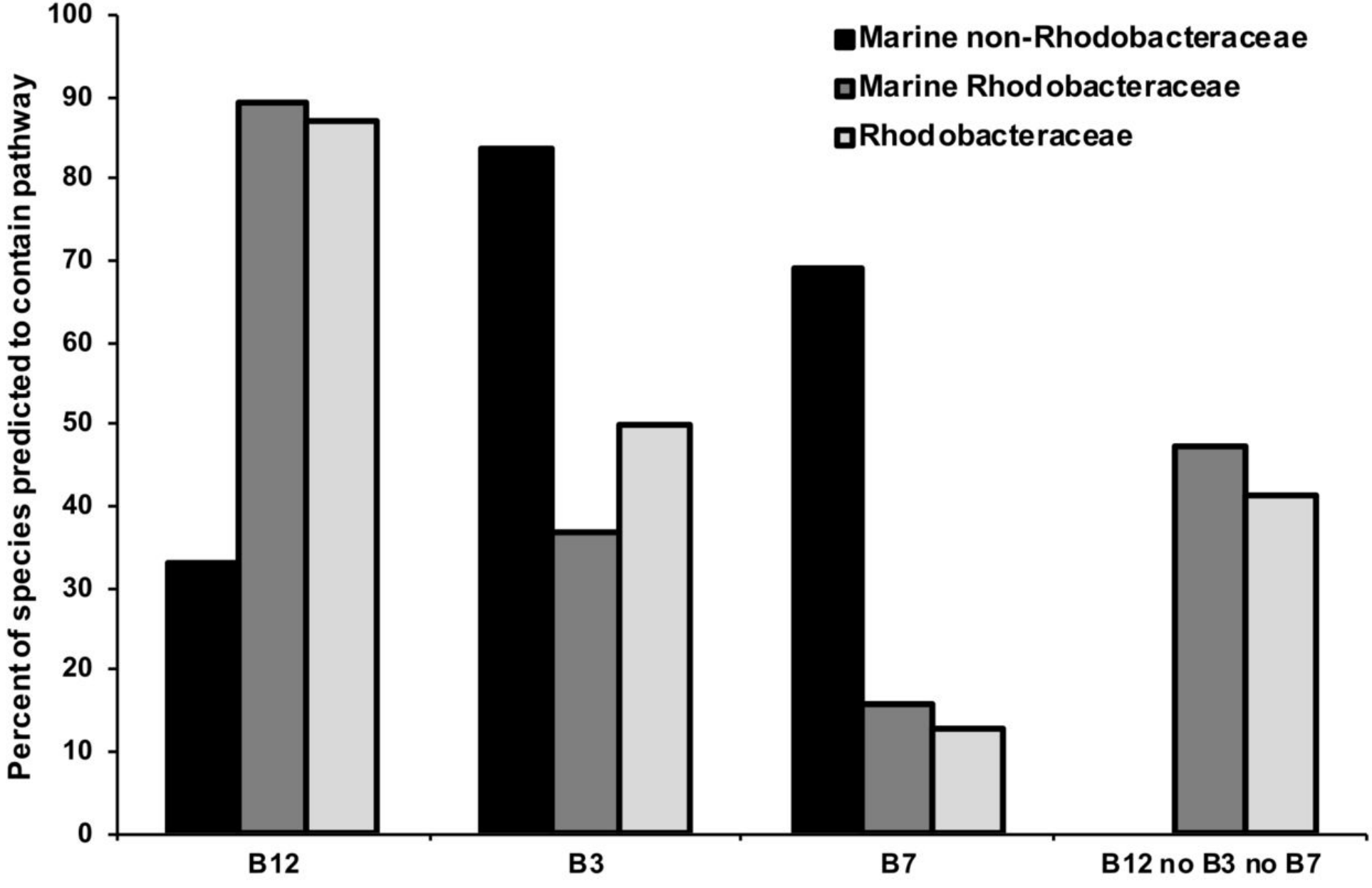

a
+ B-vitamins
- Cobalamin
- Thiamine
- Biotin
- PABA
- Niacin
- B-vitamins
O. tauri
D. shibae

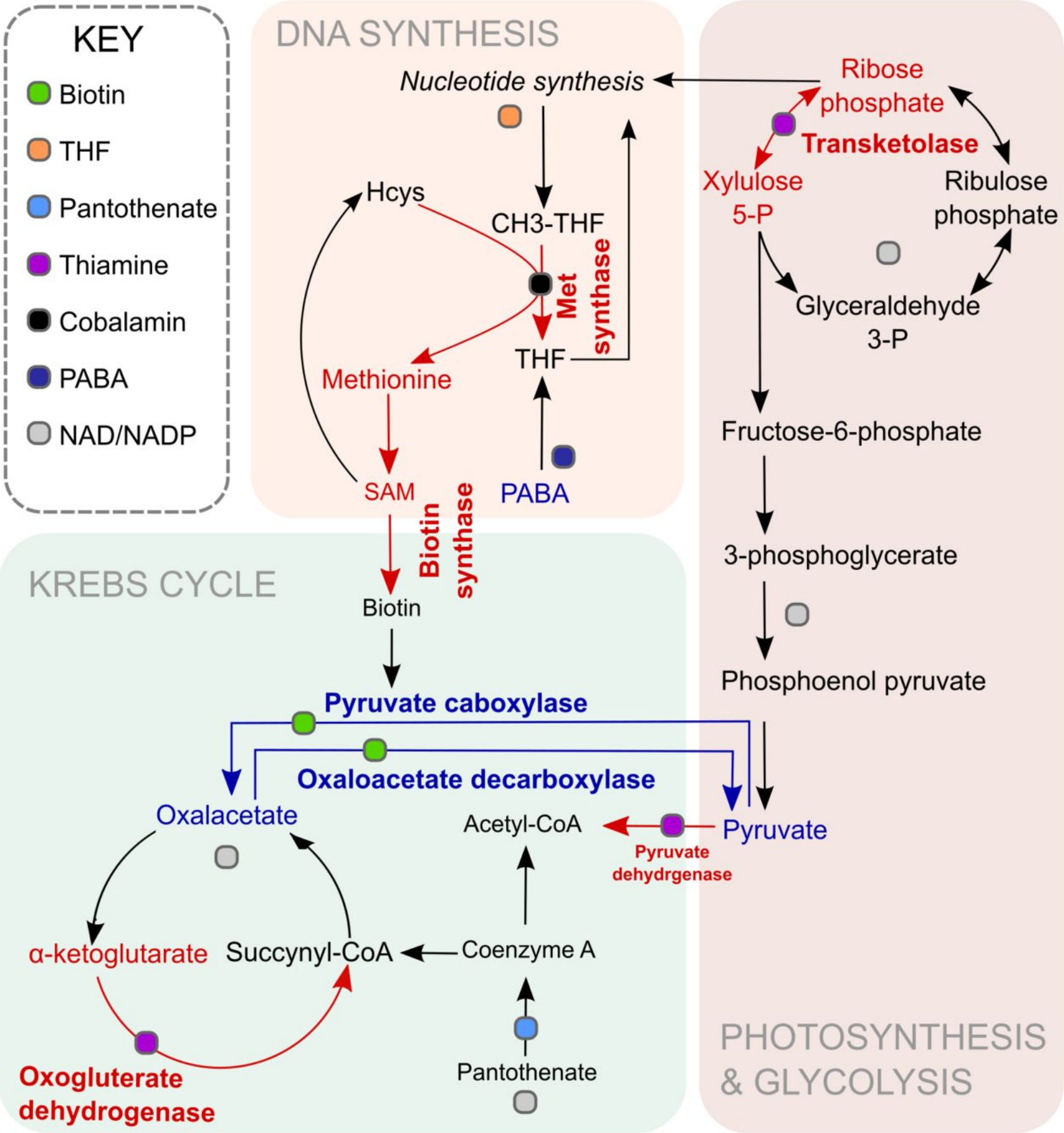

b
PABA
Chorismate
PAB-AB
PabA
PabB
4-Amino-4-deoxychorismate
PabC
4-Aminobenzoic acid
Biotin
Pimeloyl-COA
BioF
7-Keto-8-amino-
pelargonic acid
BioA
BIO1
7,8-Diaminopelargonic acid
BioD
Dethiobiotin
BioB
Biotin
nadB
nadX
nadA
nadC
Aspartate
IA
Quinolinate
Non-enzymatic
2-Amino-
3-carboxylmuconate
semialdehyde
Niacin
HAAO
3-Hydroxy-
anthranilate
KYNU
KMO
3-Hydroxy-L
-kynurenine
Kynurenine
hpaB
Anthranilate
Niacin
Key
Significant protein
hit in O. tauri
Significant protein
hit in D. shibae


a
150
125
100
75
50
25
0
O. tauri cells per ml (millions)
Mono-culture
Co-culture
+ B-vitamins
- Cobalamin
- Thiamine
- B-vitamins
b
550
500
450
150
100
50
0
D. shibae cells per ml (millions)
Mono-culture
Co-culture
+ B-vitamins
- Biotin
- Aminobenzoic acid
- Niacin
- B-vitamins


a
Log bacteria:algal cells
more D. shibae
more O. tauri
10k:1
1k:1
100:1
10:1
1:1
1:10
1:100
1:1k
1:10k
1:100k
0
5
10
15
20
25
Days
b
Log bacteria:algal cells
more D. shibae
more O. tauri
10k:1
1k:1
100:1
10:1
1:1
1:10
+1% gluc., pABA, B3 & B7
B1 & B12
No addition
5
10
15
20
25
Days


a
Sub. 1
Sub. 2
Sub. 3
Sub. 4
1000
100
10
1
0.1
0.01
O. tauri cells/ml (millions)
10k:1
100:1
1:1
1:100
1:10k
-B12
Starting log bacterial/algal cell ratio
b
10:1
1:1
1:10
Log bacterial/algal cell ratio


Marine non-Rhodobacteraceae
Marine Rhodobacteraceae
Rhodobacteraceae
Percent of species predicted to contain pathway
100
90
80
70
60
50
40
30
20
10
0
B12
B3
B7
B12 no B3 no B7


KEY
Biotin
THF
Pantothenate
Thiamine
Cobalamin
PABA
NAD/NADP
DNA SYNTHESIS
Nucleotide synthesis
Hcys
CH3-THF
Met synthase
THF
Methionine
SAM
PABA
Biotin synthase
Biotin
KREBS CYCLE
Pyruvate caboxylase
Oxaloacetate decarboxylase
Oxalacetate
Acetyl-CoA
Pyruvate
Pyruvate dehydrgenase
α-ketoglutarate
Succynyl-CoA
Coenzyme A
Pantothenate
Oxogluterate dehydrogenase
Ribose phosphate
Transketolase
Xylulose 5-P
Ribulose phosphate
Glyceraldehyde 3-P
Fructose-6-phosphate
3-phosphoglycerate
Phosphoenol pyruvate
PHOTOSYNTHESIS & GLYCOLYSIS