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

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

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Subject category: Microbe-microbe and microbe-host interactions

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24 **Abstract**

25 *Ostreococcus tauri*, a picoeukaryotic alga that contributes significantly to primary production in
26 oligotrophic waters, has a highly streamlined genome, lacking the genetic capacity to grow without
27 the vitamins thiamine (B₁) and cobalamin (B₁₂). Here we demonstrate that the B₁₂ and B₁ auxotrophy
28 of *O. tauri* can be alleviated by co-culturing with a heterotrophic bacterial partner *Dinoroseobacter*
29 *shibae*, a member of the *Rhodobacteraceae* family of alpha-proteobacteria, genera of which are
30 frequently found associated with marine algae. *D. shibae* lacks the complete pathway to synthesise
31 three other B vitamins: niacin (B₃), biotin (B₇), and *p*-aminobenzoic acid (a precursor for folate, B₉),
32 and the alga is in turn able to satisfy the reciprocal vitamin requirements of its bacterial partner in a
33 stable long-term co-culture. Bioinformatics searches of 197 representative marine bacteria with
34 sequenced genomes identified just 9 species that had a similar combination of traits (ability to make
35 vitamin B₁₂, but missing one or more genes for niacin and biotin biosynthesis enzymes), all of which
36 were from the *Rhodobacteraceae*. Further analysis of 70 species from this family revealed the
37 majority encoded the B₁₂ pathway, but only half were able to make niacin, and fewer than 13% biotin.
38 These characteristics may have either contributed to or resulted from the tendency of members of this
39 lineage to adopt lifestyles in close association with algae. This study provides a nuanced view of
40 bacterial-phytoplankton interactions, emphasising the complexity of the sources, sinks and dynamic
41 cycling between marine microbes of these important organic micronutrients.

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

47 **Introduction**

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

56 Analysis of several *Ostreococcus* species reveals highly reduced genomes, although they retain
57 the majority of metabolic attributes essential for autotrophic growth, including a complete set of genes
58 for transport and assimilation of ammonium, nitrate and urea (6). However, like many other algae
59 their growth is dependent on the presence of certain organic micronutrients in the environment,
60 specifically thiamine (vitamin B₁) (7) and cobalamin (vitamin B₁₂) (8), both of which are required as
61 cofactors for enzymes involved in central metabolism. Genomic evidence revealed that *O. tauri* has
62 only a partial biosynthetic pathway for thiamine, suggesting it may have lost its ability to synthesise
63 this cofactor *de novo* (9, 10). The B₁₂-biosynthetic pathway, comprising over 20 enzyme-catalysed
64 steps, is found only in certain prokaryotes (11). The requirement for this vitamin by B₁₂-dependent
65 algae is as a cofactor for the essential B₁₂-dependent enzyme, methionine synthase (METH), rather
66 than the inability to synthesise the vitamin. Those species that can grow independently of an external
67 supply of B₁₂ encode an alternative methionine synthase enzyme (METE) that does not use B₁₂ as a
68 cofactor (8, 12). *O. tauri* encodes *METH* but not *METE*, and thus requires vitamin B₁₂ for growth (8).

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

74 algae such as *O. tauri*, and were below the detection threshold in some regions (14). Addition of
75 vitamin B₁₂ (together with Fe and N) enhanced chlorophyll *a* levels threefold (beyond stimulating
76 effects of adding just Fe + N) in the eastern boundary of the South Atlantic Gyre (15). This highlights
77 the crucial role of this compound in controlling phytoplankton growth in natural marine microbial
78 communities, and demonstrates that the vitamin auxotrophy of *O. tauri* is representative of the natural
79 world, rather than a particularity of this species.

80 Although small populations may persist under limiting micronutrient conditions, many PPEs
81 including *Ostreococcus* periodically form characteristic blooms, implying that levels of vitamins must
82 increase. Prokaryotes are the ultimate and only source of vitamin B₁₂ (11). However, structural
83 diversity in the forms of B₁₂ produced by different prokaryote taxa has important implications in
84 terms of their bioavailability to eukaryote auxotrophs. In particular, evidence indicates that B₁₂-
85 synthesising cyanobacteria produce a variant, pseudocobalamin, that is considerably less bioavailable
86 to eukaryotic algae (16). This suggests cobalamin-producing heterotrophic bacteria and archaea are
87 likely to produce the majority of the cofactor that is bio-available for algae (12, 17). Further, certain
88 bacterial phyla are more likely to be found associated with algae. In an analysis of over 40 different
89 species of both macro and microalgae, six phyla (Bacteroidetes, Proteobacteria, Firmicutes,
90 Actinobacteria, Verrucomicrobia and Planctomycetes) accounted for the majority of bacteria (18), and
91 both alpha- and gamma-Proteobacteria are almost invariably found in studies of algal-associated
92 microbiomes (19). In particular, species from the family *Rhodobacteraceae* (20) have been shown to
93 deliver vitamins and other metabolites to marine algae. For example, *Dinoroseobacter shibae*, a
94 cosmopolitan obligately aerobic bacterium, originally isolated as a symbiont to cultured marine
95 dinoflagellates (21), could supply thiamine and B₁₂ to its hosts in exchange for a source of fixed
96 carbon (22). Similarly, *Ruegeria pomeroyi* supported the B₁₂ requirements of the diatom *Thalassiosira*
97 *pseudonana* in exchange for organosulphur compounds (23). Analysis of environmental isolates of
98 another diatom, *Pseudo-nitzschia multiseriis*, showed association with a number of bacteria one of
99 which, a *Sulfitobacter* species, promoted algal cell division by providing indole acetic acid (24).

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

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

112

113

114 **Materials & methods**

115 *Algal and bacterial strains and culture conditions*

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

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

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

141 All axenic and co-cultures containing *O. tauri* were grown at 18°C with a 16:8 hour light:dark
142 cycle (irradiance of 150 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in unmodified L1 medium (30) unless otherwise stated.
143 For experiments testing exchange of B vitamins, monocultures of *D. shibae* and *O. tauri* were pre-
144 washed 3 times in L1 medium and then starved of vitamins for 7 days prior to the start of the
145 experiment. B-vitamins were added to cultures at the following concentrations: 0.40 nM cobalamin,
146 2.05 nM biotin, 296 nM thiamine, 812 nM niacin and 291 nM *p*-aminobenzoic acid. These are
147 concentrations known to be sufficient for unhindered algal cell growth in L1 medium. However, it is
148 likely that these amounts are in excess of requirements, and the effect on cellular metabolism of
149 providing a different ratio of these metabolites has not been demonstrated in dedicated experimental
150 analyses.

151

152 *Measuring cell densities of O. tauri and D. shibae*

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

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

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

169

170 *Bioinformatics Analysis*

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

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

190 Results

191

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

194 Figure 1a shows the growth requirements of *O. tauri* and *D. shibae* in minimal medium with different
195 supplements. *O. tauri* requires both vitamins B₁₂ and B₁ (7, 8), as cofactors for cobalamin-dependent
196 methionine synthase (B₁₂) and several enzymes of intermediary carbon metabolism including
197 transketolase (B₁). *D. shibae* DFL-12 on the other hand encodes the genes for the biosynthesis of B₁₂
198 and B₁ (22), and can grow without either supplement. However, *D. shibae* is known to need
199 exogenous sources of three other B vitamins: biotin (vitamin B₇), niacin (B₃) and *p*-aminobenzoic acid
200 a precursor for folate (B₉) biosynthesis, whereas *O. tauri* does not require these molecules for growth.
201 All three of these compounds have vital roles in central metabolism: biotin is a co-factor for enzymes
202 necessary for essential carboxylation, transcarboxylation and decarboxylation reactions, folate is
203 required for enzymes of 1-carbon metabolism, and niacin is a precursor to the hydrogen carriers
204 NAD⁺ & NADP⁺, redox carriers used ubiquitously within the cell (reviewed in 37). Despite these
205 fundamental roles, the exact genetic basis underlying the auxotrophy of *D. shibae* for these vitamins is
206 currently unknown. We therefore carried out a detailed assessment of the genomes of the two
207 organisms to investigate this, and whether they may be able to support one another's growth in co-
208 culture.

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

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

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

233 The compound *p*-aminobenzoic acid, an intermediate in the synthesis of folate and its
234 bioactive forms, is produced from chorismate (45). In bacteria, two enzymes are required for this
235 process. The first, aminodeoxychorismate synthase (ADCS) is made up of two protein sub-units,
236 PabA and PabB (46). Sub-unit II of the ADCS complex (PabA) acts as a glutamine amidotransferase,
237 transferring the amino group from glutamine to sub-unit I (PabB), which is then used to aminate the
238 chorismate molecule (46). *Arabidopsis thaliana*, some yeasts, and certain algae have been shown to
239 encode a bi-functional enzyme (PAB-AB) that performs both of these steps (47). In both bacteria and
240 plants, the final enzyme in the pathway is 4-amino-4-deoxychorismate lyase (ADCL, PabC) that
241 catalyses the reversible conversion of aminodeoxychorismate into *p*-aminobenzoic acid and pyruvate
242 (48). *O. tauri* has significant hits for PAB-AB and PABC (Figure 1b and supporting material in Table
243 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

272 95 with a cocktail of antibiotics over several subcultures reduced the load considerably, but one
273 bacterium, *Zeaxanthibacterium* sp. (*Flavobacteriaceae*), was always present. Although
274 supplementation with B₁ & B₁₂ was still required, indicating that it was not providing the vitamins to
275 *O. tauri*, we nonetheless wanted to separate the algal and bacterial cells to avoid confounding factors.
276 Since these are essentially the same dimensions (~1 μm in diameter), we explored the possibility of
277 using fluorescence activated cell sorting (FACS). Initially, cells were gated on their size and shape,
278 and then gated further based on their pulse width/pulse area, as described in Methods. This process
279 could distinguish between single cells and doublets or triplets, indicative of bacteria attached to algae.
280 Once settings were established that allowed only single cells to flow through the channel, chlorophyll
281 fluorescence was used to separate algal cells from bacteria (Figure S2). Cells were treated again with
282 antibiotics for 14 days, following which no bacteria were detected by light microscopy or growth on
283 marine broth plates.

284 Given the non-overlapping B-vitamin dependencies of *O. tauri* and *D. shibae*, we then
285 investigated whether a stable co-culture of the two organisms could be established in medium
286 containing none of the five B vitamins, to instigate the transfer of complementary micronutrients.
287 Cultures were maintained for three sub-cultures (each of 21-25 day duration) to ensure that any rescue
288 effect was long-term and stable. At stationary phase, total cell numbers for both *O. tauri* and *D.*
289 *shibae* were taken and compared to control mono-cultures grown in parallel (Figure 2). Maximal
290 growth was evident when *O. tauri* and *D. shibae* were grown in monoculture with all the necessary B
291 vitamins. However, a significant increase in *O. tauri* cell density was observed when the alga was
292 grown in co-culture with *D. shibae* (Figure 2a, white bars) in medium lacking thiamine, B₁₂ or all B-
293 vitamins compared with the respective axenic monocultures (grey bars) (Student's t-test p<0.05),
294 although the rescue effect varied in magnitude. We found that whilst *D. shibae* was able to satisfy the
295 requirements of *O. tauri* for B₁₂, the rescue effect was weaker when thiamine was omitted, with the
296 maximum carrying capacity achieved only ~30% of that when the vitamin was included in the growth
297 medium. In turn, co-culturing with *O. tauri* satisfied the requirements of *D. shibae* for each of niacin,
298 biotin and *p*-aminobenzoic acid (Figure 2b), with statistically significant differences observed
299 compared to growth in monoculture under the equivalent deficiency profiles (Student's t-test P<0.05

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

303

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

306 The previous experiment indicated that growth yields of both *O. tauri* (OTH95) and *D. shibae* DFL-
307 12 were lower in co-culture compared to mono-cultures. The observation that carrying capacities of
308 organisms engaged in mutualisms are different to mono-culture is common, and has been termed
309 regulation (51). This is particularly well documented for plants in association with rhizobial
310 symbionts, where the balance of host to mutualist is carefully maintained, and allows a carefully
311 maintained exchange of resources (e.g. 52). For algal and bacterial systems in mutualism regulation
312 has been observed (53) but often the signalling mechanism remains unclear (reviewed in 54).

313 To determine whether *O. tauri* and *D. shibae* actively regulate each other's growth, we inoculated the
314 two organisms at different relative proportions into minimal medium without niacin, *p*-aminobenzoic
315 acid or B₁₂; both thiamine and biotin were included to enable reasonable growth rates and minimise
316 the time needed for each experiment (omission of these vitamins had the greatest inhibitory effect on
317 *O. tauri* and *D. shibae* cell density in co-culture, respectively (Figure 2)). For those cultures with
318 higher initial algal numbers, the starting inoculation density for the algae was set to $\sim 2 \times 10^6$ cells per
319 ml, with bacterial numbers adjusted accordingly to obtain the different ratios. When bacteria were
320 initially in excess, the starting inoculation density of bacterial cells was $\sim 1 \times 10^6$ cells per ml. Growth
321 of the cultures were monitored over time by cell counting as described in the Methods. We found that
322 regardless of starting numbers of either algal or bacterial cells, after 21-25 days in co-culture a $\sim 1:1$
323 ratio of the two organisms was reached (Figure 3a). *D. shibae* cells have a faster growth rate than *O.*
324 *tauri*, and so the 1:1 equilibrium was reached within 8 days when starting cultures had a higher ratio
325 of algae to bacteria, whereas 25 days were required for *O. tauri* cells to reach parity from an initial
326 inoculum with more bacterial cells.

327 Previously, we had shown that a similar co-culture could be established between the B₁₂-
328 dependent freshwater green alga *Lobomonas rostrata* with the soil bacterium *Mesorhizobium loti* (53).
329 An exogenous supply of either vitamin B₁₂ or a fixed carbon source disrupted the nature of the co-
330 culture. We therefore attempted to disrupt the *O. tauri*/*D. shibae* interaction through the addition of
331 different B vitamins and a carbon source that favour bacterial growth. When the suite of vitamins
332 required by the bacteria were provided exogenously into the algal-bacterial co-culture together with a
333 source of fixed carbon, the ratio of bacteria:algae altered significantly in favour of *D. shibae*,
334 releasing the co-cultures from the established 1:1 ratio (Figure 3b). Addition of just thiamine and B₁₂
335 to the co-cultures also encouraged the growth of bacteria slightly relative to controls, even though
336 these conditions satisfy the vitamin requirements of *O. tauri* and would be expected to favour the
337 growth of the alga. This suggests that *O. tauri* is unable to regulate the growth of *D. shibae* when not
338 engaged in mutualism with the bacteria. Nevertheless, when conditions for mutualism were
339 maintained in minimal medium over successive subcultures (i.e. without supplementation with fixed
340 carbon or B vitamins), *O. tauri* and *D. shibae* continued to grow in a stable self-sustaining mutualistic
341 relationship over four consecutive sub-cultures regardless of starting inoculum, resulting in a ratio of
342 around 1:1 for the two organisms (Figure 4).

343

344 *Niacin and biotin auxotrophy is enriched in B₁₂-biosynthesising bacteria belonging to the*
345 *Rhodobacteraceae*

346 The co-culture between *O. tauri* (OTH95) and *D. shibae* DFL-12 demonstrates a novel role for the
347 vitamin cofactors niacin, biotin and *p*-aminobenzoic acid in nutrient exchange. To assess the broader
348 relevance of this observation, we investigated whether the potential to form mutualistic interactions
349 with eukaryotic marine algae based on reciprocal B vitamin exchanges is a likely common
350 characteristic of algae-associated bacteria. A survey of reports in the literature of bacteria found
351 associated with marine algae from 7 different locations identified a total of 28 genera (Table S8).
352 Strikingly, in each location species of the *Rhodobacteraceae* family (order Rhodobacterales) were
353 found (asterisked in Table S8). We chose 70 species from this family that had sequenced genomes,
354 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 B₁₂ 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 B₁₂ 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 B₁₂ 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 B₁₂, but not B₃ or B₇ (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

383 progressive trophic levels is out-dated (56, 57). Instead, studies on aquatic microbial interactions are
384 unveiling a suite of different lifestyles and cross-feeding interactions at the heart of these complex
385 communities. Vitamins have emerged as important players in associations between aquatic microbes
386 (e.g. 23, 24, 56, 57), since by definition they are required obligately by auxotrophs for survival, so the
387 demands for them must be met by other species capable of their biosynthesis, even if not directly (58).
388 The findings presented here are the first physiological demonstration of a bilateral B-vitamin
389 exchange between aquatic microorganisms. Perhaps particularly important is our finding that *O. tauri*
390 was able to provide *D. shibae* with niacin, the vitamin for which *D. shibae* exhibited the highest
391 dependency. Very few studies have attempted to determine the concentration of niacin in oceanic
392 environments: at the time of writing the only available paper is from half a century ago (59) and states
393 niacin was undetectable in the Alaskan surface waters sampled. Our *in silico* survey found 20% of
394 marine bacteria sampled do not have the genetic capacity to synthesise it *de novo* (Supplementary
395 Dataset S2), but niacin auxotrophy is significantly enriched in the *Rhodobacteraceae* (35/70 species
396 sampled), whilst in contrast these are more likely to synthesise vitamin B₁₂ than marine bacteria
397 generally. Two *Roseobacter* species, *R. litoralis* and *R. denitrificans*, have previously been shown
398 experimentally to require an exogenous source of niacin and biotin for growth (22). These bacteria
399 were originally isolated from the surface of green seaweeds, and were not found in open waters. More
400 recently, another member of the *Rhodobacteraceae* (20), a *Sulfitobacter* species named SA11, was
401 identified as a key symbiont with the toxic bloom forming alga *Pseudo-nitzschia* (24). Previous
402 studies have also identified niacin auxotrophy in host-associated bacteria, including in pathogenic and
403 enteric bacteria (60). For example, *Shigella flexneri*, the pathogen responsible for shigellosis in
404 primates, is a niacin auxotroph, having lost the *nadA* and *nadB* genes responsible for synthesis of
405 quinolinate from aspartate, a precursor to niacin (61). The central role played by the bioactive forms
406 of niacin as redox carriers and signalling molecules (62) makes it a crucial micronutrient for cell
407 growth and survival in all organisms. The inability of some microbes to synthesise it is therefore
408 perhaps surprising, especially considering that it requires just 3 enzymatic steps from central
409 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 B₁₂ 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 K_s values of thiamine for several algal species, which have been reported to be up to four
417 orders of magnitude higher than those for B₁₂ (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 huxleyi* 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.

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

476

477 **Acknowledgements**

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

485

486 **Conflict of Interest Statement**

487 The authors declare no conflict of interest

488

489

490 **References**

491

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659

660 **Figure legends**

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

668

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

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

676

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

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

680 25 days. **B.** The effect of adding-back nutrients on the established ratio. Ratio of *O. tauri* to *D. shibae*
681 cells over the course of one culture when a stable co-culture is inoculated into medium favouring the
682 bacteria (supplemented with 1% glucose and vitamins *p*-aminobenzoic acid (*p*ABA), B₃ and B₇),
683 medium favouring the alga (with B₁ and B₁₂) or no nutrient addback.

684

685 **Figure 4. Rescue effect of *O. tauri* and *D. shibae* for required B vitamins is stable and long-term.**

686 **A.** Log cell density of *O. tauri* cells at stationary phase over 4 sub-cultures that were inoculated at
687 differing initial ratios to *D. shibae*. **B.** The average ratio of *D. shibae*: *O. tauri* at each different
688 starting bacterial: algal ratio at stationary phase. Error bars indicate standard error for mean of ratios
689 at stationary phase across all sub-cultures (n=4).

690

691 **Figure 5. Distribution of pathways for synthesis of vitamins B3, B7 and B12 in selected bacterial**

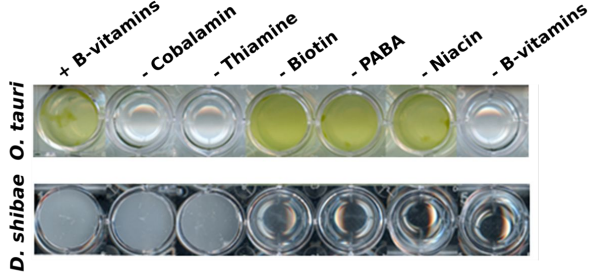
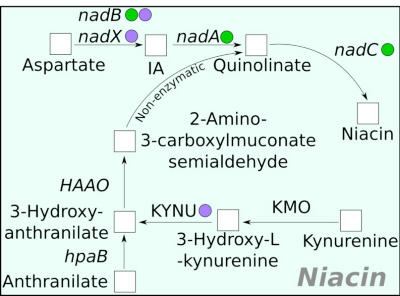
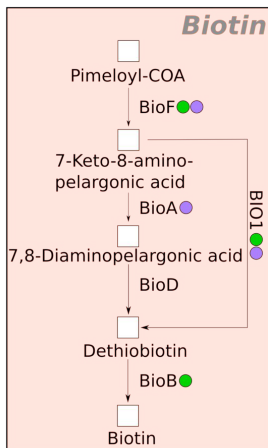
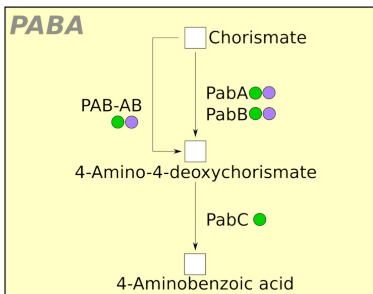
692 **species.** A range of bacterial species with sequenced genomes were subjected to a bioinformatics
693 pipeline to predict their vitamin biosynthesis capabilities (16). A set of 197 verified marine bacteria
694 was obtained from the GOLD database (51), and a selection of 70 members of the *Rhodobacteraceae*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

699

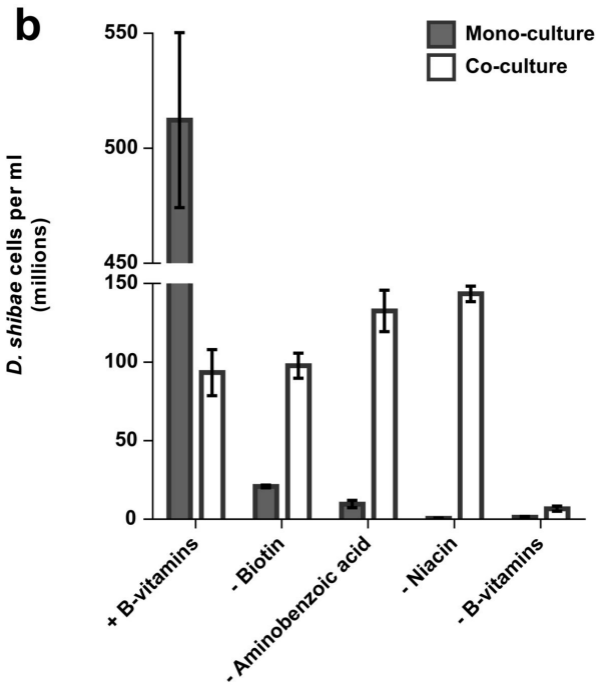
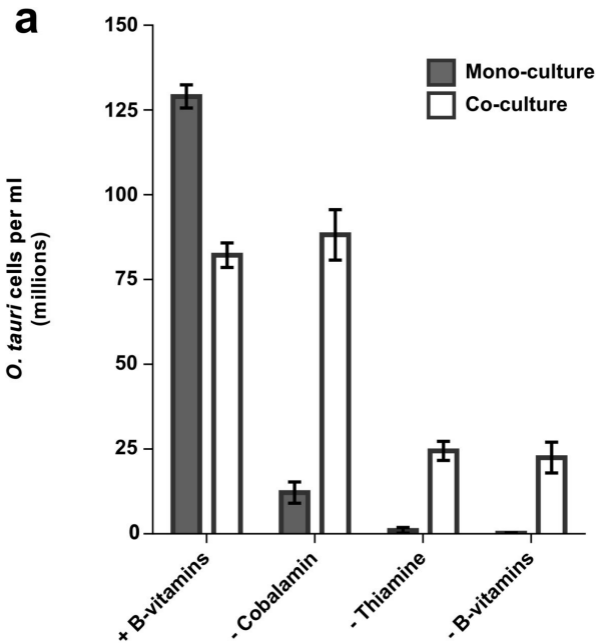
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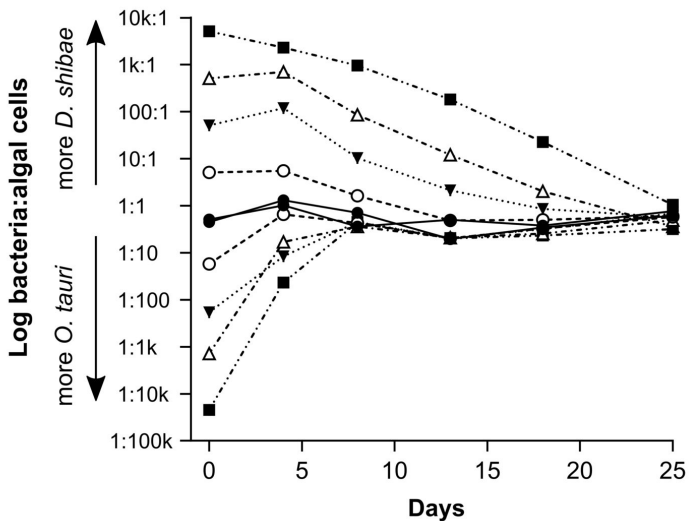
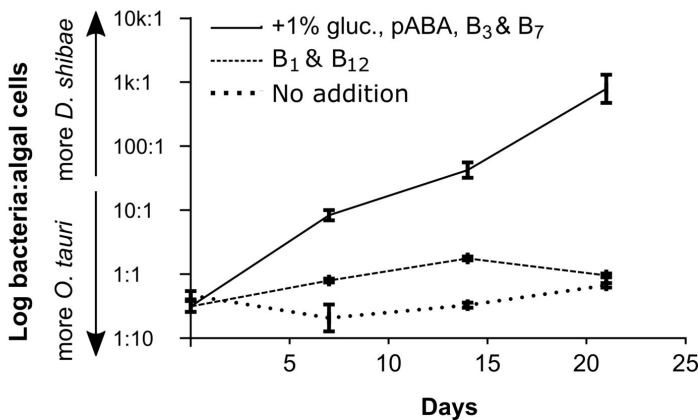
701 **Figure 6. The meta-metabolome of the *O. tauri*-*D. shibae* partnership.**

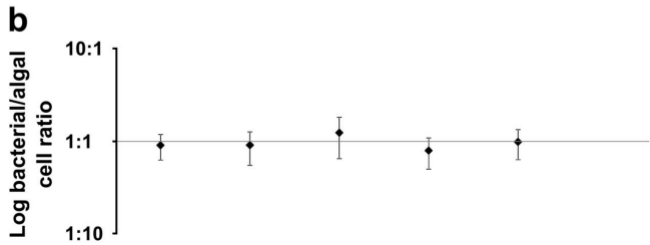
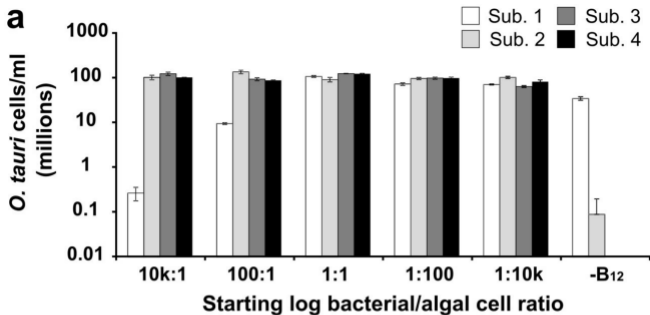
702 Red arrows indicate reactions dependent on vitamins provided by *D. shibae* in the model system, blue
703 arrows indicate reactions dependent on vitamins produced by *O. tauri*. The provision of niacin, is
704 arguably the most important aspect of this mutualism as many reactions in the *D. shibae*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

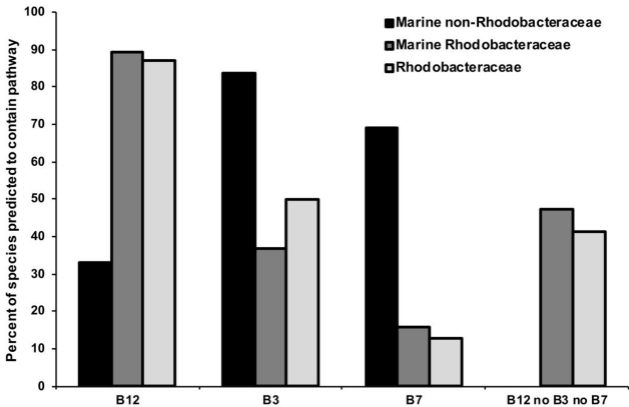
a**b****Key**

- Significant protein hit in *O. tauri*
- Significant protein hit in *D. shibae*



a**b**

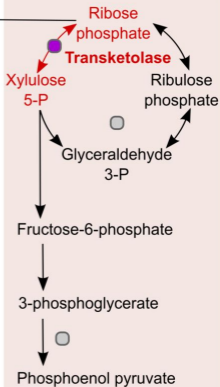
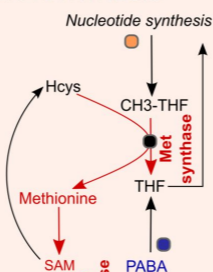




KEY

- Biotin
- THF
- Pantothenate
- Thiamine
- Cobalamin
- PABA
- NAD/NADP

DNA SYNTHESIS



KREBS CYCLE

