

## LETTERS

# Algae acquire vitamin B<sub>12</sub> through a symbiotic relationship with bacteria

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Vitamin B<sub>12</sub> (cobalamin) was identified nearly 80 years ago as the anti-pernicious anaemia factor in liver<sup>1</sup>, and its importance in human health and disease has resulted in much work on its uptake<sup>2</sup>, cellular transport<sup>3</sup> and utilization<sup>4</sup>. Plants do not contain cobalamin because they have no cobalamin-dependent enzymes. Deficiencies are therefore common in strict vegetarians<sup>5</sup>, and in the elderly, who are susceptible to an autoimmune disorder that prevents its efficient uptake<sup>6</sup>. In contrast, many algae are rich in vitamin B<sub>12</sub>, with some species, such as *Porphyra yezoensis* (Nori), containing as much cobalamin as liver<sup>7</sup>. Despite this, the role of the cofactor in algal metabolism remains unknown, as does the source of the vitamin for these organisms. A survey of 326 algal species revealed that 171 species require exogenous vitamin B<sub>12</sub> for growth, implying that more than half of the algal kingdom are cobalamin auxotrophs. Here we show that the role of vitamin B<sub>12</sub> in algal metabolism is primarily as a cofactor for vitamin B<sub>12</sub>-dependent methionine synthase, and that cobalamin auxotrophy has arisen numerous times throughout evolution, probably owing to the loss of the vitamin B<sub>12</sub>-independent form of the enzyme. The source of cobalamin seems to be bacteria, indicating an important and unsuspected symbiosis.

Vitamin B<sub>12</sub> is one of nature's most complex metabolites, requiring at least 19 separate enzymatic steps for its synthesis from uroporphyrinogen III, the common precursor of all tetrapyrroles including haem and chlorophyll. These enzymes have been characterized in prokaryotic organisms only<sup>8</sup>. Many marine algae are known to be extremely rich in vitamin B<sub>12</sub>, but the source remains a matter of controversy. Although there have been reports that some algae are able to synthesize cobalamin *de novo*<sup>9</sup>, many other algae have been found to require exogenous cobalamin for growth in culture<sup>10</sup>, implying that they are unable to make it themselves. The vitamin B<sub>12</sub> requirements of different species of algae grown in axenic culture were evaluated in several early projects, but there has been no systematic examination across the algal kingdom. We therefore compiled the data in the literature for more than 300 species of algae (Supplementary Table S1). To assess the validity of the data, we grew a number of representative species from each phylum (highlighted in Supplementary Table S1) in minimal medium and tested their vitamin B<sub>12</sub> requirements. In every case our results coincided with those previously published. Table 1 presents a summary of our analysis by algal group. Of the 326 species surveyed, over half require vitamin B<sub>12</sub> for growth, demonstrating that these organisms are not true autotrophs. Furthermore, this requirement shows no relationship to established algal lineages: all the phyla contain species that require the vitamin and species that do not. This pattern is also mirrored within individual genera such as *Lobomonas* in the Chlorophyta, *Peridinium* in the Dinophyta, *Pavlova* in the Haptophyta and *Thalassiosira* in the Heterokontophyta. This suggests that vitamin B<sub>12</sub> auxotrophy has arisen independently numerous times during evolution, and that these algae cannot synthesize vitamin B<sub>12</sub> *de novo*. This

poses the question, do the remaining algae synthesize the vitamin, or have they dispensed with it altogether like higher plants?

Recently, the genome sequences of three algae have been released: *Chlamydomonas reinhardtii* (see <http://www.jgi.doe.gov/>), a green alga (Chlorophyta); *Cyanidioschyzon merolae*<sup>11</sup>, a red alga (Rhodophyta); and the diatom (Heterokontophyta) *Thalassiosira pseudonana*<sup>12</sup>. Our own growth experiments confirm that the first two organisms do not require vitamin B<sub>12</sub>, whereas *T. pseudonana* shows a clear dependence on it (Supplementary Table S1). *C. reinhardtii* has been proposed to synthesize the vitamin *de novo*, because when the organism was transferred from a medium containing vitamin B<sub>12</sub> to unsupplemented medium, the cells still contained traces of the vitamin<sup>13</sup>. However, a search of the *C. reinhardtii* genome found no genes with sequence similarity to those encoding vitamin B<sub>12</sub> biosynthetic enzymes. Similarly, there are no biosynthetic genes in the *C. merolae* genome. *T. pseudonana*, on the other hand, contains a gene with sequence similarity to *cbiP*, which encodes cobyrinic acid *a,c*-diamide synthase. However, when we carried out a polymerase chain reaction with reverse transcription (RT-PCR) analysis, we failed to detect transcripts of this gene (Fig. 1a), which suggests that it is not expressed. Furthermore, this organism contains no genes for any of the other 18 enzymes specific for vitamin B<sub>12</sub> biosynthesis.

It is conceivable that cobalamin is synthesized through an alternative pathway to that found in bacteria. To test this, five vitamin B<sub>12</sub>-independent algae (Supplementary Table S1), including *C. reinhardtii*, were grown for at least five subcultures in unsupplemented medium and assayed for the presence of vitamin B<sub>12</sub> using a bioassay with a detection limit of 1 ng ml<sup>-1</sup>. In every case, there was no measurable cobalamin in cell extracts. The most likely explanation for the earlier report of vitamin B<sub>12</sub> biosynthesis in *C. reinhardtii*<sup>13</sup> is that the cobalamin present in the cells had been taken up from the supplemented medium. Coupled with the data from the three published genomes, it can be concluded that the pathway for vitamin

**Table 1 | A summary of the vitamin B<sub>12</sub> requirements of different algal phyla**

Phylum	Species surveyed	Require B <sub>12</sub>	Do not require B <sub>12</sub>
Chlorophyta	154	49	105
Glaucocestophyta	1	1	0
Rhodophyta	13	12	1
Cryptophyta	8	7	1
Dinophyta	30	26	4
Euglenophyta	15	13	2
Haptophyta	22	14	8
Heterokontophyta	83	49	34
Total	326	171	155

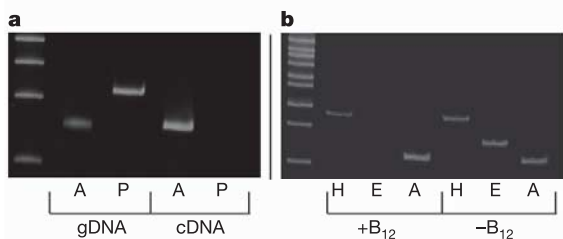
Data from the individual species detailed in Supplementary Table S1 are compiled under the different algal groups. The first three groups have simple plastids, resulting from primary endosymbiosis, whereas the remaining groups have complex plastids, due to secondary and tertiary endosymbioses<sup>28</sup>.

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B<sub>12</sub> biosynthesis is not present in algae, supporting the generally held view that the ability to synthesize the vitamin did not make the prokaryotic to eukaryotic transition<sup>8</sup>. Cyanobacteria (the ancestors of chloroplasts) and the  $\alpha$ -subgroup of proteobacteria (which gave rise to mitochondria) contain members that have the capacity to synthesize vitamin B<sub>12</sub>, as do the Archaea. However, none of the sequenced genomes from lower eukaryotes (including the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the protista *Entamoeba histolytica* and *Dictyostelium discoideum*) contains genes for a functional vitamin B<sub>12</sub> biosynthetic pathway.

Vitamin B<sub>12</sub> auxotrophy in individual algal species is thus likely to have arisen because the cofactor became essential for their metabolism, rather than from the loss of a functional biosynthetic pathway. To address this further, we investigated the role of the biological form of vitamin B<sub>12</sub> in algae. We searched the three algal genomes for genes that encode vitamin B<sub>12</sub>-dependent enzymes. The major difference between the cobalamin-dependent and -independent algae was found to be associated with methionine metabolism. *C. reinhardtii* and *C. merolae* contain vitamin B<sub>12</sub>-independent methionine synthase genes (*metE*). In contrast, the vitamin B<sub>12</sub>-dependent alga *T. pseudonana* contains the gene for the vitamin B<sub>12</sub>-dependent methionine synthase (*metH*) only. Interestingly, *C. reinhardtii* contains both isoforms of methionine synthase. We used RT-PCR to investigate whether the two *C. reinhardtii* genes were expressed. The results clearly demonstrate that *metH* is expressed both in the presence and in the absence of vitamin B<sub>12</sub> (Fig. 1b, tracks marked H), whereas *metE* is expressed only in the absence of the vitamin (track E). It appears that *C. reinhardtii* preferentially uses the vitamin B<sub>12</sub>-dependent form of methionine synthase, which has a higher rate of catalysis<sup>14</sup>, but in the absence of the vitamin the alga is able to survive by inducing the expression of *metE*.

The metabolic consequences of vitamin B<sub>12</sub> deprivation in humans is a reduction in methionine synthase activity, causing changes in the balance of intracellular folate derivatives, which ultimately interferes with nucleic acid biosynthesis<sup>15</sup>. The biochemical signature of this condition is increased amounts of the substrate homocysteine in blood plasma<sup>16</sup>. The relationship between methionine metabolism and vitamin B<sub>12</sub> in algae was investigated further using *Lobomonas rostrata*, a vitamin B<sub>12</sub>-dependent green alga closely related to *C. reinhardtii*. Instead of the vitamin, the alga was grown in the presence of methionine and folate. In the absence of supplements (row I in Fig. 2a), or with methionine (row II) or folate (row III) individually, the cells died after they had been subcultured three



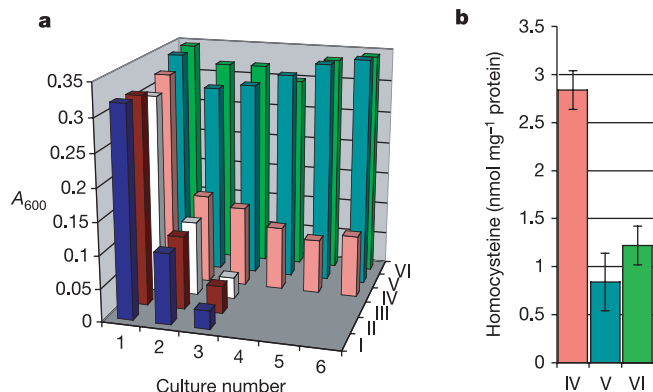
**Figure 1 | RT-PCR analysis of genes involved in vitamin B<sub>12</sub> metabolism.** **a**, Analysis of the gene for cobyric acid *a,c*-diamide synthase (*cbiP*) from *Thalassiosira pseudonana*. PCR was carried out with either genomic DNA (gDNA) or cDNA as template. Track A used primers to the constitutively expressed actin gene; track P used primers to the *cbiP* gene. The lack of a band in track P with cDNA suggests that the cobyric acid *a,c*-diamide synthase gene is not expressed. **b**, Analysis of the vitamin B<sub>12</sub>-dependent (*metH*) and -independent (*metE*) methionine synthase genes from *Chlamydomonas reinhardtii* grown in the presence (+B<sub>12</sub>, 10  $\mu\text{g l}^{-1}$ ) or absence (-B<sub>12</sub>) of vitamin B<sub>12</sub>. Track H used primers for *metH*; track E used primers for *metE*; Track A used primers for the constitutively expressed actin gene. The lack of a band in track E (+B<sub>12</sub>) reveals that *metE* is not expressed in the presence of vitamin B<sub>12</sub>. Details of primers used are given in Supplementary Methods.

times. However, methionine and folate added together (row IV) rescued vitamin B<sub>12</sub> auxotrophy, although the rate of growth was slower than in the presence of vitamin B<sub>12</sub> (rows V and VI). Amino acid analysis of *L. rostrata* cells grown in the absence of vitamin B<sub>12</sub> showed that homocysteine accumulates in the cell (Fig. 2b), demonstrating that the role of the vitamin in this organism is to make methionine. Thus, the most likely explanation for cobalamin dependency in *L. rostrata* is that it has lost the *metE* gene.

In their natural environment, vitamin B<sub>12</sub>-dependent algae must be able to obtain the vitamin from an external source. Reported levels of free cobalamin are generally about 2–6  $\text{ng l}^{-1}$  (up to 4 pM) in fresh water<sup>17</sup> and up to 3  $\text{ng l}^{-1}$  (~2 pM) in sea water<sup>18</sup>. Our growth studies suggested that this would be insufficient to support algal growth. For example, the marine red alga *Porphyridium purpureum* (Supplementary Fig. S1), the dinoflagellate *Amphidinium operculatum* (Supplementary Fig. S2) and the freshwater euglenoid *Euglena gracilis* (Supplementary Fig. S3) would not grow in minimal media made with natural filter-sterilized sea water or pond water, unless supplemented with at least 10  $\text{ng l}^{-1}$  (~7 pM) vitamin B<sub>12</sub>. For comparison, most algal culture media contains 10  $\mu\text{g l}^{-1}$  vitamin B<sub>12</sub>. These results demonstrate that the concentration of free cobalamin in the environment is limiting. Given that vitamin B<sub>12</sub> dependency has arisen numerous times in the algal kingdom, there must be an alternative, readily accessible source.

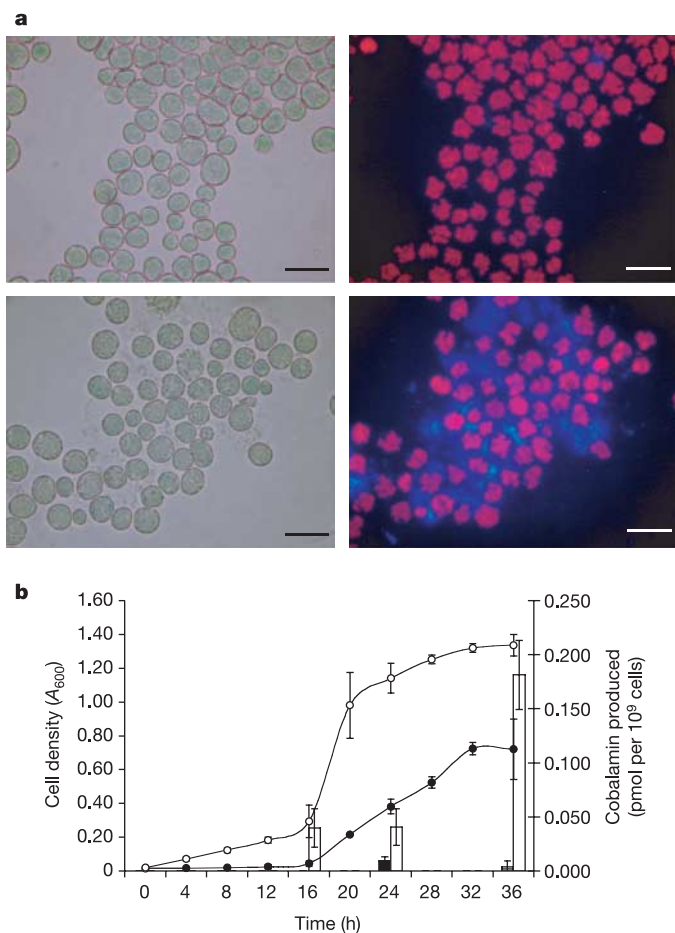
Bacteria have previously been implicated in supplying B vitamins to algae (for example, see ref. 17). Indeed, many algal cultures are maintained in culture collections non-axenically. One such culture of *A. operculatum* was obtained from the Culture Collection of Algae and Protozoa (Oban, UK). The algal cells in this culture grew without the addition of exogenous vitamin B<sub>12</sub>. A species of bacterium was isolated from the culture medium and found to be able to synthesize vitamin B<sub>12</sub> *de novo*. The 16S ribosomal RNA gene was amplified by PCR, sequenced and found to be identical to that of *Halomonas* sp. To establish whether this bacterium was responsible for supplying *A. operculatum* with vitamin B<sub>12</sub>, it was added back to axenic cultures of *A. operculatum* and *P. purpureum*. In both cases, *Halomonas* sp. was able to support growth of the algae to the same extent as vitamin B<sub>12</sub> (Supplementary Figs S1 and S2).

Because the medium of these co-cultures did not contain an organic carbon source, the bacteria were presumably using the



**Figure 2 | Effect of vitamin B<sub>12</sub> deprivation on the green alga *L. rostrata*.** **a**, Growth of *L. rostrata* in Jaworski's medium for six subcultures. I, no supplements; II, 10 mM methionine; III, 1  $\mu\text{M}$  folate; IV, 1  $\mu\text{M}$  folate and 10 mM methionine; V, 10  $\mu\text{g l}^{-1}$  vitamin B<sub>12</sub>, 1  $\mu\text{M}$  folate and 10 mM methionine; VI, 10  $\mu\text{g l}^{-1}$  vitamin B<sub>12</sub>. The values represent the optical density of the cultures measured at 600 nm ( $A_{600}$ ) after 7 days in continuous light. **b**, Homocysteine analysis of *L. rostrata* cells grown under the indicated conditions in **a** (IV, V and VI). The analysis was repeated three times; error bars represent standard deviation of three replicates. The amount of homocysteine increases in the absence of vitamin B<sub>12</sub> because methionine synthase is inactive.

products of algal photosynthesis to grow, suggesting that this is a mutualistic relationship. Examination of cells of *P. purpureum* grown in co-culture with *Halomonas* sp. by microscopy clearly shows the bacterium associated with the extracellular muciferous layer of *P. purpureum* (Fig. 3a). The algal and bacterial cells remain together through multiple washing steps during DAPI staining, indicating that the bacterium is tightly associated with this muciferous layer. A further indication of the intimate relationship was obtained when *Halomonas* sp. was grown in minimal medium in the presence and absence of fucoidin, a commercially available algal extract. The extract increased the rate of bacterial growth, and at the same time significantly increased the amount of vitamin B<sub>12</sub> produced from the bacterial cells (Fig. 3b). This suggests that vitamin B<sub>12</sub> biosynthesis is upregulated in *Halomonas* sp. in the presence of algal extracts, and that the products of algal metabolism affect the rate of bacterial growth.



**Figure 3 | Interactions between bacteria and algae grown in co-culture.** **a**, The association of the red alga *P. purpureum* with *Halomonas* sp. The top left panel is a light microscope image of an axenic *P. purpureum* culture after DAPI staining (see Supplementary Methods). The top right panel is an epifluorescence image of the top left panel. The red chlorophyll epifluorescence masks that produced by DAPI-stained DNA in the algal cells. The bottom left panel is a light microscope image of *P. purpureum* cells grown in co-culture with *Halomonas* sp. after DAPI staining. The bottom right panel is an epifluorescence image of the bottom left panel. In this case there is more blue epifluorescence owing to DAPI staining of bacterial DNA. Scale bars, 30  $\mu$ m. **b**, Growth curve with *Halomonas* sp. in ASWH medium grown in the presence (open circles) or absence (filled circles) of fucoidin, a commercially available algal extract. The bars represent the amount of cobalamin produced per  $10^9$  cells. The presence of fucoidin increases both the rate of bacterial growth and the amount of vitamin B<sub>12</sub> produced from the bacterial cells. Error bars represent standard deviation of three replicates.

In conclusion, the data show that over half of all algal species surveyed require vitamin B<sub>12</sub> for growth, because, like animals, they require it to make methionine. There is no evolutionary relationship for vitamin B<sub>12</sub>-dependence in the algae, so it must have arisen numerous times during evolution. There is no evidence of vitamin B<sub>12</sub> synthesis in any algal lineage, so vitamin B<sub>12</sub> auxotrophy must have resulted from a change in algal metabolism, most likely the loss of the vitamin B<sub>12</sub>-independent methionine synthase MetE. Data from sequenced algal genomes further support this hypothesis. Most prokaryotes contain both isoforms of methionine synthase, whereas animals have evolved to contain MetH only, and plants MetE only. Therefore, early eukaryotes probably contained both isoforms of methionine synthase, and only later lost one of the genes. Interestingly, the genome of *T. pseudonana* also contains a gene for the vitamin B<sub>12</sub>-dependent enzyme methylmalonyl CoA mutase, and this enzyme has recently been purified from *Pleurochrysis carterae*<sup>19</sup>. Furthermore, *E. gracilis* has been suggested to contain a vitamin B<sub>12</sub>-dependent type II ribonucleotide reductase<sup>20</sup>. The presence of these other cobalamin-dependent enzymes may create a further selective pressure to retain vitamin B<sub>12</sub> in the metabolism of some species with complex plastids (Table 1).

We have demonstrated that the source of vitamin B<sub>12</sub> for microalgae is through a direct interaction with bacteria. We propose that the nature of this interaction is symbiotic, with the algae supplying fixed carbon in return for vitamin B<sub>12</sub>. Recent studies have shown that macroalgae also have bacteria closely associated with them<sup>21</sup>, suggesting that they too may acquire vitamin B<sub>12</sub> by this means. Owing to their colonization of the oceans, algae are responsible for approximately 50% of the world's atmospheric carbon fixation<sup>22</sup>. Our data indicate that more than half of these are dependent on bacteria for an essential micronutrient. There is also emerging evidence for tantalizingly similar interactions between algae and bacteria for the acquisition of other micronutrients<sup>23,24</sup>. The recognition of these bitrophic interactions will necessitate a change in our understanding of algal communities, and is likely to have profound implications for the exploitation of algae, both as a food source and for biotechnological applications.

## METHODS

Additional methods are given in Supplementary Information.

**Growth of algae.** The media and conditions used for the growth of different algal strains are provided in the Supplementary Information. With the exception of *Euglena* minimal medium and *Cyanidium* medium<sup>25</sup>, used to assess the vitamin B<sub>12</sub> requirements of *Euglena gracilis* and *Cyanidium caldarium*, respectively, the recipes for all of the other media used in this study can be found on the CCAP website (<http://www.ife.ac.uk/CCAP/>). To assess the growth of different species at natural vitamin B<sub>12</sub> concentrations, algal growth media were made exactly as described, except that distilled water was replaced by natural water, and the media filter-sterilized through a 0.2- $\mu$ m filter rather than being autoclaved.

**Assessment of vitamin B<sub>12</sub> requirement of algal species.** To assess the vitamin B<sub>12</sub> requirement of algal species, 1 ml of culture was used to inoculate 50 ml fresh medium that contained no vitamin B<sub>12</sub>. The cells were grown for 3–28 days (depending on the species) and then subcultured into fresh medium. This process was repeated five times. If the algal cells died at any point during this process, but remained alive in control medium to which vitamin B<sub>12</sub> was added, the species was regarded as requiring vitamin B<sub>12</sub>. If the cells did not die, the culture was checked to ensure that it was axenic by light microscopy. In addition, the algal culture was plated onto ASWH agar (see Supplementary Information) for marine algae and LB agar for freshwater algae, and checked for bacterial growth. The vitamin B<sub>12</sub> content was then measured in cells pelleted from a 50-ml culture. The cell pellet was resuspended in 200  $\mu$ l sterile distilled water, heated to 100 °C for 10 min, cooled on ice, and then centrifuged at 13,000 g for 2 min. The supernatant was collected and assayed for vitamin B<sub>12</sub> by bioassay using *Salmonella typhimurium* strain AR3612 (ref. 26).

**Supplementation of *L. rostrata* medium with methionine.** *Lobomonas rostrata* was grown in 50 ml Jaworski's medium for 7 days in continuous light with shaking in the presence of 10  $\mu$ g l<sup>-1</sup> vitamin B<sub>12</sub>, 10 mM methionine and 1  $\mu$ M folic acid. The alga was subcultured six times. After each 7-day period, a 1-ml sample of culture was removed and the optical density measured at 600 nm. For

homocysteine analysis, a 40-ml sample of culture, grown for 7 days, was centrifuged at 5,000 g for 20 min. The supernatant was discarded, and the pellet resuspended in 1 ml distilled water. The cells were sonicated with 6 × 20 s bursts separated by 30 s, on ice, in the presence of 100 µl glass beads (213–300 µm). The cell debris and glass beads were removed by centrifuging the sample at 13,000 g for 5 min at 4 °C, and the supernatant was used for homocysteine analysis (see Supplementary Methods).

**Isolation and identification of bacteria.** Bacteria were isolated from non-axenic algal cultures by spreading the culture onto ASWH agar plates and incubating them at 30 °C for 36 h. To isolate bacteria from fresh water, a 200-µl sample of the water was spread onto LB agar plates, and the plates were incubated at 20 °C for 48 h. DNA was extracted from bacterial cells, and the 16S rRNA gene was amplified by PCR using degenerate primers as described previously<sup>27</sup>. The amplified product was gel purified and sequenced directly using the same primers that were used for PCR amplification. The sequence was compared to other sequences in the ribosomal database at Michigan State University (<http://rdp.cme.msu.edu>) to identify the bacterial species.

**Generation of axenic *A. operculatum* cultures.** *Amphidinium operculatum* cultures were grown in artificial sea water (ASW) at 18 °C on a 16 h/8 h light/dark cycle for 28 days without shaking. A 1-ml aliquot of this culture was used to inoculate ASW containing a cocktail of antibiotics (100 µg ml<sup>-1</sup> carbenicillin, 100 µg ml<sup>-1</sup> kanamycin and 100 µg ml<sup>-1</sup> streptomycin). The *A. operculatum* cells were subcultured three times, for a total of 84 days, in this medium before being transferred back to ASW without antibiotics. To ensure that the final culture was axenic, the algal cells were viewed by light microscopy and plated onto ASWH agar plates. Bacterial contamination was not observed in either case.

**Growth experiments with *Halomonas* sp.** *Halomonas* sp. was grown aerobically, in ASWH media, at 30 °C in the presence or absence of 0.01% w/v fucoidin (Sigma). The optical density was measured every 4 h. After 16, 24 and 36 h a sample was taken from the culture: 100 µl was used for serial dilutions on ASWH agar plates, and the cells from the remaining 10 ml of medium were assayed for vitamin B<sub>12</sub> (as above).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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