environmental microbiology

Environmental Microbiology (2012) **14**(6), 1466–1476 doi:10.1111/j.1462-2920.2012.02733.x

Mutualistic interactions between vitamin B_{12}-dependent algae and heterotrophic bacteria exhibit regulation

Elena Kazamia,[1] Hjördis Czesnick,[1] Thi Thanh Van Nguyen,[1] Martin Tom Croft,[1] Emma Sherwood,[1] Severin Sasso,[1] Sarah James Hodson,[2] Martin James Warren[2] and Alison Gail Smith[1]*

[1]*Department of Plant Sciences, University of Cambridge, Cambridge, UK.*

[2]*Department of Biosciences, University of Kent, Canterbury, UK.*

Summary

Many algae are auxotrophs for vitamin B_{12} (cobalamin), which they need as a cofactor for B_{12}-dependent methionine synthase (METH). Because only prokaryotes can synthesize the cobalamin, they must be the ultimate source of the vitamin. In the laboratory, a direct interaction between algae and heterotrophic bacteria has been shown, with bacteria supplying cobalamin in exchange for fixed carbon. Here we establish a system to study this interaction at the molecular level. In a culture of a B_{12}-dependent green alga *Chlamydomonas nivalis*, we found a contaminating bacterium, identified by 16S rRNA analysis as *Mesorhizobium* sp. Using the sequenced strain of *M. loti* (MAFF303099), we found that it was able to support the growth of B_{12}-dependent *Lobomonas rostrata*, another green alga, in return for fixed carbon. The two organisms form a stable equilibrium in terms of population numbers, which is maintained over many generations in semi-continuous culture, indicating a degree of regulation. However, addition of either vitamin B_{12} or a carbon source for the bacteria perturbs the equilibrium, demonstrating that the symbiosis is mutualistic and facultative. *Chlamydomonas reinhardtii* does not require B_{12} for growth because it encodes a B_{12}-independent methionine synthase, METE, the gene for which is suppressed by addition of exogenous B_{12}. Co-culturing *C. reinhardtii* with *M. loti* also results in reduction of *METE* expression, demonstrating that the bacterium can deliver the vitamin to this B_{12}-independent alga. We discuss the implications of this for the widespread distribution of cobalamin auxotrophy in the algal kingdom.

Received 5 November, 2011; revised 28 February, 2012; accepted 3 March, 2012. *For correspondence. E-mail as25@cam.ac.uk; Tel. (+44) 1223 333952; Fax (+44) 1223 333953.

Introduction

Despite their photosynthetic lifestyle, an estimated half of all microalgal species are auxotrophs for the organic micronutrient vitamin B_{12} (Croft *et al.*, 2005). Vitamin B_{12} (cobalamin) is a water-soluble vitamin that is required by many eukaryotic organisms, but is synthesized by prokaryotes only (Warren *et al.*, 2002). It is a complex Co^{2+}-containing modified tetrapyrrole that acts as a cofactor for enzymes involved in C1 metabolism and certain radical reactions. In prokaryotes, there are over 20 enzymes that have a cobalamin cofactor (Marsh, 1999), while in animals there are just two B_{12}-dependent enzymes, methylmalonyl-CoA mutase, involved in odd-chain-fatty-acid metabolism in the mitochondria, and methionine synthase (METH), which catalyses the C1 transfer from methylhydrofolate to homocysteine to make methionine (Drummond *et al.*, 1993). Vitamin B_{12} is not found in, or required by, land plants or fungi, because they contain no cobalamin-dependent enzymes (Eichel *et al.*, 1995), and instead encode a B_{12}-independent form of methionine synthase (METE). In contrast, the vitamin is widespread throughout the algal kingdom, and algae such as the seaweed *Porphyra yezoensis* (nori), are an extremely rich dietary source of cobalamin (Watanabe *et al.*, 2002). However, algae do not synthesize cobalamin, and so must acquire it from the environment; those algae that are non-requirers do not synthesize vitamin B_{12}, but some can use it if it is available. We have surveyed several complete algal genome sequences, and together with growth assays and expression studies, have found that vitamin B_{12}-independent species all encode a functional METE, whereas this is absent from auxotrophic species (Helliwell *et al.*, 2011). Interestingly in two of the latter, *Volvox carteri* and *Gonium pectorale METE* pseudogenes were present, providing evidence of the evolution of B_{12}-dependence in action. Some algae, such as *Chlamydomonas reinhardtii*, possess both isoforms of

the enzyme, and can use vitamin B_{12} if it is available, in which case the *METE* gene is repressed, a situation analogous to that in many eubacteria, including *Escherichia coli* (Whitfield *et al*., 1970). It is important to note that methionine synthase is involved not just in synthesis of the amino acid methionine, but also in C1 metabolism, in particular in folate cycling. Thus, B_{12}-dependent algae can grow if supplemented with both methionine and folate, but not methionine alone (Croft *et al*., 2005), due to the phenomenon of folate-trapping, a characteristic also seen in humans with B_{12} deficiency (Scott, 1999). Because vitamin B_{12} auxotrophy is so widespread in the algal kingdom, and with no phylogenetic relationship between auxotrophs (Croft *et al*., 2005), the loss of *METE* must have happened multiple times throughout evolution. This implies that there is a readily available supply of cobalamin in the environment to allow so many algal species to thrive.

The source of the vitamin remains the subject of debate. In axenic cultures in the laboratory, growth of vitamin B_{12}-dependent algae is proportional to the amount of vitamin available (Carlucci and Silbernagel, 1969; Carlucci and Cuhel, 1977; Graneli and Risinger, 1994). A minimum of 7 pM (10 ng l^{-1}) is required for growth in culture of most species (Croft *et al*., 2005), but measurements of free cobalamin in solution in natural habitats rarely find levels above this concentration. For example, in seawater vitamin B_{12} levels typically vary between 0 and 3 pM (Provasoli and Carlucci, 1974; Panzeca *et al*., 2009). Furthermore, enrichment experiments show that B-vitamins can have a significant effect on phytoplankton dynamics, as addition of B_{12} was shown to stimulate phytoplankton growth in both temperate coastal waters (Sañudo-Wilhelmy *et al*., 2006; Gobler *et al*., 2007), in the Southern Ocean's Gerlache Straight (Panzeca *et al*., 2006), and in the Ross Sea (Bertrand *et al*., 2007). That half of all the algal species have an absolute requirement for vitamin B_{12} suggests that these species are able to persist and thrive alongside non-requirers, especially during such times as algal blooms, in an environment that appears to contain only trace amounts of the free bioavailable cofactor in solution.

Because only prokaryotes can synthesize cobalamin, they must be the ultimate source of the micronutrient. We have shown that, in the laboratory, B_{12}-producing heterotrophic bacteria are able to supply cobalamin to algae in co-cultures, in return for photosynthate (Croft *et al*., 2005). A marine bacterium, *Halomonas* sp., was isolated from cultures of the dinoflagellate *Amphidinium carterae* and from blades of *Porphyra miniata* (a red alga), and could support the growth of several marine algae in minimal media (Croft *et al*., 2005). Similarly, the symbiotic bacterium *Dinoroseobacter shibae* was shown to deliver both vitamin B_{12} and vitamin B_1 (thiamine) to its dinoflagellate host (Wagner-Döbler *et al*., 2010). Numerous other symbiotic interactions between bacteria and algae have been reported in recent years. Uptake of Fe by algae has been demonstrated to be enhanced by the presence of oligotrophic bacteria (Keshtacher-Liebson *et al*., 1995). Several clades of *Marinobacter* were found in close association with dinoflagellates and coccolithophores, producing a specific siderophore that promotes algal assimilation of iron, which they did not synthesize as free-living bacteria. In return, the bacteria use organic carbon from the algae (Amin *et al*., 2009). Co-inoculation of presumed bacterial contaminants isolated from a culture of *Chlorella ellipsoidea* with the alga resulted in 0.5–3 times greater algal growth than that of *C. ellipsoidea* alone (Park *et al*., 2008). Finally, many green algae cannot develop normally when grown under axenic conditions. *Monostroma oxyspermum*, for example, proliferates unicellularly in an aseptic culture, but develops into a normal foliaceous gametophyte in the presence of select marine bacteria (Matsuo *et al*., 2003).

Algal–bacterial symbiosis centred on vitamin B_{12} provision is one of simplest such examples, as it appears to be driven by loss of a single gene, *METE* (Helliwell *et al*., 2011), and thus can be readily studied at the molecular level. To this end we wanted to establish a laboratory model of a co-culture system that is genetically tractable and lends itself to easy molecular and physiological manipulations. In this paper we report on such a system by describing new symbiotic associations between the green alga *Lobomonas rostrata* and the soil bacterium *Mesorhizobium loti*. We investigate the stability of the co-cultures, and have started to probe the nature of the symbiotic interaction between these organisms.

Results

Identification of algal and bacterial partners

Chlamydomonas reinhardtii does not require vitamin B_{12} for growth, because it has both isoforms of methionine synthase, *METE* and *METH* (Croft *et al*., 2005; Helliwell *et al*., 2011). In contrast, the related *Chlamydomonas nivalis* (CCAP 11/128) was reported to have an obligate requirement for vitamin B_{12} (Provasoli and Carlucci, 1974). A culture of *C. nivalis* was obtained from the Culture Collection of Algae and Protozoa (CCAP), Oban, UK, but it was able to grow without addition of vitamin B_{12} to the medium. Examination of the culture revealed the presence of bacterial cells. The 16S rRNA gene from the bacterium was amplified by PCR and sequenced, and the bacterium identified as closely related to *Mesorhizobium loti*. *Mesorhizobium loti* is a soil bacterium known to be able to synthesize vitamin B_{12}. This was confirmed by inspection of the genome sequence of *M. loti* (Kaneko

et al., 2000), which was found to encode the complete aerobic pathway of cobalamin biosynthesis (Warren *et al.*, 2002; Fig. S1A). To determine if *M. loti* was able to supply *C. nivalis* with vitamin B_{12}, we attempted to make the culture of *C. nivalis* axenic by addition of a combination of antibiotics. These had no effect on the alga directly, which grew if the medium contained exogenous vitamin B_{12}, but in its absence the algal cells died after the first subculture, suggesting that the bacterium is able to supply *C. nivalis* with vitamin B_{12}. However, we were not able to recover an axenic culture of *C. nivalis*, so it was not possible to determine the vitamin B_{12} requirements of this strain of *C. nivalis* with complete confidence.

We therefore focused on another B_{12}-dependent green alga *L. rostrata* (SAG 45-1), which like *Chlamydomonas* is a member of the Volvocales family, and for which an axenic culture was available (Croft *et al.*, 2005). We investigated whether its growth could be supported by three different rhizobial species: *Mesorhizobium loti* (strain MAFF 303099) *Rhizobium leguminosarum* (strain RL 3841) and *Sinorhizobium meliloti* (strain RM 1021), all of which have sequenced genomes (Kaneko *et al.*, 2000; Galibert *et al.*, 2001; Young *et al.*, 2006). As for *M. loti,* the other two strains encode genes for the complete aerobic cobalamin biosynthetic pathway (Fig. S1B and C).

We grew *L. rostrata* in co-culture with each of the three bacterial strains on autotrophic medium (TP^+, see *Experimental procedures*), which did not contain a carbon source for the bacteria nor vitamin B_{12} required by the alga. Neither organism was able to grow on the medium in isolation. However, in co-culture each of the bacterial species was able to support *L. rostrata* growth to some extent, although only those with *M. loti* continued after six subcultures (Fig. 1). For that reason, the majority of the subsequent work focused on growth of *L. rostrata* with *M. loti.* To establish the efficacy of the apparent symbiosis, the growth of the two species in co-culture was compared with that of axenic cultures of each organism (Fig. 2). The average rate of growth, *K*, of *L. rostrata* in co-culture is slower ($K = 0.219$) than in medium containing vitamin 100 ng l^{-1} B_{12} ($K = 0.303$), and the final carrying capacity reached in batch culture is about 10-fold lower (see *Experimental procedures* for *K* calculation). Similarly, in co-culture with *L. rostrata*, *M. loti* did not reach cell densities recorded on medium containing a carbon source and grew more slowly in the initial stages (Fig. 2B). Because the nature of the carbon source provided by the algae for bacterial growth is unknown, a suitable growth comparison for bacteria on a carbon source is difficult. We tested various carbon sources (Fig. S2A). Mannitol, glycerol and glucose were all effective, but only glycerol both sustained the bacterial population over time, and did not affect the growth of algae (Fig. S2B).

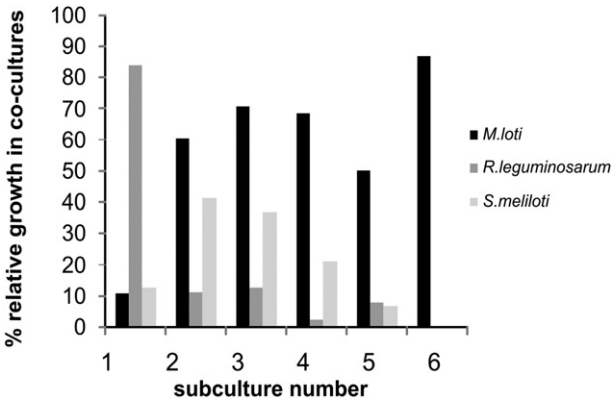

Fig. 1. Growth of *Lobomonas rostrata* in co-culture with rhizobial strains. The B_{12}-dependent green alga *L. rostrata* was maintained in co-cultures in autotrophic medium (TP^+, see *Experimental procedures*) with three different sequenced rhizobial strains: *M. loti* MAFF303099 (black bars), *R. leguminosarum* bv viciae 3841 (dark grey), and *S. meliloti* RM 1021 (light grey). Growth was measured by cell counts taken 12 days after each subculture. The data are presented as a percentage of growth relative to a control culture of axenic *L. rostrata* supplemented with 100 ng l^{-1} cyanocobalamin. After six subcultures only the cultures with *M. loti* had viable algal cells, and in fact could be propagated indefinitely.

What is the extent of the species' interdependence?

It is clear from these initial experiments that the relationship between *L. rostrata* and *M. loti* grown on TP^+ medium is mutualistic, as neither species is able to survive in the medium without the other. We probed this further by inoculating co-cultures with different ratios of algal and bacterial cells, from $1:10^6$ to $10^5:1$ and propagating in semi-continuous manner by removing 20% of culture each day and replacing with the same amount of fresh medium. Remarkably, in all cases, within 5–7 days the *L. rostrata* and *M. loti* equilibrated at a ratio of between 1:10 and 1:30 irrespective of the starting ratios (Fig. 3), indicating that there is a degree of regulation of the symbiosis. This ratio of population densities was established and maintained over several weeks. The same results were observed in batch culture, which we were able to maintain at equilibrium indefinitely by subculturing in mid-log phase. We probed the stability of the observed equilibrium by challenging the basis for the symbiotic interaction. To an established co-culture we either added vitamin B_{12} (100 ng l^{-1}), to eliminate the algal necessity for a bacterial symbiont, or conversely, added a carbon source, which would stimulate bacterial growth independently of the algae. In a control treatment, algae and bacteria were allowed to continue growing on medium that required a symbiosis. In the cobalamin 'add-back' treatment, *L. rostrata* grew significantly faster than the bacteria, whose population was kept at levels lower than control, as evidenced by an increase in the ratio of algal : bacterial cells (Fig. 4, open circles). When a carbon

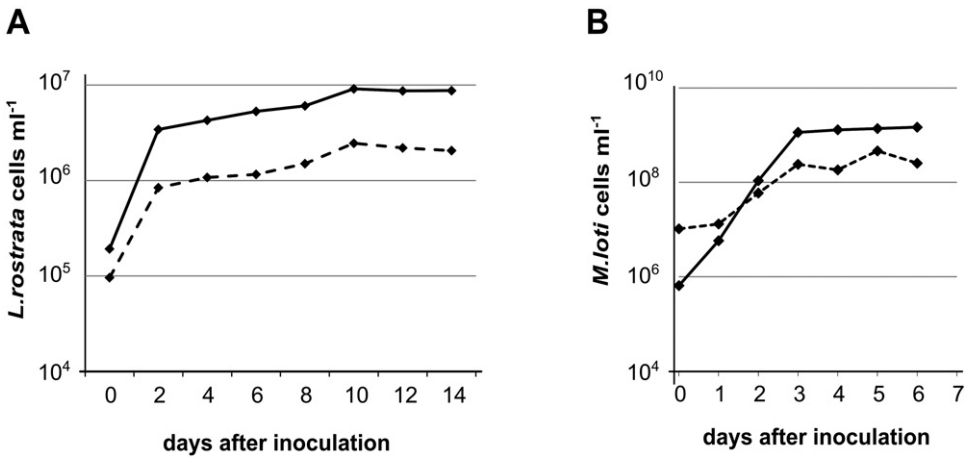

Fig. 2. Co-cultures have lower carrying capacity than supplemented cultures in autotrophic medium.
A. Growth of *L. rostrata* in co-culture (dashed lines) compared with axenic cultures supplemented with 100 ng l^{-1} vitamin B_{12} (solid line). The carrying capacity is clearly lower in co-cultures.
B. Growth of *M. loti* in co-culture (dashed line) compared with axenic cultures supplemented with 0.1% glycerol (solid line). Graphs are on logarithmic scale, so no error bars are shown, but the range was within 20%.

source was added to co-culture, the equilibrium was disrupted in favour of the bacterial population (Fig. 4, open squares), indicating that the regulation of algal and bacterial cell numbers in the co-cultures is likely to be due to exchanged nutrients, rather than some other generic limitation such as for nitrogen or phosphate.

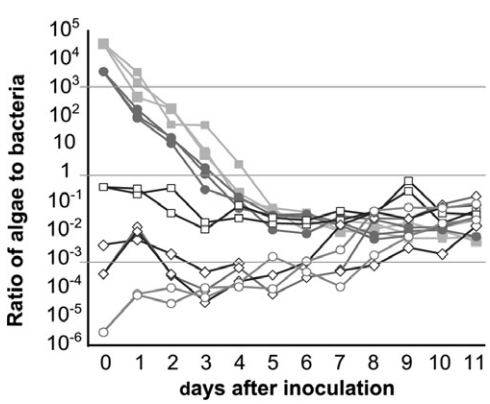

Fig. 3. Algal and bacterial growth in co-cultures shows a degree of regulation. Co-cultures were initiated in TP^{+} medium with different ratios of algal to bacterial cells (corresponding to approximately 10^4:1, 10^3:1, 1:1, 1:10^3 and 1:10^5, each with three biological replicates), and then maintained semi-continuously, with 20% of growth medium replenished daily. Each of the points represents the ratio of cell numbers for each biological repeat plotted on a logarithmic scale. Within 7 days and thereafter the ratio of algae to bacteria is observed to converge to approximately 1:30, regardless of the starting population densities. The experiments where starting population ratios of agae: bacteria were > 1 are indicated by closed squares. Open squares denote the progress of cultures whose initial concentration of bacteria was greater than the algal density.

Bacterial lysis is unlikely to be responsible for delivery of the vitamin

We wanted to determine to what extent there was a specific delivery of nutrients, or whether the exchange was merely the result of bacterial lysis or shedding of algal cell wall material. First, we tested whether physical contact is required for the provision of the vitamin in a simple immobilization experiment. Cells of *M. loti* were enclosed in a

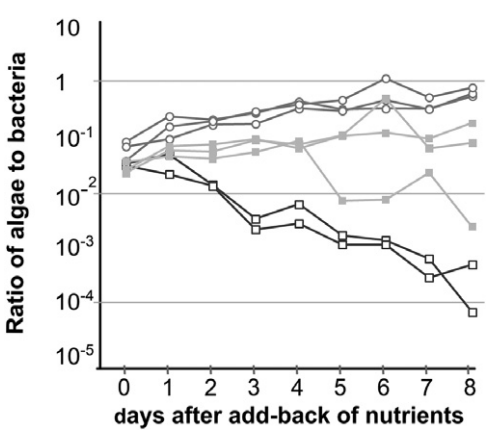

Fig. 4. Symbiosis between algae and bacteria is facultative. Co-cultures at equilibrium (described in Fig. 3) were divided into three treatments, with three biological repeats for each treatment. Control cultures (symbols are closed squares) continued to be propagated semi-continuously, by replenishing 20% of medium every day for 8 days. A carbon source (glycerol, 0.1%) was added to three flasks (denoted with open circle symbols), and 100 ng l^{-1} vitamin B_{12} was added to the last three (symbols are open squares). Cell counts were monitored as before, and the ratios plotted on a logarithmic scale. The ratios of algal:bacterial cells are seen to diverge following nutrient supplementation.

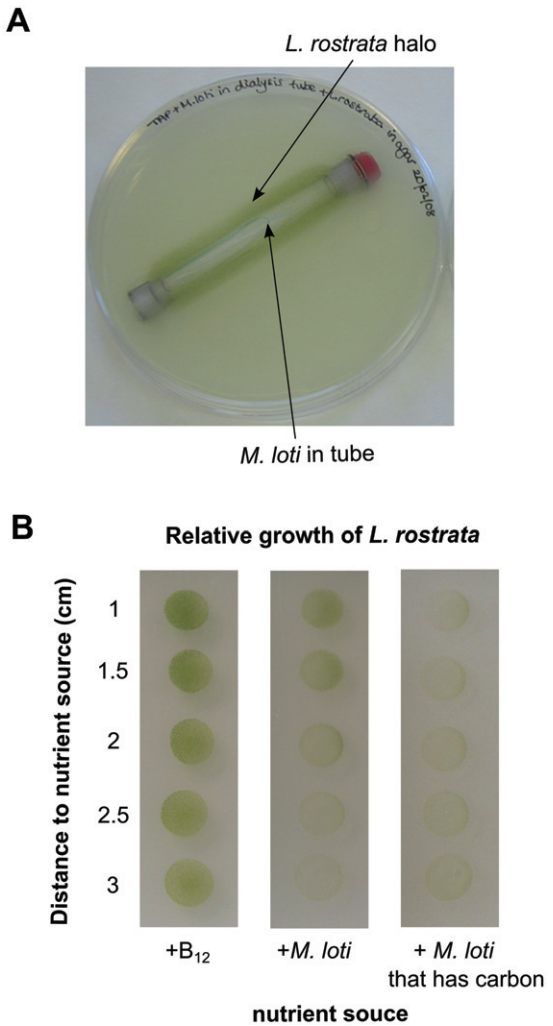

Fig. 5. Algae and bacteria do not require physical contact for the exchange of nutrients.
A. Dialysis tubing (with a cut-off of 12–14 kDa) containing a culture of *M. loti* was embedded in 1.5% TAP agar infused with a low concentration of *L. rostrata* grown on TAP medium without vitamin B_{12}. After 5 days a halo of *L. rostrata* growth was observed around the dialyser tube. The rest of the plate remained pale green as *L. rostrata* was unable to grow and divide.
B. Aliquots (50 µl) of *L. rostrata* cells (grown on TP^+ medium and suspended in 0.5% agar) were spotted onto TAP medium and then aliquots of a nutrient source cyanocobalamin (100 ng l^{-1}) or *M. loti* ± carbon source) were spotted at increasing distances from the algal cells. The plates were photographed after 16 days.

dialyser tube that had a cut-off of 12–14 kDa, so that nutrients could be exchanged, but it did not allow passage of the bacterial cells. The dialyser tube was embedded in an agar plate that contained a low concentration of *L. rostrata* cells starved of vitamin B_{12}. After 5 days, a halo of algal growth was clearly seen around the dialyser tube (Fig. 5A), indicating that the bacterial cells had produced sufficient vitamin B_{12} for it to diffuse through the membrane and allow algal cell growth. This result indicates that direct physical contact is not required. To investigate the exchange of nutrients between the organisms further, we devised a slightly modified experimental set-up. *L. rostrata* cells (30 µl, 5×10^5 cells ml^{-1}, i.e. ~ 10^4 cells per spot) were spotted onto a TP^+ agar plate (1.5%), with drops of B_{12} solution (5 µl of 100 ng l^{-1}) placed at increasing distances, then the extent of algal growth monitored. Fig. 5B (first panel) shows the results after 16 days: algal growth is clearly visible, and although the density of cells decreases with increasing distance from the vitamin supply, there is essentially an even distribution of cells across the spot, indicating that the vitamin can diffuse easily through the agar. In contrast, when aliquots of *M. loti* cells (~ 10^5 cells) were spotted a similar distance, growth of *L. rostrata* was observed, but this time there was a clear gradient across the spot of algal cell growth (Fig. 5B, second panel). Experiments were also conducted using different concentrations of a B_{12} source placed equidistant from the algal cells, with similar results (data not shown). When the agar plates contained a carbon source, little or no algal growth was observed (Fig. 5B, third panel); in other words insufficient B_{12} was provided for the algae when the bacteria did not require a carbon source. Taken together, these observations provide a strong indication that there is a specific interaction between the two organisms, and it is not simply the result of coincidental availability of nutrients.

It is nonetheless possible that there is sufficient B_{12} released from bacterial lysis, which is immediately taken up by algal cells. We therefore tested this with a mutant of *C. reinhardtii* (*thi1*; CC23), which is defective in thiamine (B_1) biosynthesis, and thus requires this vitamin (a minimum of 400 ng l^{-1}) as a nutritional supplement (Eversole, 1956). Although *M. loti* MAFF303099 is able to synthesize thiamine, in co-cultures with *thi1* the bacteria were not able to support the growth of the algal thiamine auxotroph (Fig. S3), indicating that while bacterial lysis might occur, this is insufficient to provide enough vitamin B_1 for the algae, and it is unlikely therefore that this is the explanation for the delivery of vitamin B_{12}.

Uptake of vitamin B_{12} by algal cells

The results from the previous section indicate that vitamin B_{12} is delivered specifically to the algal cells. To study this further, we wanted to determine the efficacy of B_{12} uptake by the algal cells. We set up axenic cultures of *L. rostrata* in heterotrophic medium (TAP), propagated in semi-continuous state by maintaining the culture in its exponential growth phase, so that the only limiting factor was the availability of vitamin B_{12}, and then supplemented them with increasing amounts of the vitamin. The results from these cultures show a clear correlation between the growth of algae and the amount of vitamin B_{12} added between 0 and 100 ng l^{-1}, after which point the vitamin is

no longer limiting (Fig. S4). At the lowest supplementation levels (10–30 ng l^{-1}), there is little or no vitamin B_{12} detectable in the medium after 5 days (Fig. 6), but interestingly, at 40 and 50 ng l^{-1} supplementation, where growth is still limited, there is measurable B_{12} in the medium. This indicates that a minimum concentration of the vitamin in the medium is necessary for optimal growth. In contrast, in the medium of an established co-culture of *L. rostrata* and *M. loti* in TAP, growth is clearly not limited, and yet at most 10 ng l^{-1} of vitamin B_{12} was detectable in the medium (Fig. 6).

Evidence that the B_{12} itself can be taken up by algal cells, rather than some other nutrient supplied by the bacteria came from co-culturing experiments with *C. reinhardtii*. Although this species does not need B_{12} for growth, it will take it up if supplied, in which case the *METE* gene for vitamin B_{12}-independent methionine synthase is switched off (Croft *et al.*, 2005), and the algal metabolism exclusively uses METH. Repression of *METE* could therefore be used to assess vitamin B_{12} uptake in this alga in co-cultures (unlike *L. rostrata,* which only has METH), but when an inoculum of *M. loti* was added to *C. reinhardtii* cells in TP^+ (which contains no carbon source) the bacterial cells did not grow at all (Fig. S3). However, addition of a carbon source allowed bacterial growth, indicating that, unlike *L. rostrata*, *C. reinhardtii* is not able to provide suitable photosynthate to the heterotrophic bacteria. Accordingly,

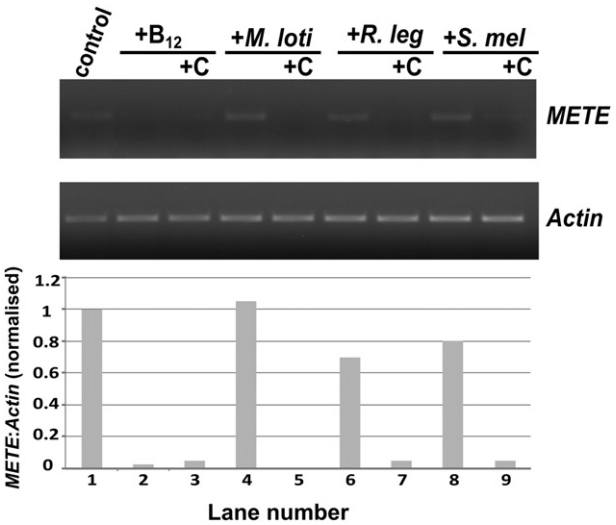

Fig. 7. Rhizobia provide vitamin B_{12} to *C. reinhardtii* if fixed carbon is supplied to the growth medium for bacterial growth. *C. reinhardtii* was grown in TP medium, either without the addition of vitamin B_{12} (control), in the presence of vitamin B_{12} supplied at 100 ng l^{-1}, or in co-cultures with bacteria. Three types of bacterial co-cultures were set up- inoculated either with *M. loti* (*M. lot*), *R. leguminosarum* (*R. leg*), or *S. meliloti* (*S. mel*). An additional source of fixed carbon (+ C) was added to half of the co-cultures (in the form of 1% mannitol). All cultures were grown for four days at 25°C, 140 r.p.m. under continuous light before RNA was extracted. The gel photograph shows the products of an RT-PCR analysis conducted using primers to the METE and actin genes. Band intensity was quantified using imaging software and the normalized ratio of METE to actin shown.

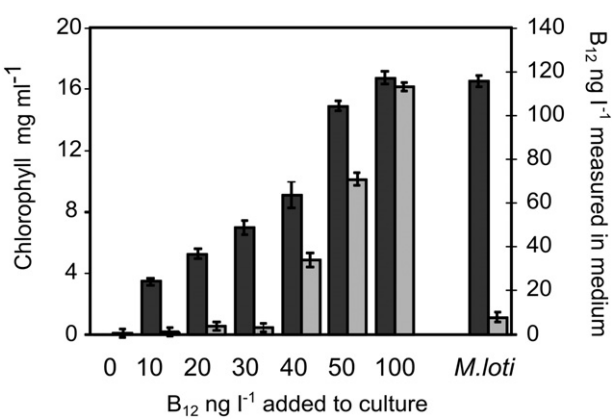

Fig. 6. Growth of *L. rostrata* in heterotrophic medium supplemented with increasing concentrations of vitamin B_{12}, or with *M. loti*.
Lobomonas rostrata maintained in the exponential phase was inoculated into fresh TAP medium containing different concentrations of vitamin B_{12} (0–100 ng l^{-1}). The dark grey bars represent relative algal growth after 5 days in culture. The light grey bars represent the concentration of vitamin B_{12} in the medium at the end of the experiment. Error bars show the standard deviation for four independent experiments. Concentrations of vitamin B_{12} below 50 ng l^{-1} limit the growth of *L. rostrata* in axenic culture. In contrast, when *L. rostrata* is grown in co-culture with *M. loti*, the algae grow to a level equivalent to 100 ng l^{-1} of vitamin B_{12} supplementation, but only a trace (less than 10 ng l^{-1}) of B_{12} is detectable in the medium.

stable co-cultures of *C. reinhardtii* and each of the three rhizobial species were set up in heterotrophic medium, then total RNA was extracted, reverse-transcribed into cDNA and used for RT-PCR with primers to the *METE* gene and actin as a control (Fig. 7). When the alga was grown alone, the *METE* transcript was detectable but addition of B_{12}, with (+C) or without a carbon source, downregulated it almost completely (lanes 2 and 3). The *METE* transcript was similarly repressed when *C. reinhardtii* was co-cultured with *M. loti,* and with the other two rhizobial species *R. leguminosarum* and *S. meliloti* (Fig. 7, lanes 4–9), although again only when a carbon source was included in the medium. These results demonstrate that although the alga is unable to supply fixed carbon to the bacteria, each of these rhizobial species can supply the vitamin to *C. reinhardtii*.

Discussion

In this work we have shown that three green algae, *C. reinhardtii*, *C. nivalis* and *L. rostrata* are able to acquire vitamin B_{12} from vitamin B_{12}-synthesizing bacteria. *L. rostrata* was chosen for further investigations of the algal–bacterial associations because it is a vitamin B_{12} auxotroph, and was available in axenic culture. All three

bacterial species belong to the Rhizobiales order, are heterotrophic, and were able to supply *L. rostrata* with vitamin B_{12} in medium that does not contain a source of organic carbon. This indicates that the bacteria were able to use algal photosynthate as a carbon source in return for supplying the alga with vitamin B_{12} – an exchange that serves as the basis for a symbiotic relationship. However, not all combinations resulted in healthy co-cultures. Although *S. meliloti* and *R. leguminosarum* supported algal growth initially, after several subcultures the algal cells clumped and died (Fig. 1). Interestingly if we used TAP medium, rather than the photoautotrophic TP^+ medium, the co-cultures could be sustained (data not shown). Similarly, healthy co-cultures of all three bacterial species with *C. reinhardtii* could be established in TAP, or after addition of a carbon source to TP^+. This demonstrates a certain degree of specificity for algal–bacterial associations, which may be manifested through the suitability of photosynthate that the alga can provide to the heterotrophic bacterial cells. We therefore narrowed our investigation to the *L. rostrata–M. loti* symbiosis.

The term symbiosis was coined by De Bary in 1879 and means 'the living together of different organisms'. It is an umbrella term describing interactions between species that range from mutualism to parasitism. In the first case, all organisms involved receive a fitness benefit from the interaction, while in the extreme case of parasitism one organism benefits at the expense of the other. Nutritional interactions are common, as it is the availability of resources in the environment that selects for the symbiosis. The algal–bacterial symbiosis that we describe presently is a case of mutualism: *L. rostrata* cannot grow without a symbiotic bacterium in the absence of vitamin B_{12}. The symbiont, in turn, cannot grow without the alga in the absence of a carbon source. There is no defined path for the evolution of mutualisms (Medina and Sachs, 2010); as a result they are variable in nature, ranging from obligate, where the symbionts cannot survive without each other, to facultative symbiosis. In the latter case it is the environment that renders the symbiosis necessary on occasion.

In co-cultures of *L. rostrata* and *M. loti*, we observed that a stable equilibrium in terms of relative population sizes is established. Regardless of the starting inoculation ratio, *L. rostrata* and *M. loti* equilibrate around a ratio of 1:30 algae to bacteria (Fig. 3), which is maintained over numerous generations, kept healthy in semi-continuous culture maintained at exponential growth. Symbioses are known to persist for an appreciable time relative to the lifespans of the partners and the process that maintains a stable proportion of symbionts to host is termed 'regulation' (Smith and Douglas, 1987). Although the mechanism behind the regulation remains unknown for the *M. loti –L. rostrata* partnership (as is also the case for many other examples of symbiosis), the constancy of the relative population sizes of partners is a common and clear-cut phenomenon that is capable of experimental manipulation (Smith and Douglas, 1987). Accordingly, we challenged the basis of the observed equilibrium through the add-back of nutrients, and observed that the equilibrium in numbers was broken (Fig. 4). Addition of a carbon source resulted in an explosion of bacterial growth, with no concomitant growth of algae. Similarly, addition of B_{12} caused the algae to proliferate, reaching a carrying capacity over and above that observed in the presence of the bacteria, with no increase in the absolute bacterial numbers. These observations argue against nutrient exchange between the organisms being simply a matter of cell lysis (or shedding of material), because this would predict that higher population densities of algae would support an increasing bacterial population, and vice versa. Further evidence against the lysis model comes from our measurements of vitamin B_{12} freely available in solution of the co-cultures. We found that the growth of *L. rostrata* in co-cultures is at least an order of magnitude higher than would be expected if the algae were grown in axenic culture on medium containing a similar amount of the vitamin (Fig. 6).

The exact nature of the molecular exchange at the heart of the proposed algal–bacterial mutualism remains to be elucidated. However, physical contact between the organisms is not necessary as shown by the experiments on semi-solid medium, where the algae are not supplied with metabolically costly cobalamin if the bacteria have a readily available carbon source (Fig. 5C). However, when grown on medium that selects for the facultative mutualism, the bacteria support algal growth in a similar fashion as cobalamin diffusing through the agar matrix. The photosynthate that is provided by the *L. rostrata* cells still remains to be determined, but many algae are known to shed cell wall components during growth (e.g. Burczyk, 1986), so it plausible that these can be used by *M. loti* as a carbon source. On the other hand, it may be that the algae specifically excrete metabolites into the medium, which then allow *M. loti* to grow and produce cobalamin in return.

The ability to growth without vitamin B_{12} in algae is correlated with the presence of the B_{12}-independent form of methionine synthase, METE, whereas B_{12}-auxotrophs lack a functional METE (Helliwell *et al.*, 2011). In this context, the observation that co-culturing *C. reinhardtii* with bacteria is sufficient to turn off the expression of the *METE* gene is highly significant. This suggests that algae that live closely associated with bacteria from which they are able to acquire vitamin B_{12} would not express the *METE* gene, as they would have a constant source of the vitamin. As such, there would be no evolutionary pressure upon them to retain a functional *METE* gene, so this gene

would be randomly lost from different algae over the course of evolution (Helliwell *et al.*, 2011). In an evolutionary context, the loss of *METE* is risky because the algae become B_{12}-dependent. In the algal kingdom the lack of evolutionary pattern of the different combinations of vitamin B_{12}-dependent and vitamin B_{12}-independent methionine synthases implies that in the last common ancestor both methionine synthases were present and *METE* was differentially either lost or retained over time. This is likely either to have occurred because the presence of the vitamin was originally abundant in the environment making METE redundant, or mutualism with bacteria offered a selective advantage such as increased growth rates. However, the case for symbiosis is evident when vitamin auxotrophy is coupled to a low environmental availability of the vitamins. Under this scenario, algal–bacterial symbiosis can be thought to have evolved through a mechanism of reciprocity of by-products (Sachs and Simms, 2006), although the role of bacteria in establishing the symbiosis remains unclear. Algal–bacterial symbioses are not limited to vitamin B_{12} auxotrophs. Other mutualisms have been described, the most noteworthy of which, prevalent in marine communities, is when in return for a continuous supply of primary photosynthesis products, bacteria supply algae with iron through production of photosensitive siderophores (Amin *et al.*, 2009). Moreover, as the notion of algal–bacterial symbiosis as a widespread natural phenomenon becomes better established, it becomes less surprising that it is often difficult to obtain axenic cultures of certain algae, as was the case with *C. nivalis*.

In conclusion, the work presented here establishes a model system with which to probe algal–bacterial associations for the exchange of the essential micronutrient, vitamin B_{12}, at the biochemical and molecular levels. Moreover, it provides a more general opportunity to increase our understanding of the evolution of mutualisms and the role of organic micronutrients in aquatic ecosystems, where algae are important primary producers.

Experimental procedures

Algal and bacterial strains and growth conditions

Chlamydomonas reinhardtii (wild-type strain 12) used for RT-PCR was a gift from Dr Saul Purton, University College London, UK. *Chlamydomonas nivalis* (CCAP 11/128) was obtained from Oban, Scotland. The *C. reinhardtii* thiamine auxotroph (*thi1*; CC23) was obtained from the Core Collection of the *Chlamydomonas* Center, Duke University, North Carolina. *Lobomonas rostrata* (SAG 45-1) was obtained from the Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG), Germany. Algae were grown heterotrophically in Tris Acetate Phosphate (TAP) medium (Harris, 1989), or in an autotrophic medium TP, in which acetate was omitted, with pH adjusted to 7.2 by hydrochloric acid. *Lobomonas rostrata* was grown autotrophically on a modified form of TP, TP^+, which is buffered with extra phosphate [7.475 mM NH_4Cl, 0.35 mM $CaCl_2.2H_20$, 0.405 mM $MgSO_4.7H_2O$, 2.65 mM KH_2PO_4, 4 mM K_2HPO_4 and 1 ml l^{-1} of Hutner's trace elements described in Merchant *et al.*, (2006)]. Cultures were maintained in continuous light with shaking (140 r.p.m.) at 25°C. When supplemented with vitamin B_{12}, the vitamin was provided in the form of cyanocobalamin (Sigma-Aldrich, UK) supplied at a concentration of 100 ng l^{-1}, unless otherwise stated. Thiamine (Sigma-Aldrich UK) was supplemented at 400 ng l^{-1}. Rhizobial strains of *S. meliloti* (RM 1021), *R. leguminosarum* (RL 3841), and *M. loti* (MAFF 303099) were gifts from Prof Allan Downie, John Innes Centre, UK. Precultures were grown in liquid Acid Minimal Salts (AMS) medium (Poole *et al.*, 1994) supplemented with 0.1% glycerol, and grown at 28°C for 4 days. Before inoculation into co-culture both *L. rostrata* and the bacteria were washed four times in TP^+ medium. The bacteria were centrifuged at 13 200 r.p.m. for 3 min, and the pellet was resuspended in fresh medium. The algae were centrifuged at 6000 r.p.m. for 8 min and the pellet resuspended in fresh medium. Thereafter algae and bacteria were combined in TP^+ medium in appropriate ratios of cell numbers, determined as below.

Population density measurements

For some experiments, algal growth was measured by monitoring levels of chlorophyll as described by Croft and colleagues (2005). For more quantitative analysis, *L. rostrata* cell numbers were determined by counting after visualization by bright-field light microscopy. Samples were killed and stained using standard Lugol's solution. A 2-chamber Mod-Fuchs Rosenthal haemocytometer was used to count 100–400 cells per sample from a known volume of culture. Bacterial growth was measured by counting the number of colony-forming units on solid TY plates (1.5% agar) after an appropriate series of dilutions (Beringer, 1974).

The growth rate K was estimated according to the equation $K = \mathrm{Ln}\ (N2/N1)/(t2 - t1)$; where

N1 and N2 = population density at time 1 ($t1$) and time 2 ($t2$) respectively (Levasseur *et al.*, 1993).

Growth of algae and bacteria on agar plates

To investigate delivery of B_{12} from *M. loti* to *L. rostrata*, growth on agar plates was employed. To test whether physical contact was necessary, a Visking Dialysis Membrane (12–14 kDa) manufactured by Medicell International, was cleaned with a large volume of 1 mM EDTA pH 8.0 and 2% sodium bicarbonate heated at 80°C for 20 min stirring frequently. It was subsequently clipped into shape and autoclaved in a large volume of distilled water. *Lobomonas rostrata* grown on TAP medium supplemented with vitamin B_{12} was poured into cooling agar at 50°C, to create a pale solution of *L. rostrata*-infused 1.5% TAP agar. A suspension of *M. loti* cells grown on AMS medium was placed into the dialysis tubing, which was then transferred into a Petri dish. The 1.5% TAP agar containing *L. rostrata* was poured into the plate, embedding the dialysis tubing in the plate as it was left to cool in the flow

hood. Plates were inspected daily and photographed. The other approach involved spotting aliquots of *L. rostrata* cells (2.5×10^4 algal cells ml^{-1} washed and suspended in 0.5% agar) onto TAP-agar plates (1.5%), and then placing spots of either B_{12} (100 ng l^{-1}) or *M. loti* (5 µl of 1×10^9 cells ml^{-1}) at increasing distances from the algal sample. In some cases, bacterial spots also contained a carbon source (glucose 0.8% w/v). Again growth was monitored daily.

Vitamin B_{12} bioassay

The vitamin B_{12} concentration of co-culture medium was assayed using the *Salmonella typhimurium* bioassay as previously described (Raux *et al*., 1996).

Reverse transcriptase (RT) PCR analysis

Total RNA was extracted from *C. reinhardtii* using phenol-chloroform extraction and lithium chloride precipitation, and stored at −80°C. After treatment with RQ1 DNase (Promega No. M610A), cDNA was prepared from 5 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and amplified by PCR. Reaction mixtures contained buffer from the Taq DNA polymerase (Bioline) with additional 2 mM $MgCl_2$ and 5% DMSO, together with 400 nM of each primer and self-made Taq DNA polymerase. Amplification conditions were as follows: 94°C for 2 min; repeated cycles of 94°C for 20 s, 65°C for 30 s, 72°C for 40 s (28 cycles for METE, 25 cycles for the actin gene); followed by 72°C for 5 min. Primers used were 14-actin-F (TGTGCTACGTGGCCCTGGACTT, 22 bp) and 15-actin-R (AGGGGCCGGACTCGTCGTACT, 21 nt) for the actin gene (expected product size: 456 bp from cDNA, 1013 bp from genomic DNA), and Cr_metE_exon_5_f (GTGGACGACCCCCGCTCTGCG, 21 nt) and 13-METE-R (GCGCCGCCATCATGGCGTTGT, 21 nt) for METE (expected: 239 bp from cDNA, 457 bp from genomic DNA). The identities of the RT-PCR products were verified by DNA sequencing. Gel images were analysed using the software Image J (version 1.40g).

Bacterial 16S rRNA gene PCR

A 50 ml aliquot of the *C. nivalis* culture was centrifuged at 5000 *g* for 10 min to collect algal and bacterial cells. The cell pellet was resuspended in 500 µl sterile distilled water, and boiled for 10 min in a heat block, then centrifuged at 13 000 *g* for 1 min to prepare a crude cell lysate. The 16S rRNA gene was amplified from this by PCR using Taq DNA polymerase (Bioline) with degenerate primers as described in Lane (1991), sequenced, and then compared to other sequences in the ribosomal database at Michigan State University (http://rdp.cme.msu.edu) to identify the bacterial species.

Acknowledgements

We thank Dr Katherine Helliwell for useful discussions, Sam Harrison for PCR analysis of CNB, Mrs Geraldine Heath for technical assistance, Dr Saul Purton (UCL) for the *Chlamydomonas* strains and Prof Allan Downie (John Innes Centre, Norwich) for the rhizobial strains.

References

Amin, S.A., Green, D.H., Hart, M.C., Küpper, F.C., Sunda, W.G., and Carrano, C.J. (2009) Photolysis of iron-siderophore chelates promotes bacterial-algal mutualism. *Proc Natl Acad Sci USA* **106:** 17071–17076.

Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84:** 188–198.

Bertrand, E.M., Saito, M.A., Rose, J.M., Riesselman, C.R., Lohan, M.C., Noble, A.E., *et al*. (2007) Vitamin B_{12} and iron colimitation of phytoplankton growth in the Ross Sea. *Limnol Oceanogr* **52:** 1079–1093.

Burczyk, J. (1986) Cell wall carotenoids in green algae which form sporopollenins. *Phytochemistry* **26:** 121–128.

Carlucci, A.F., and Cuhel, R.L. (1977) Vitamins in the South Polar Seas: distribution and significance of dissolved and particulate vitamin B_{12}, thiamine, and biotin in the Southern Indian Ocean. In *Adaptations Within Antarctic Ecosystems, Proceedings of Third SCAR Symposium on Antarctic Biology*. Llano, G. (ed.). Houston, USA: Gulf Printing, pp. 115–128.

Carlucci, A.F., and Silbernagel, S.B. (1969) Effect of vitamin concentrations on growth and development of vitamin-requiring algae. *J Phycol* **5:** 64–67.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., and Smith, A.G. (2005) Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. *Nature* **438:** 90–93.

Drummond, J.T., Huang, S., Blumenthal, R.M., and Matthews, R.G. (1993) Assignment of enzymatic function to specific protein regions of cobalamin-dependent methionine synthase from *Escherichia coli*. *Biochemistry* **32:** 9290–9295.

Eichel, J., González, J.C., Hotze, M., Matthews, R.G., and Schröder, J. (1995) Vitamin-B_{12}-independent methionine synthase from a higher plant (Catharanthus Roseus). *Eur J Biochem* **230:** 1053–1058.

Eversole, R.A. (1956) Biochemical mutants of *Chlamydomonas reinhardtii*. *Am J Bot* **43:** 404–407.

Galibert, F., Finan, T.M., Long, S.R., Puhler, A., Abola, P., Ampe, F., *et al*. (2001) The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293:** 668–672.

Gobler, C.J., Norman, C., Panzeca, C., Taylor, G.T., and Sañudo-Wilhelmy, S.A. (2007) Effect of B vitamins (B_1, B_{12}) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. *Aquat Microb Ecol* **49:** 181–194.

Graneli, E., and Risinger, L. (1994) Effects of cobalt and vitamin B_{12} on the growth of *Chrysochromulina polylepis*. *Mar Ecol Prog Ser* **113:** 177–183.

Harris, E.H. (1989) *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*. San Diego, USA: Academic Press.

Helliwell, K.E., Wheeler, G.L., Leptos, K.C., Goldstein, R.E., and Smith, A.G. (2011) Insights into the evolution of Vitamin B_{12} auxotrophy from sequenced algal genomes. PMID: 21551270.

Kaneko, T., Nakamura, Y., Sato, S., Asamizu, E., Kato, T., Sasamoto, S., *et al*. (2000) Complete genome structure of

the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* (supplement). *DNA Res* **7:** 381–406.
Keshtacher-Liebson, E., Hadar, Y., and Chen, Y. (1995) Oligotrophic bacteria enhance algal growth under iron-deficient conditions. *Appl Environ Microbiol* **61:** 2439–2441.
Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematic*. Stackebrandt, E., and Goodfellow, M. (eds). New York, USA: John Wiley and Sons, pp. 115–175.
Levasseur, M., Thompson, P.A., and Harrison, P.J. (1993) Physiological acclimation of marine phytoplankton to different nitrogen sources. *J Phycol* **29:** 587–595.
Marsh, E.N.G. (1999) Coenzyme B_{12} (cobalamin)-dependent enzymes. In *Essays in Biochemistry*. Vol. 34. Ballou, D.P. (ed.). London, UK: Portland Press, pp. 139–154.
Matsuo, Y., Suzuki, M., Kasai, H., Shizuri, Y., and Harayama, S. (2003) Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. *Environ Microbiol* **5:** 25–33.
Medina, M., and Sachs, J.L. (2010) Symbiont genomics, our new tangled bank. *Genomics* **95:** 129–137.
Merchant, S.M., Allen, M.D., Kropat, J., Moseley, J.L., Long, J.C., Tottey, S., and Terauchi, A.M. (2006) Between a rock and a hard place: trace element nutrition in Chlamydomonas. *Biochim Biophys Acta* **1763:** 578–594.
Panzeca, C., Tovar-Sanchez, A., Agustí, S., Reche, I., Duarte, C.M., Taylor, G.T., and Sañudo-Wilhelmy, S.A. (2006) B vitamins as regulators of phytoplanktondynamics. *Eos Trans Am Geophys Union* **87:** 593–596.
Panzeca, C., Beck, A.J., Tovar-Sanchez, A., Segovia-Zavala, J., Taylor, G.T., Gobler, C.J., and Sañudo-Wilhelmy, S.A. (2009) Distributions of dissolved vitamin B_{12} and Co in coastal and open-ocean environments. *Estuar Coast Shelf Sci* **85:** 223–230.
Park, Y., Kyoung-Wo, J., Kyungyong, L., Sang-Eun, J., and Tae-Jin, C. (2008) Growth promotion of *Chlorella ellipsoidea* by co-inoculation with *Brevundimonas* sp. isolated from the microalga. *Hydrobiologia* **598:** 219–228.
Poole, P.S., Schofiel, N.A., Reid, C.J., Drew, E.M., and Walshaw, D.L. (1994) Identification of chromosomal genes located downstream of dctD that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum*. *Microbiology* **140:** 2797- 2809.
Provasoli, L., and Carlucci, A.F. (1974) Vitamins and growth regulators. In *Algal Physiology and Biochemistry*. Stewart, W.D.P. (ed.). Malden, USA: Blackwell Science, pp. 741–787.
Raux, E., Lanois, A., Levillayer, F., Warren, M.J., Brody, E., Rambach, A., and Thermes, C. (1996) *Salmonella typhimurium* cobalamin (vitamin B_{12}) biosynthetic genes: functional studies in *S. typhimurium* and *Escherichia coli*. *J Bacteriol* **17:** 753–767.
Sachs, J.L., and Simms, E.L. (2006) Pathways to mutualism breakdown. *Trends Ecol Evol* **21:** 585–592.
Sañudo-Wilhelmy, S.A., Gobler, C.J., Okbamichael, M., and Taylor, G.T. (2006) Regulation of phytoplankton dynamics by vitamin B_{12}. *Geophys Res Lett* **33:** 10–13.
Scott, J. (1999) Folate and vitamin B_{12}. *Proc Nutr Soc* **58:** 441–448.
Smith, D.C., and Douglas, A.E. (1987) *The Biology of Symbiosis*. London, UK: Edward Arnold.
Wagner-Döbler, I., Ballhausen, B., Berger, M., Brinkoff, 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.
Warren, M.J., Raux, E., Schubert, H.L., and Escalante-Semerena, J.C. (2002) The biosynthesis of adenosylcobalamin (vitamin B_{12}). *Nat Prod Rep* **19:** 390–412.
Watanabe, F., Takenaka, S., Kittaka-Katsura, H., Ebara, S., and Miyamoto, E. (2002) Characterization and bioavailability of vitamin B_{12}- compounds from edible algae. *J Nutr Sci Vitaminol* **48:** 325–331.
Whitfield, C.D., Steers, E.J., and Weissbach, H. (1970) Purification and properties of 5- methyltetrahydropteroyltriglutamate-homocysteine transmethylase. *J Biol Chem* **245:** 390–401.
Young, J.P.W., Crossman, L.C., Johnston, A.W.B., Thomson, N.R., Ghazouli, Z.F., Hull, K.H., *et al*. (2006) The genome of Rhizobium leguminosarum has recognizable core and accessory components. *Genome Biol* **7:** R34.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Chromosomal map of sequenced rhizobial strains showing vitamin B_{12} synthesis genes.
A. *Mesorhizobium loti* MAFF303099. Genes for the enzymes of the aerobic pathway for cobalamin biosynthesis (Warren *et al*., 2002) were identified by BLAST searches of the genomes at NCBI (http://www.ncbi.nlm.nih.gov/sites/genome), using default parameters, and confirmed by sequence alignments and Pfam analysis. No hits were obtained for the anaerobic pathway enzymes. Genes on opposite strand are denoted in purple.
B. Chromosomal map of sequenced *Sinorhizobium meliloti* 1201 strain showing genes for aerobic vitamin B_{12} biosynthesis pathway. See Fig. 2A for details.
C. Chromosomal map of sequenced *Rhizobium leguminosarum bv. viciae* 3841 strain showing genes for aerobic vitamin B_{12} biosynthesis pathway. See Fig. 2A for details.
Fig. S2. Growth of algae and bacteria on different carbon sources.
A. *M. loti* cultures were grown on TP^+ medium supplemented with 0.5% glucose, 0.1% glycerol, 0.5% mannitol, 0.1% succinate or 0.1% malate. Shown are the mean growth curves with error bars representing standard deviation ($n = 3$). Bacterial cultures after 5 days growth on glucose, succinate and malate 'crashed', with no viable cells recovered subsequently. Bacteria grown on mannitol and glycerol were still viable after 10 days. Mannitol and glycerol were therefore chosen for further investigation.
B. *L. rostrata* was grown on TP^+ medium supplemented with 100 ng l^{-1} vitamin B_{12} with or without a carbon source. The 'control' line describes average growth in the absence of a carbon source. Growth is also shown on 1% glycerol and 0.5% mannitol. The algae are able to use mannitol as a growth source, whereas glycerol does not affect growth.

Fig. S3. *M. loti* does not support the growth of a thiamine-dependent mutant of *C. reinhardtii.* Growth of *C. reinhardtii* thiamine auxotroph mutant (*thi1*; CC23) was observed on autotrophic (A) and heterotrophic (B) medium at 25°C, 140 r.p.m. under continuous light. The algae were either supplemented with 400 ng l^{-1} thiamine (+ B_1), or with *M. loti*. In the latter case bacterial cells were grown on solid medium and added as indicated (with *M. loti*). The bacteria were unable to rescue the growth of the algal mutant on either medium, although they are able to use acetate, and so grew successfully (shown by the cloudy solution) on TAP.

Fig. S4. *L. rostrata* maintained in the exponential phase was inoculated into fresh TAP medium that contained different concentrations of vitamin B_{12} (0–10 ng l^{-1}). The bars represent relative algal growth (measured by chlorophyll amount) after 5 days in culture.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.