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Exploring the onset of B_{12}-based mutualisms using a recently evolved *Chlamydomonas* auxotroph and B_{12}-producing bacteria

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Summary

Cobalamin (vitamin B_{12}) is a cofactor for essential metabolic reactions in multiple eukaryotic taxa, including major primary producers such as algae, and yet only prokaryotes can produce it. Many bacteria can colonize the algal phycosphere, forming stable communities that gain preferential access to photosynthate and in return provide compounds such as B_{12}. Extended coexistence can then drive gene loss, leading to greater algal–bacterial interdependence. In this study, we investigate how a recently evolved B_{12}-dependent strain of *Chlamydomonas reinhardtii*, metE7, forms a mutualism with certain bacteria, including the rhizobium *Mesorhizobium loti* and even a strain of the gut bacterium *E. coli* engineered to produce cobalamin. Although metE7 was supported by B_{12} producers, its growth in co-culture was slower than the B_{12}-independent wild-type, suggesting that high bacterial B_{12} provision may be necessary to favour B_{12} auxotrophs and their evolution. Moreover, we found that an *E. coli* strain that releases more B_{12} makes a better mutualistic partner, and although this trait may be more costly in isolation, greater B_{12} release provided an advantage in co-cultures. We hypothesize that, given the right conditions, bacteria that release more B_{12} may be selected for, particularly if they form close interactions with B_{12}-dependent algae.

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Introduction

The study of interactions within microbial communities is garnering increased attention as researchers continue to uncover the extent of microbial interdependence (Gude and Taga, 2019; Gralka *et al*., 2020), which is frequently driven by nutrient exchange. These connections extend to microbes across different domains of life, such as between humans and gut bacteria (Round and Mazmanian, 2009), and plants and their companions in the rhizosphere, including rhizobial bacteria (Udvardi and Poole, 2013) and arbuscular mycorrhizal fungi (Chen *et al*., 2018). Similarly, photosynthetic algae often support a range of heterotrophic bacteria in their phycosphere, a region near the algal cell surface analogous to the rhizosphere (Krohn-Molt *et al*., 2017; Seymour *et al*., 2017; Kimbrel *et al*., 2019). In return the algae receive specific compounds such as growth factors (Seyedsayamdost *et al*., 2011) or vitamins (Croft *et al*., 2005). For example, the marine alga *Ostreococcus tauri* can support the bacterium *Dinoroseobacter shibae* in co-culture by providing photosynthate, niacin, biotin and *p*-aminobenzoic acid, and obtain cobalamin and thiamine in return (Cooper *et al*., 2019). Cobalamin (vitamin B_{12}) is a structurally complex cobalt-containing corrinoid molecule that is of particular interest in algal–bacterial interactions because it is only made by prokaryotes and yet more than half of microalgae require it for growth (Croft *et al*., 2005; Tang *et al*., 2010). B_{12} transfer among species, therefore, has an important role in maintaining community structure and function (Heal *et al*., 2017; Gómez-Consarnau *et al*., 2018; Sharma *et al*., 2019).

Nutrient amendment experiments of aquatic ecosystems have revealed that B_{12} or B_{12}-producers frequently limit phytoplankton growth (Bertrand *et al*., 2007; Koch *et al*., 2011; Paerl *et al*., 2015; Joglar *et al*., 2020; Barber-Lluch *et al*., 2021), and laboratory experiments have

investigated the effect of B_{12} supply on phytoplankton physiology (Bertrand *et al*., 2012; Heal *et al*., 2019; Koch and Trimborn, 2019; Nef *et al*., 2019). Studies have revealed the effects of B_{12} on the methionine cycle, C1 metabolism, and cell growth and division in *Euglena gracilis* (Shehata and Kempner, 1978; Carell and Seeger, 1980), *Tisochrysis lutea* (Nef *et al*., 2019), *Thalassiosira pseudonana* (Heal *et al*., 2019), and *Chlamydomonas reinhardtii* (Bunbury *et al*., 2020). In these species, B_{12} acts as a cofactor for the enzyme methionine synthase (METH), although some, like *C. reinhardtii*, also encode a B_{12}-independent isoform (METE). It is the presence or absence of the METE isoform that is the best determinant of algal B_{12} dependence (Helliwell, 2017), and while B_{12} dependence is widespread, its complex phylogenetic distribution points to multiple instances of METE gene loss (Helliwell *et al*., 2011; Ellis *et al*., 2017).

There are several possible hypotheses for bacterial B_{12} excretion, ranging from as simple as B_{12} release on bacterial cell death (Haines and Guillard, 1974; Droop, 2007), to B_{12} export systems that may be regulated by algae (Kazamia *et al*., 2012; Grant *et al*., 2014; Cruz-López and Maske, 2016; Peaudecerf *et al*., 2018). The BtuBFCD complex in Gram-negative bacteria and the BtuFCD complex in Gram-positive bacteria are the best characterized prokaryotic systems for B_{12} uptake (Rodionov *et al*., 2003; Degnan *et al*., 2014a), but although there is speculation that many vitamin transporters may be bidirectional, this has not been confirmed for cobalamin (Romine *et al*., 2017). The molecular machinery in algae is also uncertain, although a protein involved in algal B_{12} uptake was identified in the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Bertrand *et al*., 2012), and subsequently in *C. reinhardtii* (Sayer *et al*. submitted), and B_{12} uptake proteins are now predicted by homology to exist in multiple algal species.

Whatever the mechanism of B_{12} release, its presence in the environment paves the way for the evolution of B_{12} 'providers' into 'cheaters', that is, organisms that benefit from but do not contribute to the nutrient pool (Morris *et al*., 2012). Partial B_{12} biosynthesis pathways are common in bacterial genomes, indicating the evolutionary benefit of dispensing with this metabolically expensive process (Shelton *et al*., 2019). 'Leakiness' of a specific process or metabolite is generally considered to be detrimental but unavoidable (Morris, 2015). However, in the context of a mutualistic cross-feeding relationship, increased leakiness may prove advantageous under certain circumstances (Stump *et al*., 2018). A hypothesis can therefore be developed, whereby it is only when B_{12}-producing bacteria are closely associated with B_{12}-requirers such as photosynthetic algae that provide something in return, that B_{12} release is evolutionarily favoured.

The unicellular chlorophyte, *C. reinhardtii*, is widely used as a model organism for researching photosynthesis and abiotic stress responses (Sasso *et al*., 2018; Salomé and Merchant, 2019), but it has also been used to study microbial symbiosis (Hom and Murray, 2014; Calatrava *et al*., 2018; Durán *et al*., 2021). Here, we use *C. reinhardtii* and B_{12}-producing bacteria to investigate the inherent capacity for a simple mutualism without previous coevolution. We then investigate how the symbiotic dynamics differ between bacteria and the *C. reinhardtii* wild-type, which is B_{12}-independent, versus its B_{12}-dependent mutant as an analogy for the commensal–mutualism switch that would occur for algae that evolve B_{12} dependence. Perturbing the mutualistic co-cultures by nutrient addition reveals how each species responds to the presence of other members of a natural community and to what extent the interaction is regulated. Finally, using two strains of the same species of bacteria that release different amounts of B_{12}, we investigate how increased B_{12} release, a disadvantage in axenic cultures, proves advantageous in the presence of B_{12}-dependent algae.

Experimental procedures

Strains

The wild-type *C. reinhardtii* strain used in this work originated from strain 12 of wild-type 137c. The two other mentioned strains, metE7 and revertant, were derived from this strain by experimental evolution: selection for rapid growth under 1000 $ng \cdot L^{-1}$ B_{12} produced a metE mutant, called S-type, via insertion of a type II transposable element (Helliwell *et al*., 2015). Subsequent excision of this transposon to repair the wild-type sequence of METE, produced the B_{12}-independent revertant strain. In one instance, imprecise excision of the transposon left 9 bp behind to produce a genetically stable B_{12}-dependent strain, metE7 (Helliwell *et al*., 2015).

Mesorhizobium loti, a soil-dwelling rhizobium that forms facultative symbiotic relationships with legumes, was used as a B_{12} producer in most co-culture experiments. Strains MAFF303099, the sequenced strain (Kaneko *et al*., 2000) and ΔbtuF and ΔbluB mutants from the STM library (Shimoda *et al*., 2008) were used. Two *Escherichia coli* strains, ED656 and ED662 (ΔbtuF), were also used as B_{12}-producers. *E. coli* strain ED656 was constructed in a similar fashion to ED741 (Young *et al*., 2021). ED656 was generated from *E. coli* MG1655 by engineering it to contain a set of B_{12} biosynthesis genes. Strain ED656 is MG1655 with (Plac)-T7RNAP/(T7P)-cobA-I-G-J-F-M-K-L-H-B-W-N-S-T-Q-J-D-bluE-C-bluF-P-U-B-cbiW-V-E-btuR-R. All the genes were cloned individually in pET3a and then subcloned together using

the 'Link and Lock' method (Deery *et al*., 2012). ED662 (hereinafter ΔbtuF) was derived from ED656 by insertion of a kanamycin resistance cassette in place of *btuF*. *E. coli* JW0154 ΔbtuF::KmR (Keio collection, *E. coli* Genetic Stock Centre) was used for the construction of ED662 (=ED656 ΔbtuF::KmR) by P1-mediated transduction (Baba *et al*., 2006). *Salmonella typhimurium* AR3612, a metE and cysG double mutant, was used in bioassays to determine the concentration of B_{12} in samples (Raux *et al*., 1996). *Sinorhizobium meliloti* RM1021, *Rhizobium leguminosarum* bv. viciae 3841 and *Pseudomonas putida* were also initially assessed for B_{12} production and release.

Algal and bacterial culture conditions

Algal colonies were maintained on Tris-acetate phosphate (TAP) (The Chlamydomonas Sourcebook) with Kropat's trace elements except for selenium (Kropat *et al*., 2011) + 1000 $ng{\cdot}L^{-1}$ cyanocobalamin (CAS 68-19-9; Millipore-Sigma) agar (1.5%) in sealed transparent plastic tubes at room temperature and ambient light. Colony transfer by spreading on the agar surface using sterile loops was performed in a laminar flow hood. To initiate liquid cultures, colonies were picked and inoculated into filter-capped cell culture flasks (NuncTM, ThermoFisher), or 24-well polystyrene plates (Corning®Costar® Merck) containing TAP or Tris minimal medium (The Chlamydomonas Sourcebook) at less than 60% volume capacity and supplemented with a range of cyanocobalamin concentrations. Cultures were grown under continuous light or a light–dark period of 16 h-8 h, at 100 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$, at a temperature of 25°C, with rotational shaking at 120 rpm in an incubator (InforsHTMultitron; Basel, Switzerland).

Bacteria were maintained as glycerol stocks stored at −80°C. To initiate bacterial culture, a small portion of the glycerol stock was removed and spread on LB and TY agar plates incubated at 37°C overnight or 28°C for 3 days for *E. coli* and *M. loti,* respectively. After confirming the lack of contamination by colony morphology, single colonies were picked and inoculated into nunc flasks containing Tris minimal medium with 0.1% glycerol. Axenic bacterial cultures were incubated under the same conditions as algal cultures, as described above.

Co-cultures of algae and bacteria were initiated by inoculating them into Tris minimal medium from log-phase axenic cultures after dilution to ensure the optical density of algae and bacteria were roughly equal, except where specified. In some cases, co-cultures were acclimated for several days before making a dilution based on the algal cell density and starting measurements.

Algal and bacterial growth measurements

Algal cell density and optical density at 730 nm were measured using a Z2 particle count analyser (Beckman Coulter) with limits of 2.974–9.001 μm, and a FluoStar Optima (BMG labtech) or Thermo Spectronic UV1 spectrophotometer (ThermoFisher), respectively. Colony-forming units (CFU) of algal cells was determined by 10-fold serial dilution of a culture aliquot in growth media and spotting 10 μl volumes on TAP + 1000 $ng{\cdot}L^{-1}$ B_{12} 1.5% agar plates, followed by incubation at 25°C, 20 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$ of continuous light for 4 days and then counting the number of colonies at 100× magnification. *M. loti* and *E. coli* CFU densities were measured similarly but on TY or LB plates with incubation at 28°C in the dark for 3 days or 37°C overnight, respectively. *E. coli* ED662 (ΔbtuF) was distinguished from ED656 by its kanamycin resistance and hence its ability to grow on plates containing 50 $\mu g{\cdot}ml^{-1}$ kanamycin. Single cells for all bacteria were too small to count with a light microscope. When bacteria were grown axenically, optical density was also used as a proxy for cell density with measurements at 600 nm on a FluoStar Optima (BMG labtech) spectrophotometer.

Vitamin B_{12} quantification

Prior to B_{12} quantification, cultures were separated into fractions. In most cases, 1.15 ml of culture was centrifuged at 10 000 g for 2 min, 1.1 ml of the supernatant (media fraction) was aliquoted into a fresh tube, and 1.1 ml of fresh media was used to resuspend the cell pellet (cell fraction). These aliquots were then boiled for 5 min to release B_{12} into solution and then mixed 1:1 with 2*M9 media. The growth response of a B_{12}-dependent strain of *Salmonella typhimurium* (AR3612) incubated for 16 h at 37°C in this mixture was quantified by measuring optical density at 600 nm (Raux *et al*., 1996). B_{12} concentration was calculated by comparing OD 600 nm of the cultures to a standard curve of known B_{12} concentrations using a fitted 4 parameter logistic model and then reported as mass of B_{12} (in pg) per mL of culture from which the cells or media were separated.

Results

B_{12}-dependent strain of C. reinhardtii *takes up B_{12} produced by heterotrophic bacterium*

ChlamydomonasC. reinhardtii is a model alga in part because it grows well on media containing only inorganic nutrients. However, Helliwell *et al*. (2015) were able to generate a vitamin B_{12}-dependent mutant (hereafter metE7) by experimental evolution in the presence of high concentrations of B_{12}. We wanted to test the extent to

which B_{12}-producing bacteria could support the growth of metE7 and whether metE7 exerted any control over bacterial B_{12} production. To identify a suitable B_{12}-producer we first chose four soil bacteria (*Mesorhizobium loti* MAFF303099, *Sinorhizobium meliloti* RM1021, *Rhizobium leguminosarum* bv. viciae 3841 and *Pseudomonas putida*), which we hypothesized might co-occur in the environment with *C. reinhardtii* and could grow under similar conditions. We cultured the strains in minimal medium (TP) with glycerol in a 12 h light-12 h dark regime. After 6 days of culture, the amount of B_{12} in the media and cell fractions was determined (Fig. S1). In the three rhizobia, the level of B_{12} was roughly equal in both fractions, indicating that a significant proportion of synthesized B_{12} is lost to the surroundings. In *P. putida*, however, which unlike the rhizobial strains encodes the outer membrane B_{12} transporter *btuB*, a substantially smaller portion of detectable B_{12} was in the media. Considering that the proportion of B_{12} in the medium was highest for *M. loti*, as well the fact that stable co-cultures form between *M. loti* and the *C. reinhardtii* relative, *Lobomonas rostrata* (Kazamia *et al*., 2012), we chose *M. loti* for further axenic and co-culture experiments.

Before establishing co-cultures, we quantified the effect of vitamin B_{12} on the growth of the *C. reinhardtii* mutant compared with both the ancestral strain from which it was derived and a revertant strain that was no longer dependent on exogenous cobalamin (see Experimental; Helliwell *et al*., 2015). The three strains were cultured for 7 days with several B_{12} concentrations. As previously demonstrated (Bunbury *et al*., 2020), metE7 maximal cell density showed a clear dose–response to vitamin B_{12}, while the growth of the ancestral and revertant strains (both containing a functional METE gene) was not significantly affected by B_{12} (Fig. 1A, Fig. S2). Similarly, the effect of B_{12} supplementation on *M. loti* was quantified for both the wild-type strain and a B_{12} synthesis mutant (bluB^{-}; Shimoda *et al*., 2008), which was confirmed to be unable to synthesize B_{12} (Fig. S3B). Neither strain was affected by B_{12} supplementation (Fig. S3A).

To study the growth and B_{12} production dynamics of *M. loti*, cultures were monitored over a 6-day period using the same conditions as for *C. reinhardtii*, but with 0.1% (v/v) added glycerol and no added B_{12}. Figure 1B illustrates the rapid growth of *M. loti* by more than 1000-fold within 4 days followed by a small decline by day 6. B_{12} concentration in the bacterial cell fraction increased but to a lesser extent than cell density, suggesting the amount of B_{12} per bacterium decreased, and the B_{12} in the media increased more slowly, but more consistently. To test whether this B_{12} could be used by metE7, we first completely removed all *M. loti* cells by filtering the *M. loti* culture through a 0.4 μm filter and adding the filtrate to a new syringe body. Axenic metE7 in late log-phase that had been precultured phototrophically with 200 ng·L^{-1} of B_{12} (an amount that is sufficient to avoid B_{12} deprivation while also preventing the accumulation of substantial B_{12} stores) was added to the *M. loti* filtrate at a final OD730 of 0.1. The 1 ml of filtered aliquots was then sampled at predetermined intervals over the next hour from this mixture of *M. loti* filtrate + metE7 to determine how much B_{12} had been taken up by the algal cells. Figure 1C shows that the concentration of B_{12} measured in the filtrate (i.e. B_{12} not taken up by metE7) declined substantially within 20 min, from almost 1000 to 400 ng·L^{-1} with little decline thereafter. This indicates that *M. loti* can produce and release significant quantities of B_{12} and this can be taken up rapidly by metE7. In contrast, we found that *M. loti* is incapable of taking up exogenous B_{12} (Fig. S3), and this may explain why B_{12} progressively accumulates over time in the media fraction of *M. loti* cultures.

metE7 and M. loti *support each other in mutualistic co-culture*

After confirming metE7 could take up B_{12} produced by *M. loti*, we wanted to see how well *M. loti* would support metE7 in a co-culture with no B_{12} or exogenous organic carbon, so that *M. loti* would depend on the photosynthate provided by metE7. For comparison with this mutualistic interaction, we also set up two commensal co-cultures containing the ancestral or revertant strains (both B_{12}-independent) with *M. loti*. Figure 2A shows that the ancestral and revertant strains were able to grow more quickly and to a higher density than metE7 from day 2 onwards (day 20 Tukey test $P < 0.001$ for ancestral and revertant co-cultures vs metE7 co-culture), suggesting that low B_{12} levels were limiting metE7 growth. Despite the lower growth of metE7, *M. loti* density was similar in all three co-cultures (day 20 ANOVA; $P > 0.05$) and significantly higher than the axenic *M. loti* culture (day 20 Tukey test; $P < 0.001$) (Fig. 2B). Nonetheless, the total amounts of B_{12} were significantly lower in the mutualistic than commensal co-cultures up until the final day, when they equalized (day 20 ANOVA; $P > 0.05$) (Figs. 2C, S4).

Co-cultures provide some insights into how B_{12} producers and consumers naturally grow and survive in the environment, but ecosystems are clearly more complex due to the presence of multiple species and the fluctuations in physical conditions and nutrient availability. We chose to manipulate nutrient influx in the mutualistic co-cultures of metE7 and *M. loti* by testing the effect of added glycerol or B_{12}. We hypothesized that in the co-culture, metE7 and *M. loti* were limited by, and so would increase in response to, the addition of organic carbon and B_{12}, respectively. We also wished to test whether the

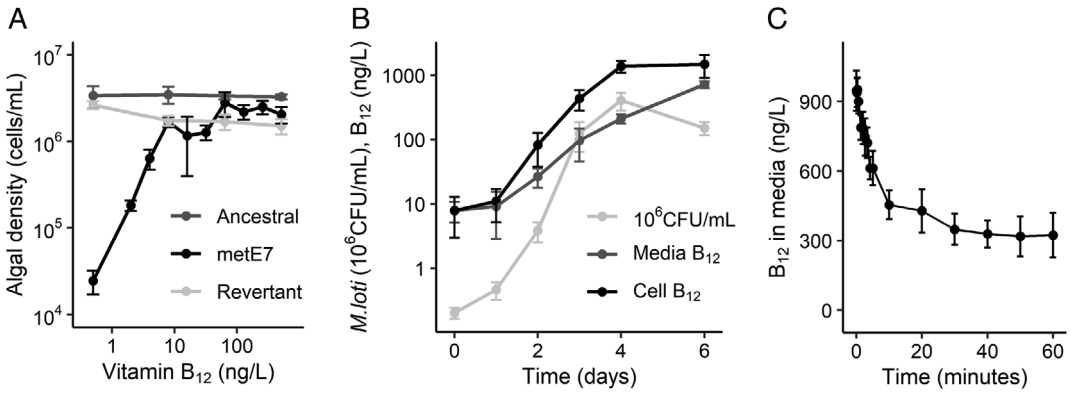

Fig. 1. B_{12}-dependent strain of *C. reinhardtii* takes up B_{12} produced by the heterotrophic bacterium *M. loti*.
A. The evolved metE7 mutant of *C. reinhardtii*, together with its ancestral line and a revertant (Helliwell *et al*. 2015), were cultured with a range of B_{12} concentrations in TP medium at 25°C with constant illumination at 100 μmol·m^{-2}·s^{-1} for 7 days, at which point the cell density in the cultures were measured.
B. *M. loti* was cultured in TP media with 0.1% glycerol at 25°C with constant illumination at 100 μmol·m^{-2}·s^{-1} and measurements of cell density and B_{12} concentration were made over 6 days.
C. An *M. loti* culture, which reached stationary phase, was filtered through a 0.4 μm filter and metE7 cells starved of B_{12} were added to the filtrate at an OD730 nm of 0.1. This culture was then further filtered at multiple time intervals over the course of 1 h to remove metE7 cells and the B_{12} concentration measured in this filtrate. Keys to the different measurements are indicated in legends within the graphs. Error bars represent standard deviations, $n >= 3$.

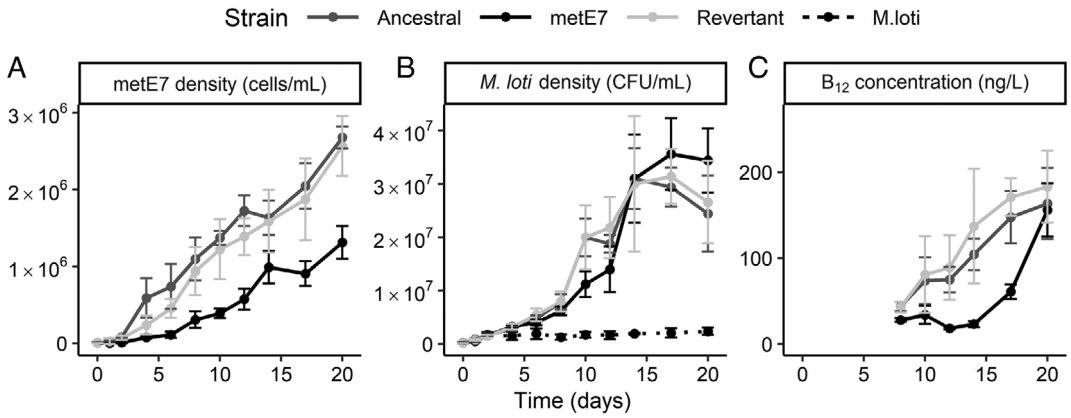

Fig. 2. Comparison of commensal and mutualistic co-cultures between various strains of *C. reinhardtii* and *M. loti*. *M. loti* was co-cultured with the revertant or ancestral lines or metE7 in TP medium at 25°C and with illumination at 100 μmol·m^{-2}·s^{-1} over a 16:8 h light dark cycle for 20 days with periodic measurements of algal and bacterial density as well as B_{12} concentration.
A. Measurement of algal density by particle counter; the ancestral and revertant line density increases at a faster rate than metE7 density.
B. *M. loti* cell density determined by plating serial dilutions of the cultures on TY media; *M. loti* density is not significantly higher in co-culture with the ancestral or revertant lines than with metE7 on almost every day. Dotted line indicates axenic growth of *M. loti* in TP medium (i.e. with no *C. reinhardtii* strain).
C. Total B_{12} concentration measured by *S. typhimurium* bioassay on aliquots of the co-cultures (media and cell fraction); B_{12} is generally higher in co-culture with the ancestral or revertant line. Dark grey = ancestral line, light grey = revertant line, black = metE7. Error bars = standard deviation, $n = 5$.

non-requirer would benefit indirectly through the increased growth and nutrient release by its partner. The co-cultures were initially grown phototrophically as before, with no B_{12} or glycerol, then split into three treatments: no nutrient addition, 200 ng·L^{-1} B_{12}, or 0.02% (v/v) glycerol. The cultures were maintained semi-continuously for 8 days by removing 10% of the culture for daily sampling and replacing it with the same volume of the respective media for each treatment.

As shown in Fig. 3A, the metE7 density increased within 1 day of adding B_{12}, but also increased in the cultures that were amended with glycerol, albeit with a delay of 1–2 days. metE7 densities in the glycerol and B_{12}-supplemented cultures appeared to reach a new, roughly stable equilibrium level approximately 10 times higher than the control co-cultures. On day 8 after nutrient addition, metE7 density was significantly different in each condition (Tukey *P*-value < 0.05 for all comparisons).

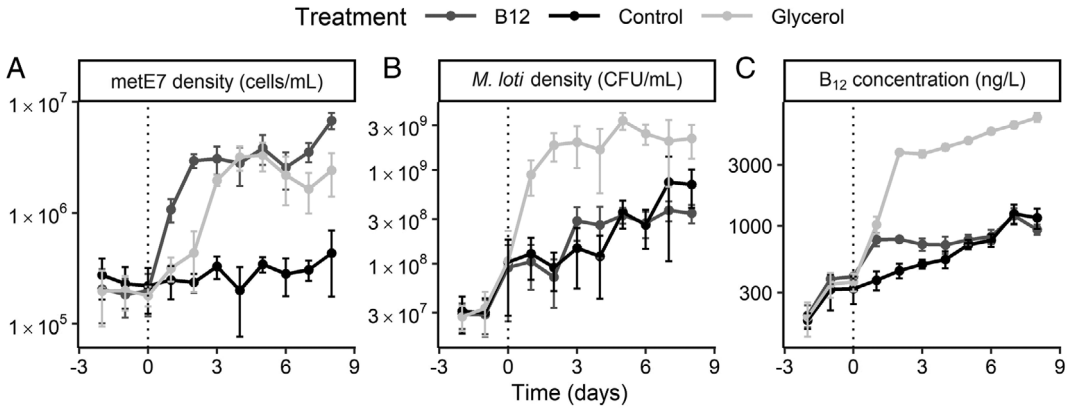

Fig. 3. metE7 and *M. loti* only partially support each other's nutrient requirements. *M. loti* and metE7 were cultured semi-continuously (10% volume replacement per day) under the same conditions as before, but with the addition of 200 ng·L^{-1} of B_{12}, 0.02% glycerol or nothing from day 0. Periodic measurements of algal and bacterial density as well as B_{12} concentration in the cells and media were made.
A. metE7 density increases in the B_{12} supplemented cultures, but also in the glycerol-supplemented cultures after a delay.
B. *M. loti* density increases in response to glycerol but not B_{12} supplementation.
C. Total B_{12} concentration increases more substantially in response to glycerol addition than B_{12} addition itself. Dark grey = 200 ng·L^{-1} of B_{12} addition, light grey = 0.02% glycerol addition, black = control. Error bars = standard deviation, $n = 4$.

Glycerol addition increased *M. loti* levels by ~10-fold relative to both the control and B_{12}-supplemented cultures (Fig. 3B) (day 8 Tukey P-value > 0.05 for control vs B_{12} and $P < 0.05$ for glycerol vs control or B_{12}). However, at no point did the *M. loti* density in the B_{12}-supplemented culture significantly differ from the control, suggesting that the increase in metE7 density did not translate into an increase in organic carbon available for *M. loti* growth. Total B_{12} increased significantly within 1 day of supplementation with either B_{12} itself or glycerol, although by day 8, B_{12} was higher only in the glycerol-supplemented cultures (Tukey P-value < 0.001) (Fig. 3C). B_{12} in the media, on the other hand, accumulated substantially only after glycerol supplementation (Fig. S5A). Therefore, glycerol addition indirectly increased metE7 density, whereas B_{12}-supplementation, which fuelled the growth of metE7, did not result in increased *M. loti* growth.

A similar study of the symbiosis between the B_{12}-dependent alga *L. rostrata* and *M. loti* found that B_{12} production increased in the presence of *L. rostrata* (Kazamia *et al*., 2012; Grant *et al*., 2014). To determine whether the same was true with metE7 we collected further data from axenic cultures of *M. loti* grown in TP medium with glycerol or co-cultures of metE7 and *M. loti* in TP medium alone. Figure S6 reveals a clear positive correlation between B_{12} concentration and *M. loti* density, but that B_{12} produced per *M. loti* cell decreases at higher densities. Rather than produce more B_{12} when grown in co-culture with metE7, *M. loti* produced 45% less B_{12} in total in co-culture than axenic cultures of a similar density ($P < 0.0001$, Fig. S6A). B_{12} in the media fraction increased less with increasing *M. loti* density in co-cultures than axenic cultures ($P < 0.0001$; Fig. S6B), presumably due to metE7 B_{12} uptake. B_{12} levels in the cell fraction, conversely, were 50% higher in co-cultures (metE7 and *M. loti*) than axenic (*M. loti* alone) cultures ($P < 0.0001$; Fig. S6C). Although we could not separate algal and bacterial fractions to measure cellular B_{12} in each, we hypothesized that the reduced B_{12} level in the media of co-cultures might lead to lower bacterial intracellular levels and hence could relieve suppression on B_{12} riboswitch-controlled B_{12} biosynthesis operons (Nahvi *et al*., 2004). We therefore tested the effect of removing B_{12} from the media of *M. loti* cultures either by replacing the media entirely or by using metE7 to take up dissolved B_{12}. B_{12}-deprived metE7 was capable of absorbing, and so removing, most of the B_{12} released by *M. loti* in a manner that B_{12}-saturated metE7 could not (Fig. S7), and yet there was no subsequent increase in B_{12} production by the bacterial cells under that condition. Similarly, entirely refreshing the media, and so removing all B_{12} from the *M. loti* culture, did not increase B_{12} production. Therefore, it seems unlikely that *M. loti* responds to metE7 B_{12} uptake by increasing B_{12} synthesis.

Greater bacterial B_{12} release increases both algal and bacterial growth in co-culture

It was not particularly surprising that metE7 did not increase *M. loti* B_{12} production, since there would be no significant advantage to the naturally B_{12}-independent *C. reinhardtii* to regulate bacterial B_{12} production. However, an evolved B_{12}-dependent alga like metE7 might have a more passive and indirect way of increasing B_{12} in its environment: B_{12}-dependent algae that happen to

co-occur with higher B_{12} providers would grow faster, produce more photosynthate and so improve the growth of the B_{12} producers. To study the effect of B_{12} provision, we first compared the wild-type strain of *M. loti* with a B_{12} uptake transporter (BtuF) mutant but found no significant difference in B_{12} production or release (Fig. S8). The B_{12}-producing rhizobial strains we had initially tested also did not have substantially different rates of B_{12} release, and due to their different growth rates and different physiologies, determining whether any effect on algal growth was due to B_{12} release would have been challenging. To address this, we took advantage of two strains of *E. coli* engineered to produce B_{12}, one of which was further modified so that it lacked BtuF.

We cultured the two *E. coli* strains, ED656 and △btuF (ED662; see Experimental procedures), in TP media with 0.1% glycerol for 4 days, under the same conditions as were used for *M. loti*, and then measured the cell density by plating on LB plates and B_{12} concentration in various fractions. Figure 4A shows that ED656 and ΔbtuF both grew to a similar density of approximately 10^8 CFU·ml^{-1}, but ΔbtuF produced 50% more B_{12} ($P < 0.01$) (Fig. 4B). Importantly, almost all this additional B_{12} was in the media fraction (Fig. 4C), such that levels were 1.5-fold higher ($P < 0.001$) for ΔbtuF than ED656, whereas the cellular B_{12} was not significantly different between *E. coli* strains (Fig. 4D). Because the growth rates of ED656 and ΔbtuF were similar, we designed a more sensitive experiment to distinguish them: after mixing both strains in different proportions the cultures were maintained over 9 days under the same conditions as described above with a 10 000-fold dilution on days 3 and 6. On days 0, 3, 6, and 9 just prior to dilution, the cells were plated on LB plates both with kanamycin and without to determine the numbers of ΔbtuF cells and ΔbtuF+ED656 cells, respectively. The proportion of ΔbtuF cells was substantially lower after 9 days than on day 0 irrespective of the starting concentration, indicating that ED656 had a faster growth rate (Fig. 4E). Since the strains are otherwise isogenic this difference can be attributed to the lack of BtuF, the presence of the kanamycin resistance cassette, or both.

Due to the higher B_{12} production and release by ΔbtuF we predicted that it would support metE7 to a greater extent than ED656. Co-culturing metE7 and either of the *E. coli* strains in TP media as before revealed that after 3 days metE7 cell density was over 100-fold greater ($P = 0.00014$) in co-culture with ΔbtuF than with ED656 (Fig. 5A). This was unlikely to be purely due to increased growth of ΔbtuF, however, because although ΔbtuF grew to a greater extent than ED656, it was only by 1.6-fold ($P = 0.046$) (Fig. 5B). This resulted in the ratio of bacterial to algal cells for ED656:metE7 being much higher than the ΔbtuF:metE7, at 10 000:1 and 170:1, respectively ($P = 0.0023$) (Fig. 5C). We also tested whether both *E. coli* strains could support metE7 without any physical interaction by spotting them out at equal densities on TP agar 10 mm away from metE7 and incubating them as before. While growth was considerably slower than in liquid co-cultures, after 30 days, it was visibly clear that both strains had supported growth of metE7, particularly the closest metE7 cells, but that ΔbtuF supported metE7 to a greater extent than ED656 (Fig. 5D).

To further investigate the difference in how well ED656 and ΔbtuF could support metE7, we initiated tricultures,

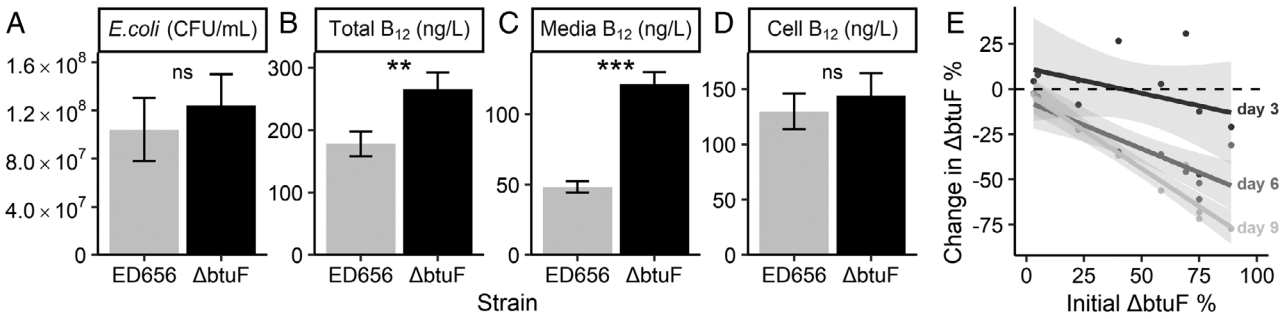

Fig. 4. Growth and B_{12} production of two *E. coli* strains engineered to synthesize vitamin B_{12}. ED656 expresses BtuF, a protein involved in B_{12} uptake, while ΔbtuF is a kanamycin resistant *btuF* knockout. *E. coli* cells were inoculated at 10^7 cells·ml^{-1} and grown for 4 days in TP media with 0.1% glycerol (v/v), at 25°C, and with illumination for a 16:8 h light: dark period at 100 μmol·m^{-2}·s^{-1}.

A. *E. coli* density in colony forming units per mL. B_{12} measured by bioassay in the (B) whole culture, (C) supernatant after centrifugation at 10 000 g for 2 min, or (D) pellet after centrifugation.

E. *E. coli* strains were grown as above but were initially inoculated at different starting percentages: 4, 20, 50, 80, or 96% ΔbtuF with the remainder made up with ED656. Cultures were maintained by diluting 10 000-fold on day 3 and 6 after CFU density measurements. CFU density of ΔbtuF and both strains combined were measured over 9 days by plating a dilution series onto LB agar plates with or without kanamycin (50 μg·ml^{-1}), respectively, and counting the colonies after an overnight incubation at 37°C. The *x* axis indicates the percentage of *E. coli* that is the ΔbtuF strain on day 0 in each culture, and the *y* axis indicates the change in that percentage on days 3, 6 and 9 (labelled on the right of the plot) compared with day 0. For panels A–D, $n = 5$, for panel E, $n = 10$.

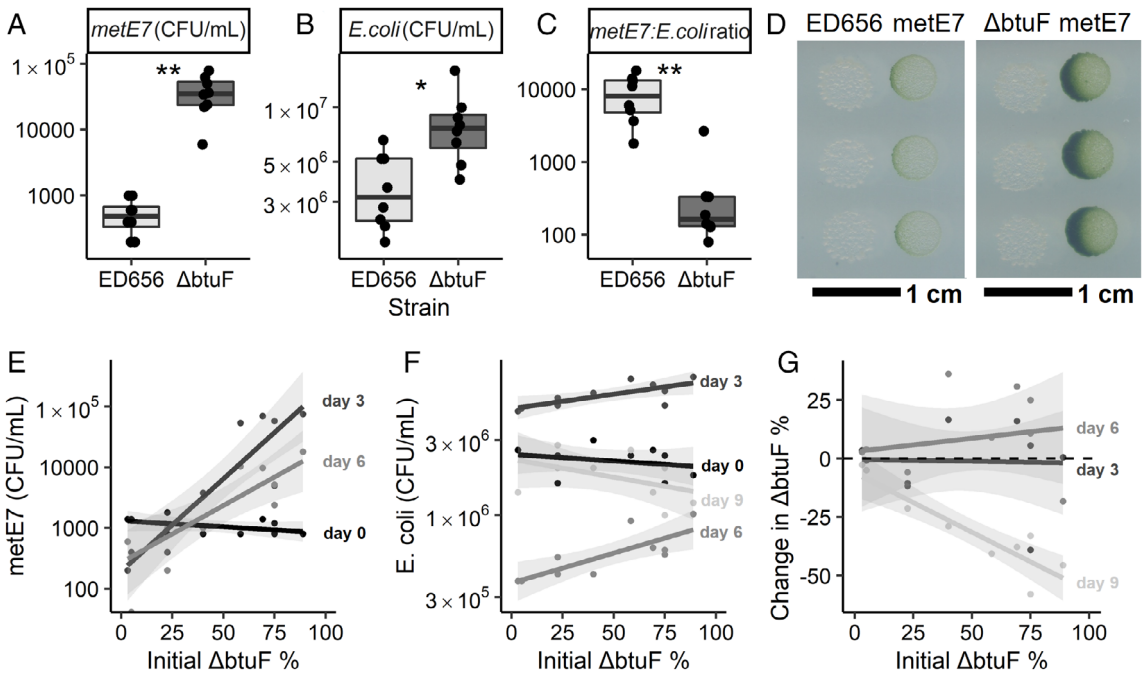

Fig. 5. The ΔbtuF mutant of *E. coli* releases more B_{12} and is better than the isogenic parent ED656 at supporting metE7. For panels A–C, metE7 was co-cultured with either *E. coli* ED656 or ΔbtuF for 3 days at 25°C with shaking at 120 rpm and constant illumination at 100 μmol·m^{-2}·s^{-1}.
A. metE7 colony forming unit (CFU) density on day 2 of co-culture with either *E. coli* strain.
B. ED656 or ΔbtuF CFU density on day three of co-culture with metE7.
C. Ratio of CFUs of *E. coli*:metE7 on day three of co-culture.
D. Photograph of 5 μl droplets of ED656 or ΔbtuF cultures at 10^5 cells·ml^{-1} spotted 10 mm from metE7 cultures at 10^5 cells·ml^{-1} on TP agar and incubated for 30 days at 25°C with constant illumination at 100 μmol·m^{-2}·s^{-1}. For panels E–G, metE7 was co-cultured for 9 days under the same conditions as above with a mix of ΔbtuF and ED656 strains of *E. coli*, where the intended starting percentage of ΔbtuF in this mix was 4, 20, 50, 80 or 96%. Cultures were maintained by diluting 10-fold on days 3 and 6 after CFU density measurements.
E. metE7 colony forming unit (CFU) density on days 0, 3, and 6 (most metE7 CFU measurements were 0 on day 9).
F. Total *E. coli* (ED656 + ΔbtuF) CFU density on days 0, 3, 6, and 9.
G. The *x* axis indicates the percentage of *E. coli* that is the ΔbtuF strain on day 0 in each culture, and the *y* axis indicates the change in that percentage on days 3, 6 and 9 (labelled on the right of the plot) compared with day 0. For panels A–C, $n = 7$–8, and for E, F, and G, $n = 10$.

all of which had the same cell density of metE7 and *E. coli*, but which had different percentages of ΔbtuF and ED656 (intended range was 4%–96% ΔbtuF). The densities of each species were determined on days 0, 3, 6, and 9 with 10-fold dilutions after measurements on days 3 and 6. After 3 days in these tricultures, it was clear that the higher the percent of ΔbtuF the higher the density that metE7 achieved (Fig. 5E; $P < 0.001$). Furthermore, there was a smaller but still significant ($P < 0.001$) positive correlation between percentage ΔbtuF and total bacterial (ΔbtuF + ED656) density (Fig. 5F). However, ΔbtuF was not able to substantially increase as a percentage of the total bacteria (Fig. 5G). By day 9, metE7 levels had collapsed, and ΔbtuF had decreased in prevalence across all starting percentages of ΔbtuF ($P < 0.01$) (Fig. 5G), indicating that ED656 was able to outcompete ΔbtuF in the presence of metE7, as it had done in the purely bacterial co-culture (Fig. 4E). These results suggest that higher B_{12} releasers could better support B_{12}-dependent algae and benefit themselves in return, but that they would likely nevertheless still be dominated by lower B_{12} releasers in a relatively homogeneous environment.

Discussion

In this study, we used an experimentally evolved B_{12}-dependent alga to ask how one that arose in the natural environment might survive when supported by B_{12}-producing bacteria. We showed that the B_{12}-dependent mutant of *C. reinhardtii*, metE7 (Helliwell *et al*., 2015), and the rhizobium *M. loti* could support one another's growth (Fig. 2). However, metE7 grew more slowly than the B_{12}-independent ancestral strain in co-culture with *M. loti*, and B_{12} addition to the co-culture increased metE7 growth (Fig. 3). Although there are several reports of regulated interactions between algae and bacteria (Seyedsayamdost *et al*., 2011; Amin *et al*., 2015; Dao *et al*., 2020), we found no evidence that *M. loti* produced more B_{12} in response to encountering metE7. Nonetheless, we subsequently found that higher B_{12} providers supported more productive co-cultures, increasing both

algal and bacterial growth, but that they were outcompeted by lower B_{12} providers. We hypothesize that only in more heterogeneous environments, which would allow higher B_{12} providers to colocalize with or attach to B_{12} auxotrophs, would productive mutualisms develop and stabilize.

metE7 was previously shown to be B_{12}-dependent (Helliwell *et al*., 2015, 2016), and *M. loti* known to produce enough B_{12} to support algal B_{12} auxotrophs (Kazamia *et al*., 2012; Helliwell *et al*., 2018; Peaudecerf *et al*., 2018; Laeverenz Schlogelhofer *et al*., 2021), but here we more accurately determined the B_{12} requirements of metE7 grown under different trophic conditions, and revealed the dynamics of *M. loti* B_{12} production and release. metE7 had a significantly lower requirement for B_{12} (EC50 $\sim$ 10 ng·L^{-1}) under phototrophic conditions than optimal mixotrophic conditions, presumably due to a slower growth rate and a lower carrying capacity (Fig. S2). This requirement is similar to laboratory cultures and environmental samples of many fresh and saltwater B_{12}-dependent algae, which mostly show half-saturation constants below 100 ng·L^{-1} (Sañudo-Wilhelmy *et al*., 2006; Tang *et al*., 2010; Helliwell, 2017). *M. loti* released sufficient B_{12} to raise the concentration in the media to almost 1000 ng·L^{-1} of B_{12} over 6 days of culture in media optimized for *C. reinhardtii*, which is considerably higher than is found in most aquatic or soil environments (Daisley, 1969; Sañudo-Wilhelmy *et al*., 2012; Barber-Lluch *et al*., 2021). It should be noted that this is $\sim$100 000-fold lower than is produced industrially by fermentation using *Propionibacterium shermanii* or *Pseudomonas denitrificans* (Acevedo-Rocha *et al*., 2019).

There is no known bacterial system for exporting corrinoids, but exogenous B_{12} addition has been shown to reduce bacterial B_{12} release, as has nutrient deprivation, suggesting some degree of regulation (Bonnet *et al*., 2010; Piwowarek *et al*., 2018). Therefore, B_{12} production per cell may have declined as *M. loti* entered nutrient-limited stationary phase (Fig. S6) or because B_{12} accumulation caused negative feedback on B_{12} synthesis, although Fig. S3 suggests this is unlikely as B_{12} addition did not affect B_{12} production. metE7 absorbed a large quantity of the B_{12} produced by *M. loti* from the medium over a short period (absorption half-time of 4 min; see Fig. 1C). Previous work has not quantified the dynamics of B_{12} uptake in *C. reinhardtii* at this resolution, but one study found that within 1 day *C. reinhardtii* cells absorbed 12 000 molecules per cell (Fumio Watanabe *et al*., 1991), considerably less than the roughly 300 000 molecules per cell we found were taken up within 1 h. Studies in *Euglena gracilis* discovered uptake of 400 000 molecules of B_{12} per cell within the first minute, which might be explained by the fact that *E. gracilis* has a cell volume that is approximately 20 times greater than *C. reinhardtii* (Shehata and Kempner, 1977; Sarhan *et al*., 1980; Craigie and Cavalier-Smith, 1982).

In co-culture with *M. loti*, metE7 grew less well than its ancestral line suggesting that its growth was B_{12}-limited, and yet the growth of *M. loti* was not significantly lower with metE7 than the B_{12}-independent lines for almost the entire growth period (Fig. 2). One potential explanation may be that the B_{12}-limited metE7 cells released (including through cell death) a greater amount of organic carbon or a different spectrum of compounds leading to a greater bacteria:algal ratio. In fact, increased cell size, cell death and an increase in starch and triacylglycerides were all found to occur in metE7 on B_{12} deprivation (Bunbury *et al*., 2020). Figure 3A clearly indicates that the addition of glycerol allows a large increase in *M. loti* growth and subsequently B_{12} production (Fig. 3C), which in turn is the likely cause of the increase in metE7 cell density. Previous studies of algal–bacterial mutualisms have investigated the effect of nutrient addition to co-cultures and have produced a variety of results. Two studies of the bacterium *D. shibae* co-cultured with different algae found that adding vitamins B_1 and B_{12}, which were required by the algae, actually improved bacterial growth by a greater amount (Cruz-López and Maske, 2016; Cooper *et al*., 2019). Our results were more similar to a study of *L. rostrata* and *M. loti*, which found that B_{12} addition improved algal growth with little to no effect on *M. loti* (Kazamia *et al*., 2012; Grant *et al*., 2014). Unlike the *L. rostrata* study, however, we found that after induction of *M. loti* growth by addition of glycerol, algal density subsequently increased, causing the bacterial:algal ratio to return towards pre-addition levels (Fig. S5).

Across much of the eubacteria, there is a highly conserved regulatory riboswitch element that is found upstream of B_{12} biosynthesis and transport genes (Rodionov *et al*., 2003). In *M. loti*, these elements are found upstream of B_{12} biosynthesis operons, and B_{12} suppresses the expression of some of these genes, likely resulting in reduced B_{12} production. We hypothesized that metE7 could compete for extracellular B_{12} and so indirectly decrease *M. loti* intracellular levels and potentially, therefore, increase B_{12} production. Although the media of dense co-cultures contained significantly less B_{12} than axenic cultures of *M. loti* with similar densities, this did not result in increased total B_{12} levels (Fig. S6). The only fraction in which B_{12} was higher in co-culture was the cellular fraction, presumably because in the co-culture this includes both algal and bacterial cells. The

short-term effects of perturbing *M. loti* cultures similarly did not indicate that either metE7 addition or B_{12} removal increased B_{12} production per *M. loti* cell (Fig. S6). The only way that the addition of metE7 appeared to affect B_{12} production was through increased growth of *M. loti*, presumably through providing a small amount of photosynthate, and this occurred irrespective of whether metE7 absorbed any B_{12} from the media.

After finding that metE7 did not induce *M. loti* to synthesize more B_{12}, in contrast to *L. rostrata* (Kazamia *et al*., 2012), it seemed unlikely that *C. reinhardtii* would have evolved any more complex measures to increase B_{12} supply such as partner selection or sanctioning (Leigh, 2010; Chomicki *et al*., 2020). However, we hypothesized that group selection might superficially resemble partner choice, because those bacteria that produced more B_{12} would increase algal growth and so could benefit from increased photosynthate if there was sufficient spatial structure to effectively exclude lower B_{12} producers. A ΔbtuF mutant of *M. loti* did not release more B_{12} than the wild type (Fig. S8) nor did *M. loti* appear to take up B_{12} (Fig. S3). In another rhizobium, *S. meliloti*, it was found that the previously annotated *BtuCDF* genes were actually involved in cobalt uptake, not cobalamin (Cheng *et al*., 2011); however, cobalamin supplementation did appear to increase the growth of *S. meliloti* (Campbell *et al*., 2006). If the homologues in *M. loti* also do not transport cobalamin, this would explain why ΔbtuF showed no difference in B_{12} release. More work will be necessary to test whether this is similar in other rhizobia, and whether the lack of B_{12} uptake predicts the ability to support algal B_{12} auxotrophs.

BtuF in *E. coli* does bind and transport B_{12} (Borths *et al*., 2002), and so we generated a B_{12}-producing strain (ED656) of *E. coli*, and a *btuF* mutant (ΔbtuF) of this strain that released more B_{12} (Fig. 4). Furthermore, ΔbtuF was better able to support metE7 in co-culture and subsequently grew better itself (Fig. 5). Although ΔbtuF resulted in greater productivity in co-culture than the parental B_{12}-producing *E. coli* strain (ED656), it also had a slower growth rate than the latter as indicated by its decreasing proportion in co-cultures with ED656 (Fig. 4E). This suggests that in a homogeneous co-culture with a B_{12}-dependent alga, ED656 would eventually dominate ΔbtuF and lead to the steady decline and possible collapse of the co-culture. Indeed this is what we found (Fig. 5E–G).

Generalizing the example mentioned above to an ecological scenario where cooperative strains are at a disadvantage to 'cheaters' (those that contribute less or overexploit a public good) is frequently labelled as a 'tragedy of the commons' or a 'prisoner's dilemma' (Hardin, 2009). These game theory concepts describe how it can be optimal at the group level for individuals to cooperate while simultaneously being optimal for each individual to cheat, such that the rational outcome (Nash equilibrium) is also the worst outcome overall (Nash, 1950). The fact that mutualistic interactions nevertheless abound in nature indicates that this dilemma is solvable, and spatial structure is often at the heart of these solutions (Stump *et al*., 2018). In multicellular hosts with microbial symbiotic partners, spatial structure is often manifested as compartmentalization, allowing hosts to control, sanction, or reward their endosymbionts (Chomicki *et al*., 2020). In aquatic microbial symbioses, it is the phycosphere of separate algal cells that can provide spatial structure for the stable establishment of different bacterial communities (Kimbrel *et al*., 2019; Durán *et al*., 2022), and vertical transmission (which favours mutualism) (Crespi, 2001) of bacteria to algal daughter cells might even be more common than in plant–rhizobia symbioses.

In our example of an algal B_{12} auxotroph, whether the alga and its associated community survive is at least partially dependent upon whether those bacteria provide sufficient B_{12}. It is therefore reasonable to hypothesize that bacteria that release more B_{12}, such as the ΔbtuF mutant discussed here, could out-compete those that release less, only if they associate with algal B_{12} auxotrophs such as metE7 in a manner that spatially excludes the lower producers. There may be similar advantages of enhanced B_{12}-producers feeding B_{12}-auxotrophs in more complex microbial communities, such as those found within the gastrointestinal microbiome, where B_{12} has been shown to be a key modulator of the ecosystem (Degnan et al., 2014b). However, the situation is further complicated by the observation that bacteria produce a range of non-cobalamin corrinoids (Bryant *et al*., 2020), which may either help specific community formation or prevent predation. Intriguingly, algae, like humans, prefer to utilize cobalamin over other corrinoids (Helliwell *et al*., 2016).

In summary, our results suggest that B_{12}-producing bacteria could support newly evolved algal B_{12} auxotrophs but not necessarily that they would favour the growth of B_{12} auxotrophs over their B_{12}-independent relatives. What precise circumstances drive the evolution of algal B_{12} auxotrophy are therefore still unclear, but complex natural communities may well be more propitious environments for B_{12} auxotrophy than the purely bipartite, reciprocal relationships studied here. Furthermore, other B_{12} auxotrophs may naturally have lower B_{12} requirements than metE7, or evolve lower requirements over time, and likely exist in environments where multiple nutrients colimit growth. Finally, natural selection tends to remove species that produce metabolites in excess of

their own needs, and so we propose that only in a more structured environment, including bacterial attachment to algae, might it be beneficial for a bacterium to produce and release more B_{12} rather than compete for its uptake.

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Author contributions

F.B. and A.G.S conceived and designed the research and drafted the manuscript. F.B., E.D. A.P.S, V.B. and E.L.H. participated in data acquisition and analysis. M.J.W. and E.D. contributed resources. All authors helped draft and critically revised the manuscript and gave final approval for publication and agree to be held accountable for the work performed therein.

References

Acevedo-Rocha, C.G., Gronenberg, L.S., Mack, M., Commichau, F.M., and Genee, H.J. (2019) Microbial cell factories for the sustainable manufacturing of B vitamins. *Curr Opin Biotechnol* **56**: 18–29.

Amin, S.A., Hmelo, L.R., van Tol, H.M., Durham, B.P., Carlson, L.T., Heal, K.R., *et al*. (2015) Interaction and signalling between a cosmopolitan phytoplankton and associated bacteria. *Nature* **522**: 98–101.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al*. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 0008.

Barber-Lluch, E., Joglar, V., Moreiras, G., Leão, J.M., Gago-Martínez, A., Fernández, E., and Teira, E. (2021) Variability of vitamin B12 concentrations in waters along the northwest Iberian shelf. *Reg Stud Mar Sci* **42**: 101608.

Bertrand, E.M., Allen, A.E., Dupont, C.L., Norden-Krichmar, T.M., Bai, J., Valas, R.E., and Saito, M.A. (2012) Influence of cobalamin scarcity on diatom molecular physiology and identification of a cobalamin acquisition protein. *Proc Natl Acad Sci U S A* **109**: E1762–E1771.

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.

Bonnet, S., Webb, E.A., Panzeca, C., Karl, D.M., Capone, D. G., and Wilhelmy, S.A.S. (2010) Vitamin B 12 excretion by cultures of the marine cyanobacteria *Crocosphaera* and *Synechococcus*. *Limnol Oceanogr* **55**: 1959–1964.

Borths, E.L., Locher, K.P., Lee, A.T., and Rees, D.C. (2002) The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. *Proc Natl Acad Sci U S A* **99**: 16642–16647.

Bryant, D.A., Hunter, C.N., and Warren, M.J. (2020) Biosynthesis of the modified tetrapyrroles—the pigments of life. *J Biol Chem* **295**: 6888–6925.

Bunbury, F., Helliwell, K.E., Mehrshahi, P., Davey, M.P., Salmon, D.L., Holzer, A., *et al*. (2020) Responses of a newly evolved auxotroph of *Chlamydomonas* to B12 deprivation. *Plant Physiol* **183**: 167–178.

Calatrava, V., Hom, E.F.Y., Llamas, Á., Fernández, E., and Galván, A. (2018) OK, thanks! A new mutualism between *Chlamydomonas* and methylobacteria facilitates growth on amino acids and peptides. *FEMS Microbiol Lett* **365**: fny021.

Campbell, G.R.O., Taga, M.E., Mistry, K., Lloret, J., Anderson, P.J., Roth, J.R., and Walker, G.C. (2006) *Sinorhizobium meliloti bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B12. *Proc Natl Acad Sci U S A* **103**: 4634–4639.

Carell, E.F., and Seeger, J.W., Jr. (1980) Ribonucleotide reductase activity in vitamin B12-deficient *Euglena gracilis*. *Biochem J* **188**: 573–576.

Chen, M., Arato, M., Borghi, L., Nouri, E., and Reinhardt, D. (2018) Beneficial services of arbuscular mycorrhizal fungi - from ecology to application. *Front Plant Sci* **9**: 1270.

Cheng, J., Poduska, B., Morton, R.A., and Finan, T.M. (2011) An ABC-type cobalt transport system is essential for growth of *Sinorhizobium meliloti* at trace metal concentrations. *J Bacteriol* **193**: 4405–4416.

Chomicki, G., Werner, G.D.A., West, S.A., and Kiers, E.T. (2020) Compartmentalization drives the evolution of symbiotic cooperation. *Philos Trans R Soc Lond B Biol Sci* **375**: 20190602.

Cooper, M.B., Kazamia, E., Helliwell, K.E., Kudahl, U.J., Sayer, A., Wheeler, G.L., and Smith, A.G. (2019) Cross-exchange of B-vitamins underpins a mutualistic interaction between *Ostreococcus tauri* and *Dinoroseobacter shibae*. *ISME J* **13**: 334–345.

Craigie, R.A., and Cavalier-Smith, T. (1982) Cell volume and the control of the *Chlamydomonas* cell cycle. *J Cell Sci* **54**: 173–191.

Crespi, B.J. (2001) The evolution of social behavior in microorganisms. *Trends Ecol Evol* **16**: 178–183.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., and Smith, A.G. (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature* **438**: 90–93.

Cruz-López, R., and Maske, H. (2016) The vitamin B1 and B12 required by the marine dinoflagellate *Lingulodinium polyedrum* can be provided by its associated bacterial community in culture. *Front Microbiol* **7**: 560.

Daisley, K.W. (1969) Monthly survey of vitamin B12 concentrations in some waters of the English Lake District: vitamin B12 in some English lakes. *Limnol Oceanogr* **14**: 224–228.

Dao, G., Wang, S., Wang, X., Chen, Z., Wu, Y., Wu, G., *et al*. (2020) Enhanced *Scenedesmus* sp. growth in response to gibberellin secretion by symbiotic bacteria. *Sci Total Environ* **740**: 140099.

Deery, E., Schroeder, S., Lawrence, A.D., Taylor, S.L., Seyedarabi, A., Waterman, J., *et al*. (2012) An enzyme-trap approach allows isolation of intermediates in cobalamin biosynthesis. *Nat Chem Biol* **8**: 933–940.

Degnan, P.H., Barry, N.A., Mok, K.C., Taga, M.E., and Goodman, A.L. (2014a) Human gut microbes use multiple transporters to distinguish vitamin B_{12} analogs and compete in the gut. *Cell Host Microbe* **15**: 47–57.

Degnan, P.H., Taga, M.E., and Goodman, A.L. (2014b) Vitamin B12 as a modulator of gut microbial ecology. *Cell Metab* **20**: 769–778.

Droop, M.R. (2007) Vitamins, phytoplankton and bacteria: symbiosis or scavenging? *J Plankton Res* **29**: 107–113.

Durán, P., Flores-Uribe, J., Wippel, K., Zhang, P., Guan, R., and Garrido-Oter, R. (2021) Characterization of the *Chlamydomonas reinhardtii* phycosphere reveals conserved features of the plant microbiota. *bioRxiv*: 2021.03.04.433956.

Durán, P., Flores-Uribe, J., Wippel, K., Zhang, P., Guan, R., Melkonian, B., *et al*. (2022) Shared features and reciprocal complementation of the *Chlamydomonas* and *Arabidopsis microbiota*. *Nat Commun* **13**: 406.

Ellis, K.A., Cohen, N.R., Moreno, C., and Marchetti, A. (2017) Cobalamin-independent methionine synthase distribution and influence on vitamin B12 growth requirements in marine diatoms. *Protist* **168**: 32–47.

Gómez-Consarnau, L., Sachdeva, R., Gifford, S.M., Cutter, L.S., Fuhrman, J.A., Sañudo-Wilhelmy, S.A., and Moran, M.A. (2018) Mosaic patterns of B-vitamin synthesis and utilization in a natural marine microbial community. *Environ Microbiol* **20**: 2809–2823.

Gralka, M., Szabo, R., Stocker, R., and Cordero, O.X. (2020) Trophic interactions and the drivers of microbial community assembly. *Curr Biol* **30**: R1176–R1188.

Grant, M.A.A., Kazamia, E., Cicuta, P., and Smith, A.G. (2014) Direct exchange of vitamin B12 is demonstrated by modelling the growth dynamics of algal-bacterial cocultures. *ISME J* **8**: 1418–1427.

Gude, S., and Taga, M.E. (2019) Multi-faceted approaches to discovering and predicting microbial nutritional interactions. *Curr Opin Biotechnol* **62**: 58–64.

Haines, K.C., and Guillard, R.R.L. (1974) Growth of vitamin B12-requiring marine diatoms in mixed laboratory cultures with vitamin B12-producing marine bacteria. *J Phycol* **10**: 245–252.

Hardin, G. (2009) The tragedy of the commons. *J Nat Resour Policy Res* **1**: 243–253.

Heal, K.R., Kellogg, N.A., Carlson, L.T., Lionheart, R.M., and Ingalls, A.E. (2019) Metabolic consequences of cobalamin scarcity in the diatom *Thalassiosira pseudonana* as revealed through metabolomics. *Protist* **170**: 328–348.

Heal, K.R., Qin, W., Ribalet, F., Bertagnolli, A.D., Coyote-Maestas, W., Hmelo, L.R., *et al*. (2017) Two distinct pools of B12 analogs reveal community interdependencies in the ocean. *Proc Natl Acad Sci U S A* **114**: 364–369.

Helliwell, K.E. (2017) The roles of B vitamins in phytoplankton nutrition: new perspectives and prospects. *New Phytol* **216**: 62–68.

Helliwell, K.E., Collins, S., Kazamia, E., Purton, S., Wheeler, G.L., and Smith, A.G. (2015) Fundamental shift in vitamin B12 eco-physiology of a model alga demonstrated by experimental evolution. *ISME J* **9**: 1446–1455.

Helliwell, K.E., Lawrence, A.D., Holzer, A., Kudahl, U.J., Sasso, S., Kräutler, B., *et al*. (2016) Cyanobacteria and eukaryotic algae use different chemical variants of vitamin B12. *Curr Biol* **26**: 999–1008.

Helliwell, K.E., Pandhal, J., Cooper, M.B., Longworth, J., Kudahl, U.J., Russo, D.A., *et al*. (2018) Quantitative proteomics of a B12-dependent alga grown in coculture with bacteria reveals metabolic tradeoffs required for mutualism. *New Phytol* **217**: 599–612.

Helliwell, K.E., Wheeler, G.L., Leptos, K.C., Goldstein, R.E., and Smith, A.G. (2011) Insights into the evolution of vitamin B12 auxotrophy from sequenced algal genomes. *Mol Biol Evol* **28**: 2921–2933.

Hom, E.F.Y., and Murray, A.W. (2014) Niche engineering demonstrates a latent capacity for fungal-algal mutualism. *Science* **345**: 94–98.

Joglar, V., Prieto, A., Barber-Lluch, E., Hernández-Ruiz, M., Fernández, E., and Teira, E. (2020) Spatial and temporal variability in the response of phytoplankton and prokaryotes to B-vitamin amendments in an upwelling system. *Biogeosciences* **17**: 2807–2823.

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*. *DNA Res* **7**: 331–338.

Kazamia, E., Czesnick, H., Van Nguyen, T.T., Croft, M.T., Sherwood, E., Sasso, S., *et al*. (2012) Mutualistic interactions between vitamin B12 -dependent algae and heterotrophic bacteria exhibit regulation. *Environ Microbiol* **14**: 1466–1476.

Kimbrel, J.A., Samo, T.J., Ward, C., Nilson, D., Thelen, M.P., Siccardi, A., *et al*. (2019) Host selection and stochastic effects influence bacterial community assembly on the microalgal phycosphere. *Algal Research* **40**: 101489.

Koch, F., Marcoval, M.A., Panzeca, C., Bruland, K.W., Sañudo-Wilhelmy, S.A., and Gobler, C.J. (2011) The effect of vitamin B 12 on phytoplankton growth and community structure in the Gulf of Alaska. *Limnol Oceanogr* **56**: 1023–1034.

Koch, F., and Trimborn, S. (2019) Limitation by Fe, Zn, co, and B12 results in similar physiological responses in two Antarctic phytoplankton species. *Front Mar Sci* **6**: 514.

Krohn-Molt, I., Alawi, M., Förstner, K.U., Wiegandt, A., Burkhardt, L., Indenbirken, D., *et al*. (2017) Insights into microalga and bacteria interactions of selected Phycosphere biofilms using metagenomic, transcriptomic, and proteomic approaches. *Front Microbiol* **8**: 1941.

Kropat, J., Hong-Hermesdorf, A., Casero, D., Ent, P., Castruita, M., Pellegrini, M., *et al*. (2011) A revised mineral nutrient supplement increases biomass and growth rate in *Chlamydomonas reinhardtii*. *Plant J* **66**: 770–780.

Laeverenz Schlogelhofer, H., Peaudecerf, F.J., Bunbury, F., Whitehouse, M.J., Foster, R.A., Smith, A.G., and Croze, O.A. (2021) Combining SIMS and mechanistic

modelling to reveal nutrient kinetics in an algal-bacterial mutualism. *PLoS One* **16**: e0251643.
Leigh, E.G. (2010) The evolution of mutualism. *J Evol Biol* **23**: 2507–2528.
Morris, J.J. (2015) Black queen evolution: the role of leakiness in structuring microbial communities. *Trends Genet* **31**: 475–482.
Morris, J.J., Lenski, R.E., and Zinser, E.R. (2012) The black queen hypothesis: evolution of dependencies through adaptive gene loss. *MBio* **3**: e00036-12.
Nahvi, A., Barrick, J.E., and Breaker, R.R. (2004) Coenzyme B12 riboswitches are widespread genetic control elements in prokaryotes. *Nucleic Acids Res* **32**: 143–150.
Nash, J.F. (1950) Equilibrium points in N-person games. *Proc Natl Acad Sci USA* **36**: 48–49.
Nef, C., Jung, S., Mairet, F., Kaas, R., Grizeau, D., and Garnier, M. (2019) How haptophytes microalgae mitigate vitamin B12 limitation. *Sci Rep* **9**: 8417.
Paerl, R.W., Bertrand, E.M., Allen, A.E., Palenik, B., and Azam, F. (2015) Vitamin B1 ecophysiology of marine picoeukaryotic algae: strain-specific differences and a new role for bacteria in vitamin cycling: marine picoeukaryote thiamine physiology. *Limnol Oceanogr* **60**: 215–228.
Peaudecerf, F.J., Bunbury, F., Bhardwaj, V., Bees, M.A., Smith, A.G., Goldstein, R.E., and Croze, O.A. (2018) Microbial mutualism at a distance: the role of geometry in diffusive exchanges. *Phys Rev E* **97**: 022411.
Piwowarek, K., Lipińska, E., Hać-Szymańczuk, E., Kieliszek, M., and Ścibisz, I. (2018) *Propionibacterium* spp.-source of propionic acid, vitamin B12, and other metabolites important for the industry. *Appl Microbiol Biotechnol* **102**: 515–538.
Raux, E., Lanois, A., Levillayer, F., Warren, M.J., Brody, E., Rambach, A., and Thermes, C. (1996) *Salmonella typhimurium* cobalamin (vitamin B12) biosynthetic genes: functional studies in *S. typhimurium* and *Escherichia coli*. *J Bacteriol* **178**: 753–767.
Rodionov, D.A., Vitreschak, A.G., Mironov, A.A., and Gelfand, M.S. (2003) Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. *J Biol Chem* **278**: 41148–41159.
Romine, M.F., Rodionov, D.A., Maezato, Y., Osterman, A.L., and Nelson, W.C. (2017) Underlying mechanisms for syntrophic metabolism of essential enzyme cofactors in microbial communities. *ISME J* **11**: 1434–1446.
Round, J.L., and Mazmanian, S.K. (2009) The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* **9**: 313–323.
Salomé, P.A., and Merchant, S.S. (2019) A series of fortunate events: introducing *Chlamydomonas* as a reference organism. *Plant Cell* **31**: 1682–1707.
Sañudo-Wilhelmy, S.A., Cutter, L.S., Durazo, R., Smail, E. A., Gómez-Consarnau, L., Webb, E.A., *et al*. (2012) Multiple B-vitamin depletion in large areas of the coastal ocean. *Proc Natl Acad Sci U S A* **109**: 14041–14045.
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**: 1045.
Sarhan, F., Houde, M., and Cheneval, J.P. (1980) The role of vitamin B 12 binding in the uptake of the vitamin by *Euglena gracilis*: B 12 uptake by *Euglena*. *J Protozool* **27**: 235–238.
Sasso, S., Stibor, H., Mittag, M., and Grossman, A.R. (2018) From molecular manipulation of domesticated *Chlamydomonas reinhardtii* to survival in nature. *Elife* **7**: e39233.
Seyedsayamdost, M.R., Case, R.J., Kolter, R., and Clardy, J. (2011) The Jekyll-and-Hyde chemistry of *Phaeobacter gallaeciensis*. *Nat Chem* **3**: 331–335.
Seymour, J.R., Amin, S.A., Raina, J.-B., and Stocker, R. (2017) Zooming in on the phycosphere: the ecological interface for phytoplankton–bacteria relationships. *Nat Microbiol* **2**: 1–12.
Sharma, V., Rodionov, D.A., Leyn, S.A., Tran, D., Iablokov, S.N., Ding, H., *et al*. (2019) B-vitamin sharing promotes stability of gut microbial communities. *Front Microbiol* **10**: 1485.
Shehata, T.E., and Kempner, E.S. (1977) Growth and cell volume of *Euglena gracilis* in different media. *Appl Environ Microbiol* **33**: 874–877.
Shehata, T.E., and Kempner, E.S. (1978) Sequential changes in cell volume distribution during vitamin B12 starvation of *Euglena gracilis*. *J Bacteriol* **133**: 396–398.
Shelton, A.N., Seth, E.C., Mok, K.C., Han, A.W., Jackson, S. N., Haft, D.R., and Taga, M.E. (2019) Uneven distribution of cobamide biosynthesis and dependence in bacteria predicted by comparative genomics. *ISME J* **13**: 789–804.
Shimoda, Y., Mitsui, H., Kamimatsuse, H., Minamisawa, K., Nishiyama, E., Ohtsubo, Y., *et al*. (2008) Construction of signature-tagged mutant library in *Mesorhizobium loti* as a powerful tool for functional genomics. *DNA Res* **15**: 297–308.
Stump, S.M., Johnson, E.C., Sun, Z., and Klausmeier, C.A. (2018) How spatial structure and neighbor uncertainty promote mutualists and weaken black queen effects. *J Theor Biol* **446**: 33–60.
Tang, Y.Z., Koch, F., and Gobler, C.J. (2010) Most harmful algal bloom species are vitamin B1 and B12 auxotrophs. *Proc Natl Acad Sci U S A* **107**: 20756–20761.
Udvardi, M., and Poole, P.S. (2013) Transport and metabolism in legume-rhizobia symbioses. *Annu Rev Plant Biol* **64**: 781–805.
Watanabe, F., Nakano, Y., Tamura, Y., and Yamanaka, H. (1991) Vitamin B12 metabolism in a photosynthesizing green alga, *Chlamydomonas reinhardtii*. *Biochim Biophys Acta (BBA)* **1075**: 36–41.
Young, T.R., Martini, M.A., Foster, A.W., Glasfeld, A., Osman, D., Morton, R.J., *et al*. (2021) Calculating metalation in cells reveals CobW acquires CoII for vitamin B12 biosynthesis while related proteins prefer ZnII. *Nat Commun* **12**: 1195.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Fig. 1. Vitamin B_{12} levels in the cell and media fraction of axenic cultures of four B12-producing bacterial strains. Bacteria were grown in TP medium +0.1% glycerol with illumination in a 12:12 h light:dark period at

100 μmol·m^{-2} ·s^{-1} and 25°C with rotational shaking at 120 rpm. After 6 days of growth, cultures were collected, centrifuged, and the pellet (cell fraction) and supernatant (medium fraction) separated and their B12 content measured. The B12 concentrations, in ng/L, are displayed as boxplots for (A) *M. loti*, (B) *S.* meliloti, (C) *R. leguminosarum*, and (D) *P. putida*. $n = 4$ biological replicates.

Supplementary Fig. 2. Assessing the B12 dependence of three lines of C. reinhardtii under different trophic conditions. The three lines include the 'ancestral' line prior to experimental evolution, 'metE7', a stable B12-dependent line, and 'revertant', a B12 independent line that had reverted from a B12- dependent line. Cultures were grown heterotrophically (TAP medium in the dark), mixotrophically (TAP medium in continuous light), and photoautotrophically (Tris minimal medium in continuous light). B12 concentrations ranged from 0.5 to 512 ng·L^{-1} and precultures of the algae, which were grown with 200 ng·L^{-1} B12, were washed thrice and inoculated at a density of roughly 100 cells·ml^{-1} . (A) Cell density was measured by particle counter after 6 days of growth for mixotrophic cultures or 8 days for heterotrophic and photoautotrophic conditions. (B) Estimated maximal density of metE7 at unlimiting B12 concentrations calculated by fitting a Monod equation to data in panel A. (C) Estimated concentration of B12 required to produce half the maximal density of metE7 cells under each trophic condition calculated by fitting a Monod equation to data in panel A. $n = 3$–4, error bars = sd.

Supplementary Fig. 3. Growth and B12 uptake of M. loti strains. The wildtype (MAFF303099) and B12 synthesis (BluB) mutant were grown in Tris minimal medium supplemented with 0.1% glycerol at 100 μmol·m^{-2} ·s^{-1}, and at a temperature of 25°C, with rotational shaking at 120 rpm over a period of 6 days with (1000 ng·L^{-1}) or without added B12. (A) Viable cells (colony forming units) of *M. loti* 303 099 increased more quickly than the BluB mutant, but there was no significant effect of B12 on growth rate of either strain. (B) The addition of B12 had no effect on the B12 recovered in the cell fraction (top panel) indicating no B12 uptake. Instead, all the added B12 remained in the media (middle panel). Red lines = 1000 ng·L^{-1} of added B12, Blue lines = no added B12, Error bars = sd, $n = 4$.

Supplementary Fig. 4. Dynamics of the ratios of B12, bacterial density and algal density during co-cultures of *M. loti* and three strains of *C. reinhardtii* (A) B12 concentration expressed as molecules of B12 per algal cell reveal very similar levels although different dynamics for the three *C. reinhardti*i strains (B) B12 concentration expressed as molecules of B12 per M. loti cell reveal lower production in co-culture with metE7 particularly around day 14 of co-culture. (C) Bacteria:algae ratio was consistently higher in the metE7 co-culture. Error bars = sd, $n = 5$.

Supplementary Fig. 5. Dynamics of B12 concentrations in the cellular and media fractions and bacteria:algae ratio in metE7 + *M. loti* co-cultures perturbed by nutrient addition. (A) B12 concentration in the media of co-cultures reveals that the highest levels were found following addition of glycerol. (B) B12 concentration in the cellular fraction reveals that glycerol addition caused significantly higher B12 production. (C) Bacteria: algae ratio initially diverged after addition of glycerol or B12 followed by a smaller convergence. Error bars = sd, $n = 4$.

Supplementary Fig. 6. *M. loti* does not increase B12 production in the presence of metE7. Several axenic cultures of *M. loti* with supplemented glycerol and co-cultures containing *M. loti* and metE7 (without glycerol) were grown in TP medium at 25°C with illumination at 100 μmol·m^{-2} ·s^{-1} over a 16:8 h light: dark cycle for up to 32 days or up until the cultures crashed. B12 measurements of the media and cell fraction were made periodically (A) Total B12 is higher in axenic *M. loti* culture than co-culture at the same *M. loti* density ($P < 0.001$) (B) B12 in the media is significantly lower in co-cultures than axenic cultures at high *M. loti* densities ($P < 0.001$). Grey = *M. loti* axenic culture, black = metE7 +- *M. loti* co-culture. N (axenic) = 106, N (co-culture) = 284, grey shaded region = 95% confidence interval.

Supplementary Fig. 7. B12 production by *M. loti* following removal of B12 from the culture media. (A) Experimental setup: Two sets of axenic *M. loti* cultures (grey) were inoculated with metE7 cells that were either saturated with (black solid) or starved (black dashed) of B12 and incubated for 1 h. All 4 cultures were then passed through a 5 μm filter, removing all metE7 cells but not *M. loti*. These *M. loti* cultures were centrifuged, and the supernatant replaced with fresh Tris-min media in treatment 'washed' (grey dashed), or otherwise resuspended without replacing the supernatant (grey solid). The resuspended, newly axenic *M. loti* cultures were grown for 3 days with illumination in a 16:8 h period at 100 μmol·m^{-2} ·s^{-1} and 25°C with rotational shaking at 120 rpm. (B) Total B12 concentration in the culture, and (C) Total B12 per *M. loti* cell. (D) B12 concentration in the supernatant after centrifuging an aliquot of the sample, and (E) media B12 per *M. loti* cell. (F) B12 concentration in the cell pellet after centrifuging an aliquot of the sample, and (G) cell B12 per *M. loti* cell. Error bars = sd, $n = 4$

Supplementary Fig. 8. Growth and B_{12} release of *M. loti* strains. The wildtype (MAFF303099) and B12 transporter mutant (*btuF*) were grown in Tris minimal medium supplemented with various concentrations of glycerol at 100 μmol·m^{-2} ·s^{-1}, and at a temperature of 25°C with rotational shaking at 120 rpm over a period of 8 days. (A) Viable cells (colony forming units) of *M. loti* MAFF 303099 increased over time at the same rate as the *M. loti btuF* mutant and both strains showed improved growth on increasing the glycerol concentration from 0.0128% (v/v) to 0.0512%, but not with a higher concentration. (B) The amount of B_{12} produced in the cells (top panel) and released into the media (middle panel) were not significantly different in the two strains, but as with the cell growth, did increase with the two higher glycerol concentrations. Blue lines = wildtype (MAFF303099), Blue lines = btuF mutant, Error bars = sd, $n = 4$.