

Exploring the onset of B₁₂-based mutualisms using a recently evolved *Chlamydomonas* auxotroph and B₁₂-producing bacteria

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Summary

Cobalamin (vitamin B₁₂) 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 B₁₂. Extended coexistence can then drive gene loss, leading to greater algal–bacterial interdependence. In this study, we investigate how a recently evolved B₁₂-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 B₁₂ producers, its growth in co-culture was slower than the B₁₂-independent wild-type, suggesting that high bacterial B₁₂ provision may be necessary to favour B₁₂ auxotrophs and their evolution. Moreover, we found that an *E. coli* strain that releases more B₁₂ makes a better mutualistic partner, and although this trait may be more costly in isolation, greater B₁₂ release provided an advantage in co-cultures.

We hypothesize that, given the right conditions, bacteria that release more B₁₂ may be selected for, particularly if they form close interactions with B₁₂-dependent algae.

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 B₁₂) 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). B₁₂ 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 B₁₂ or B₁₂-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

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investigated the effect of B₁₂ 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 B₁₂ 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, B₁₂ acts as a cofactor for the enzyme methionine synthase (METH), although some, like *C. reinhardtii*, also encode a B₁₂-independent isoform (METE). It is the presence or absence of the METE isoform that is the best determinant of algal B₁₂ dependence (Helliwell, 2017), and while B₁₂ 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 B₁₂ excretion, ranging from as simple as B₁₂ release on bacterial cell death (Haines and Guillard, 1974; Droop, 2007), to B₁₂ 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 B₁₂ 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 B₁₂ uptake was identified in the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricorutum* (Bertrand *et al.*, 2012), and subsequently in *C. reinhardtii* (Sayer *et al.* submitted), and B₁₂ uptake proteins are now predicted by homology to exist in multiple algal species.

Whatever the mechanism of B₁₂ release, its presence in the environment paves the way for the evolution of B₁₂ 'providers' into 'cheaters', that is, organisms that benefit from but do not contribute to the nutrient pool (Morris *et al.*, 2012). Partial B₁₂ 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 B₁₂-producing bacteria are closely associated with B₁₂-requirers such as photosynthetic algae that provide something in return, that B₁₂ 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 B₁₂-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 B₁₂-independent, versus its B₁₂-dependent mutant as an analogy for the commensal-mutualism switch that would occur for algae that evolve B₁₂ 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 B₁₂, we investigate how increased B₁₂ release, a disadvantage in axenic cultures, proves advantageous in the presence of B₁₂-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·L⁻¹ B₁₂ 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 B₁₂-independent revertant strain. In one instance, imprecise excision of the transposon left 9 bp behind to produce a genetically stable B₁₂-dependent strain, metE7 (Helliwell *et al.*, 2015).

Mesorhizobium loti, a soil-dwelling rhizobium that forms facultative symbiotic relationships with legumes, was used as a B₁₂ 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 B₁₂-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 B₁₂ 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 Δ btuF) was derived from ED656 by insertion of a kanamycin resistance cassette in place of *btuF*. *E. coli* JW0154 Δ btuF::KmR (Keio collection, *E. coli* Genetic Stock Centre) was used for the construction of ED662 (=ED656 Δ btuF::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·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 (Nunc™, 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 μ mol·m⁻²·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 μ m, 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 μ l volumes on TAP + 1000 ng·L⁻¹ B_{12} 1.5% agar plates, followed by incubation at 25°C, 20 μ mol·m⁻²·s⁻¹ of continuous light for 4 days and then counting the number of colonies at 100 \times 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 (Δ btuF) was distinguished from ED656 by its kanamycin resistance and hence its ability to grow on plates containing 50 μ g·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 \times 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

Chlamydomonas C. 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 B₁₂-producing bacteria could support the growth of metE7 and whether metE7 exerted any control over bacterial B₁₂ production. To identify a suitable B₁₂-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 B₁₂ in the media and cell fractions was determined (Fig. S1). In the three rhizobia, the level of B₁₂ was roughly equal in both fractions, indicating that a significant proportion of synthesized B₁₂ is lost to the surroundings. In *P. putida*, however, which unlike the rhizobial strains encodes the outer membrane B₁₂ transporter *btuB*, a substantially smaller portion of detectable B₁₂ was in the media. Considering that the proportion of B₁₂ 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 B₁₂ 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 B₁₂ concentrations. As previously demonstrated (Bunbury et al., 2020), metE7 maximal cell density showed a clear dose–response to vitamin B₁₂, while the growth of the ancestral and revertant strains (both containing a functional METE gene) was not significantly affected by B₁₂ (Fig. 1A, Fig. S2). Similarly, the effect of B₁₂ supplementation on *M. loti* was quantified for both the wild-type strain and a B₁₂ synthesis mutant (bluB⁻; Shimoda et al., 2008), which was confirmed to be unable to synthesize B₁₂ (Fig. S3B). Neither strain was affected by B₁₂ supplementation (Fig. S3A).

To study the growth and B₁₂ 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 B₁₂. 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. B₁₂ concentration in the bacterial cell fraction increased but to a lesser extent than cell density, suggesting the amount of B₁₂ per bacterium decreased, and the B₁₂ in the media increased more slowly, but more consistently. To test whether this B₁₂ 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 B₁₂ (an amount that is sufficient to avoid B₁₂ deprivation while also preventing the accumulation of substantial B₁₂ 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 B₁₂ had been taken up by the algal cells. Figure 1C shows that the concentration of B₁₂ measured in the filtrate (i.e. B₁₂ 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 B₁₂ and this can be taken up rapidly by metE7. In contrast, we found that *M. loti* is incapable of taking up exogenous B₁₂ (Fig. S3), and this may explain why B₁₂ 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 B₁₂ produced by *M. loti*, we wanted to see how well *M. loti* would support metE7 in a co-culture with no B₁₂ 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 B₁₂-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 B₁₂ 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 B₁₂ 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 B₁₂ 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 B₁₂. 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 B₁₂, respectively. We also wished to test whether the

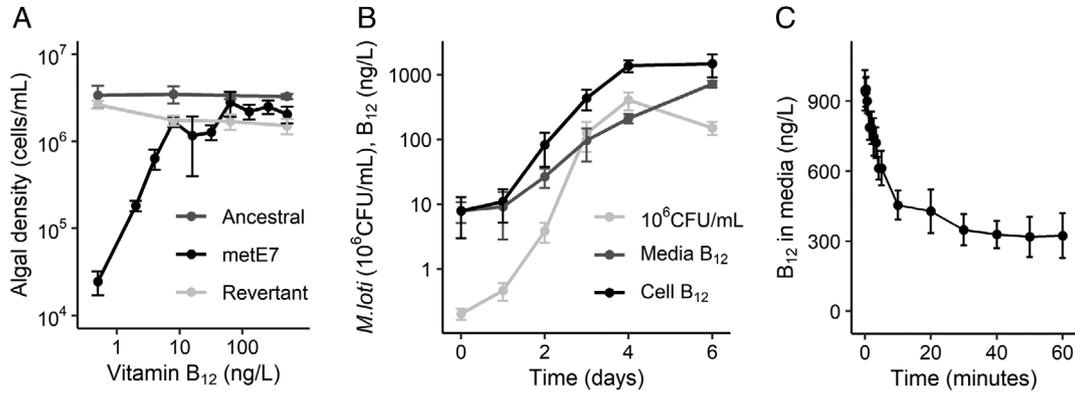


Fig. 1. B₁₂-dependent strain of *C. reinhardtii* takes up B₁₂ produced by the heterotrophic bacterium *M. loti*.

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 B₁₂ 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 B₁₂ 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 B₁₂ 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 B₁₂ concentration measured in this filtrate. Keys to the different measurements are indicated in legends within the graphs. Error bars represent standard deviations, *n* > 3.

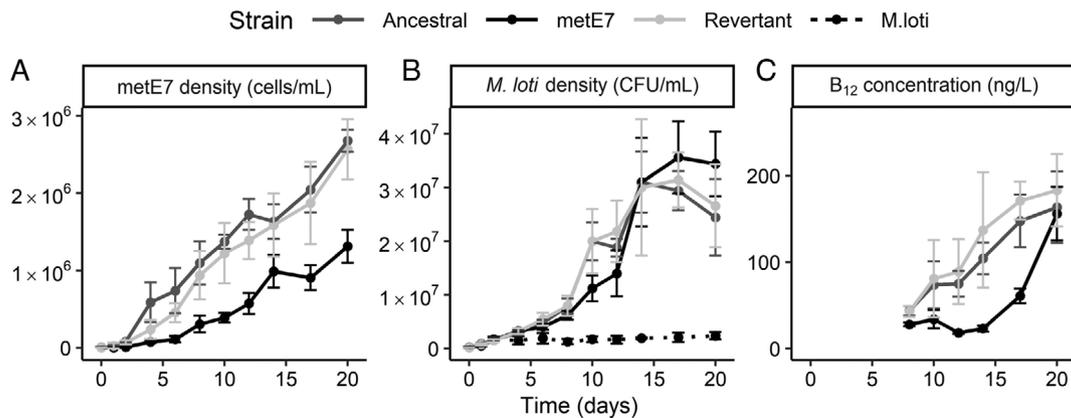


Fig. 2. Comparison of commensal and mutualistic co-cultures between various strains of *C. reinhardtii* and *M. loti*. *M. loti* was co-cultured with the revertant or ancestral lines or metE7 in TP medium at 25°C and with illumination at 100 μmol·m⁻²·s⁻¹ over a 16:8 h light dark cycle for 20 days with periodic measurements of algal and bacterial density as well as B₁₂ 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 B₁₂ concentration measured by *S. typhimurium* bioassay on aliquots of the co-cultures (media and cell fraction); B₁₂ 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-requirement would benefit indirectly through the increased growth and nutrient release by its partner. The co-cultures were initially grown phototrophically as before, with no B₁₂ or glycerol, then split into three treatments: no nutrient addition, 200 ng·L⁻¹ B₁₂, 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 B₁₂, 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 B₁₂-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).

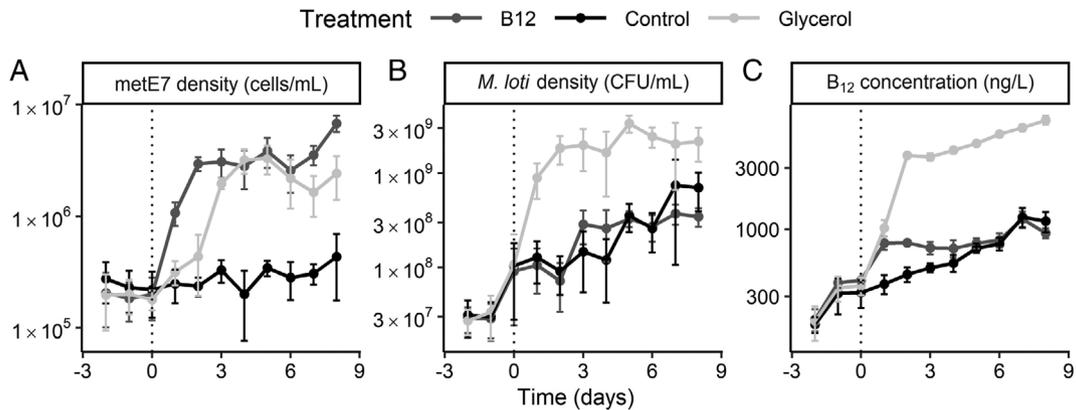


Fig. 3. *metE7* and *M. loti* only partially support each other's nutrient requirements. *M. loti* and *metE7* were cultured semi-continuously (10% volume replacement per day) under the same conditions as before, but with the addition of 200 ng·L⁻¹ of B₁₂, 0.02% glycerol or nothing from day 0. Periodic measurements of algal and bacterial density as well as B₁₂ concentration in the cells and media were made. A. *metE7* density increases in the B₁₂ supplemented cultures, but also in the glycerol-supplemented cultures after a delay. B. *M. loti* density increases in response to glycerol but not B₁₂ supplementation. C. Total B₁₂ concentration increases more substantially in response to glycerol addition than B₁₂ addition itself. Dark grey = 200 ng·L⁻¹ of B₁₂ 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 B₁₂-supplemented cultures (Fig. 3B) (day 8 Tukey *P*-value > 0.05 for control vs B₁₂ and *P* < 0.05 for glycerol vs control or B₁₂). However, at no point did the *M. loti* density in the B₁₂-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 B₁₂ increased significantly within 1 day of supplementation with either B₁₂ itself or glycerol, although by day 8, B₁₂ was higher only in the glycerol-supplemented cultures (Tukey *P*-value < 0.001) (Fig. 3C). B₁₂ in the media, on the other hand, accumulated substantially only after glycerol supplementation (Fig. S5A). Therefore, glycerol addition indirectly increased *metE7* density, whereas B₁₂-supplementation, which fuelled the growth of *metE7*, did not result in increased *M. loti* growth.

A similar study of the symbiosis between the B₁₂-dependent alga *L. rostrata* and *M. loti* found that B₁₂ 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 B₁₂ concentration and *M. loti* density, but that B₁₂ produced per *M. loti* cell decreases at higher densities. Rather than produce more B₁₂ when grown in co-culture with *metE7*, *M. loti* produced 45% less B₁₂ in total in co-culture than axenic cultures of a similar density (*P* < 0.0001, Fig. S6A). B₁₂ 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* B₁₂ uptake. B₁₂ 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 B₁₂ in each, we hypothesized that the reduced B₁₂ level in the media of co-cultures might lead to lower bacterial intracellular levels and hence could relieve suppression on B₁₂ riboswitch-controlled B₁₂ biosynthesis operons (Nahvi *et al.*, 2004). We therefore tested the effect of removing B₁₂ from the media of *M. loti* cultures either by replacing the media entirely or by using *metE7* to take up dissolved B₁₂. B₁₂-deprived *metE7* was capable of absorbing, and so removing, most of the B₁₂ released by *M. loti* in a manner that B₁₂-saturated *metE7* could not (Fig. S7), and yet there was no subsequent increase in B₁₂ production by the bacterial cells under that condition. Similarly, entirely refreshing the media, and so removing all B₁₂ from the *M. loti* culture, did not increase B₁₂ production. Therefore, it seems unlikely that *M. loti* responds to *metE7* B₁₂ uptake by increasing B₁₂ synthesis.

Greater bacterial B₁₂ release increases both algal and bacterial growth in co-culture

It was not particularly surprising that *metE7* did not increase *M. loti* B₁₂ production, since there would be no significant advantage to the naturally B₁₂-independent *C. reinhardtii* to regulate bacterial B₁₂ production. However, an evolved B₁₂-dependent alga like *metE7* might have a more passive and indirect way of increasing B₁₂ in its environment: B₁₂-dependent algae that happen to

co-occur with higher B₁₂ providers would grow faster, produce more photosynthate and so improve the growth of the B₁₂ producers. To study the effect of B₁₂ provision, we first compared the wild-type strain of *M. loti* with a B₁₂ uptake transporter (BtuF) mutant but found no significant difference in B₁₂ production or release (Fig. S8). The B₁₂-producing rhizobial strains we had initially tested also did not have substantially different rates of B₁₂ release, and due to their different growth rates and different physiologies, determining whether any effect on algal growth was due to B₁₂ release would have been challenging. To address this, we took advantage of two strains of *E. coli* engineered to produce B₁₂, one of which was further modified so that it lacked BtuF.

We cultured the two *E. coli* strains, ED656 and Δ 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 B₁₂ concentration in various fractions. Figure 4A shows that ED656 and Δ btuF both grew to a similar density of approximately 10^8 CFU·ml⁻¹, but Δ btuF produced 50% more B₁₂ ($P < 0.01$) (Fig. 4B). Importantly, almost all this additional B₁₂ was in the media fraction (Fig. 4C), such that levels were 1.5-fold higher ($P < 0.001$) for Δ btuF than ED656, whereas the cellular B₁₂ was not significantly different between *E. coli* strains (Fig. 4D). Because the growth rates of ED656 and Δ btuF 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 Δ btuF cells and Δ btuF+ED656 cells, respectively. The proportion of Δ btuF 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 B₁₂ production and release by Δ btuF 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 Δ btuF than with ED656 (Fig. 5A). This was unlikely to be purely due to increased growth of Δ btuF, however, because although Δ btuF 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 Δ btuF: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 Δ btuF supported metE7 to a greater extent than ED656 (Fig. 5D).

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

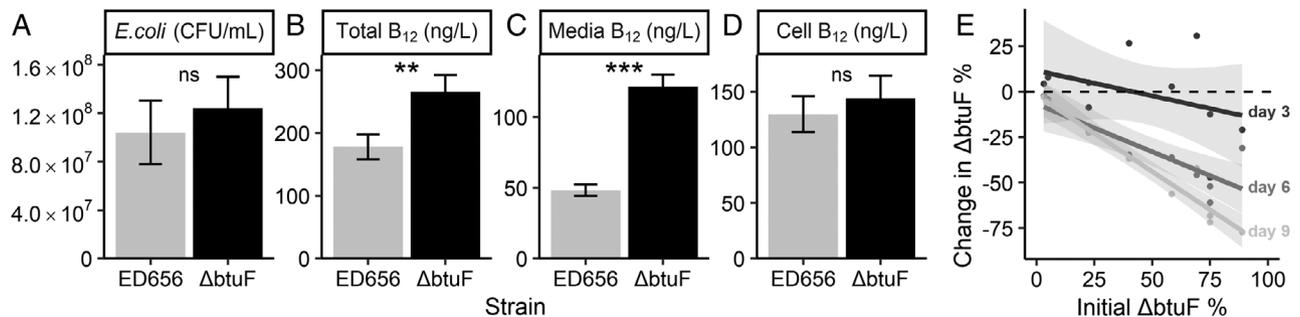


Fig. 4. Growth and B₁₂ production of two *E. coli* strains engineered to synthesize vitamin B₁₂. ED656 expresses BtuF, a protein involved in B₁₂ uptake, while Δ btuF is a kanamycin resistant *btuF* knockout. *E. coli* cells were inoculated at 10^7 cells·ml⁻¹ and grown for 4 days in TP media with 0.1% glycerol (v/v), at 25°C, and with illumination for a 16:8 h light: dark period at $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

A. *E. coli* density in colony forming units per mL. B₁₂ 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% Δ btuF 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 Δ btuF and both strains combined were measured over 9 days by plating a dilution series onto LB agar plates with or without kanamycin ($50 \mu\text{g}\cdot\text{ml}^{-1}$), respectively, and counting the colonies after an overnight incubation at 37°C. The x axis indicates the percentage of *E. coli* that is the Δ btuF 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, $n = 10$.

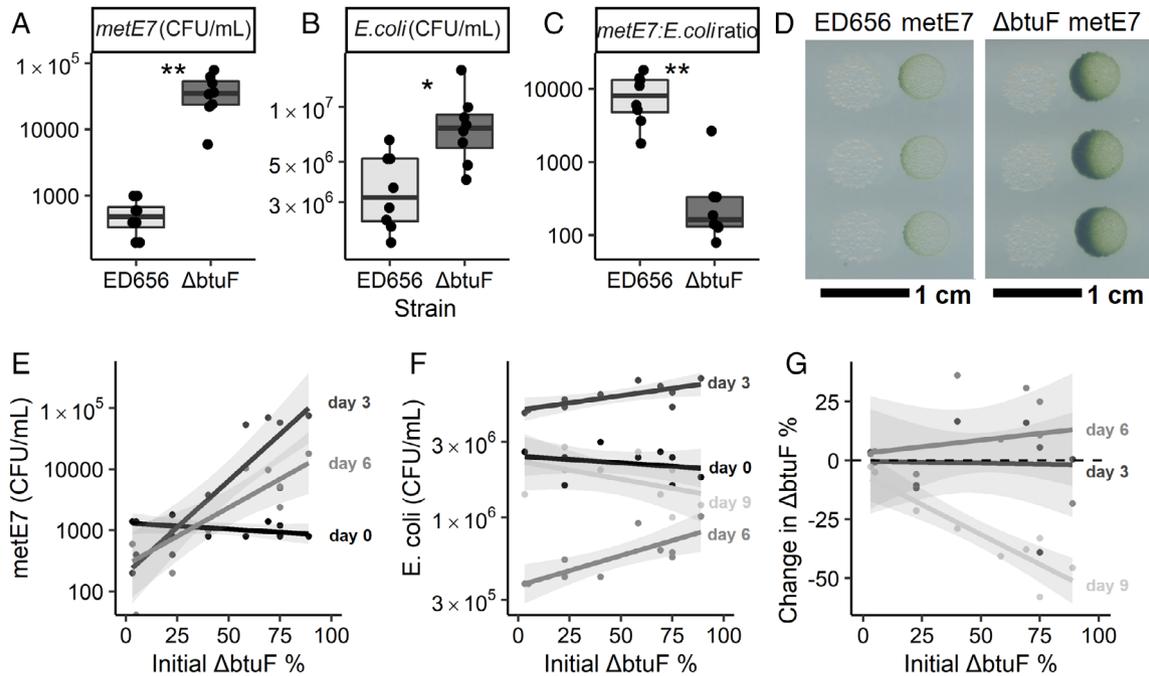


Fig. 5. The Δ btuF mutant of *E. coli* releases more B_{12} and is better than the isogenic parent ED656 at supporting *metE7*. For panels A–C, *metE7* was co-cultured with either *E. coli* ED656 or Δ btuF for 3 days at 25°C with shaking at 120 rpm and constant illumination at $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

A. *metE7* colony forming unit (CFU) density on day 2 of co-culture with either *E. coli* strain.

B. ED656 or Δ btuF 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 μ l droplets of ED656 or Δ btuF 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 $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For panels E–G, *metE7* was co-cultured for 9 days under the same conditions as above with a mix of Δ btuF and ED656 strains of *E. coli*, where the intended starting percentage of Δ btuF 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 + Δ btuF) CFU density on days 0, 3, 6, and 9.

G. The x axis indicates the percentage of *E. coli* that is the Δ btuF 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 Δ btuF and ED656 (intended range was 4%–96% Δ btuF). 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 Δ btuF 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 Δ btuF and total bacterial (Δ btuF + ED656) density (Fig. 5F). However, Δ btuF was not able to substantially increase as a percentage of the total bacteria (Fig. 5G). By day 9, *metE7* levels had collapsed, and Δ btuF had decreased in prevalence across all starting percentages of Δ btuF ($P < 0.01$) (Fig. 5G), indicating that ED656 was able to outcompete Δ btuF 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 algae 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 out-competed by lower B₁₂ providers. We hypothesize that only in more heterogeneous environments, which would allow higher B₁₂ providers to colocalize with or attach to B₁₂ auxotrophs, would productive mutualisms develop and stabilize.

metE7 was previously shown to be B₁₂-dependent (Helliwell *et al.*, 2015, 2016), and *M. loti* known to produce enough B₁₂ to support algal B₁₂ 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 B₁₂ requirements of metE7 grown under different trophic conditions, and revealed the dynamics of *M. loti* B₁₂ production and release. metE7 had a significantly lower requirement for B₁₂ (EC50 ~ 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 B₁₂-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 B₁₂ to raise the concentration in the media to almost 1000 ng·L⁻¹ of B₁₂ 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 ~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 B₁₂ addition has been shown to reduce bacterial B₁₂ release, as has nutrient deprivation, suggesting some degree of regulation (Bonnet *et al.*, 2010; Piwowarek *et al.*, 2018). Therefore, B₁₂ production per cell may have declined as *M. loti* entered nutrient-limited stationary phase (Fig. S6) or because B₁₂ accumulation caused negative feedback on B₁₂ synthesis, although Fig. S3 suggests this is unlikely as B₁₂ addition did not affect B₁₂ production. metE7 absorbed a large quantity of the B₁₂ 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 B₁₂ 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 B₁₂ 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 B₁₂-limited, and yet the growth of *M. loti* was not significantly lower with metE7 than the B₁₂-independent lines for almost the entire growth period (Fig. 2). One potential explanation may be that the B₁₂-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 B₁₂ deprivation (Bunbury *et al.*, 2020). Figure 3A clearly indicates that the addition of glycerol allows a large increase in *M. loti* growth and subsequently B₁₂ 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 B₁ and B₁₂, 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 B₁₂ 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 B₁₂ biosynthesis and transport genes (Rodionov *et al.*, 2003). In *M. loti*, these elements are found upstream of B₁₂ biosynthesis operons, and B₁₂ suppresses the expression of some of these genes, likely resulting in reduced B₁₂ production. We hypothesized that metE7 could compete for extracellular B₁₂ and so indirectly decrease *M. loti* intracellular levels and potentially, therefore, increase B₁₂ production. Although the media of dense co-cultures contained significantly less B₁₂ than axenic cultures of *M. loti* with similar densities, this did not result in increased total B₁₂ levels (Fig. S6). The only fraction in which B₁₂ 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 B₁₂ removal increased B₁₂ production per *M. loti* cell (Fig. S6). The only way that the addition of metE7 appeared to affect B₁₂ 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 B₁₂ from the media.

After finding that metE7 did not induce *M. loti* to synthesize more B₁₂, in contrast to *L. rostrata* (Kazamia et al., 2012), it seemed unlikely that *C. reinhardtii* would have evolved any more complex measures to increase B₁₂ 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 B₁₂ would increase algal growth and so could benefit from increased photosynthate if there was sufficient spatial structure to effectively exclude lower B₁₂ producers. A Δ btuF mutant of *M. loti* did not release more B₁₂ than the wild type (Fig. S8) nor did *M. loti* appear to take up B₁₂ (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 Δ btuF showed no difference in B₁₂ release. More work will be necessary to test whether this is similar in other rhizobia, and whether the lack of B₁₂ uptake predicts the ability to support algal B₁₂ auxotrophs.

BtuF in *E. coli* does bind and transport B₁₂ (Borths et al., 2002), and so we generated a B₁₂-producing strain (ED656) of *E. coli*, and a *btuF* mutant (Δ btuF) of this strain that released more B₁₂ (Fig. 4). Furthermore, Δ btuF was better able to support metE7 in co-culture and subsequently grew better itself (Fig. 5). Although Δ btuF resulted in greater productivity in co-culture than the parental B₁₂-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 B₁₂-dependent alga, ED656 would eventually dominate Δ btuF 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 B₁₂ auxotroph, whether the alga and its associated community survive is at least partially dependent upon whether those bacteria provide sufficient B₁₂. It is therefore reasonable to hypothesize that bacteria that release more B₁₂, such as the Δ btuF mutant discussed here, could out-compete those that release less, only if they associate with algal B₁₂ auxotrophs such as metE7 in a manner that spatially excludes the lower producers. There may be similar advantages of enhanced B₁₂-producers feeding B₁₂-auxotrophs in more complex microbial communities, such as those found within the gastrointestinal microbiome, where B₁₂ 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 B₁₂-producing bacteria could support newly evolved algal B₁₂ auxotrophs but not necessarily that they would favour the growth of B₁₂ auxotrophs over their B₁₂-independent relatives. What precise circumstances drive the evolution of algal B₁₂ auxotrophy are therefore still unclear, but complex natural communities may well be more propitious environments for B₁₂ auxotrophy than the purely bipartite, reciprocal relationships studied here. Furthermore, other B₁₂ auxotrophs may naturally have lower B₁₂ 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 B₁₂ 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.

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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 B₁₂ 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

$100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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 $\text{ng}\cdot\text{L}^{-1}$, 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 $512 \text{ ng}\cdot\text{L}^{-1}$ and precultures of the algae, which were grown with $200 \text{ ng}\cdot\text{L}^{-1}$ B12, were washed thrice and inoculated at a density of roughly $100 \text{ cells}\cdot\text{ml}^{-1}$. (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 $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and at a temperature of 25°C , with rotational shaking at 120 rpm over a period of 6 days with ($1000 \text{ ng}\cdot\text{L}^{-1}$) 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 = $1000 \text{ ng}\cdot\text{L}^{-1}$ 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. reinhardtii* 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 $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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 $5 \mu\text{m}$ 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 $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ 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 B₁₂ 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 $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 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 B₁₂ 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, $n = 4$.