Physiological and Molecular Responses of a Newly Evolved Auxotroph of Chlamydomonas to B$_{12}$ Deprivation

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One-sentence summary: Study of an artificially evolved strain of the model alga Chlamydomonas provides insight into how algal B$_{12}$ auxotrophy has arisen naturally but also reveals the challenges to becoming B$_{12}$ dependent.

Author contributions

F.B., P.M. and A.G.S designed the research; F.B., D.L.S. and A.H. performed the research; N.S., D.L.S. and M.P.D. contributed new reagents or analytic tools; F.B., D.L.S. and A.H. analysed the data; F.B., A.G.S., K.E.H. and P.M. wrote the paper with input from all authors.
Abstract

The corrinoid B\textsubscript{12} is synthesised only by prokaryotes yet is widely required by eukaryotes as an enzyme cofactor. Microalgae have evolved B\textsubscript{12} dependence on multiple occasions and we previously demonstrated that experimental evolution of the non-B\textsubscript{12}-requiring alga \textit{Chlamydomonas reinhardtii} in media supplemented with B\textsubscript{12} generated a B\textsubscript{12}-dependent mutant (hereafter metE7). This clone provides a unique opportunity to study the physiology of a nascent B\textsubscript{12} auxotroph. Our analyses demonstrate that B\textsubscript{12} deprivation of metE7 disrupts C1 metabolism, causes an accumulation of starch and triacylglycerides, and leads to a decrease in photosynthetic pigments, proteins, and free amino acids. B\textsubscript{12} deprivation also caused a substantial increase in reactive oxygen species (ROS), which preceded rapid cell death. Surprisingly, survival could be improved without compromising growth by simultaneously depriving the cells of nitrogen, suggesting a type of cross protection. Significantly, we found further improvements in survival under B\textsubscript{12} limitation and an increase in B\textsubscript{12} use efficiency after metE7 underwent a further period of experimental evolution, this time in coculture with a B\textsubscript{12}-producing bacterium. Therefore, although an early B\textsubscript{12}-dependent alga would likely be poorly adapted to coping with B\textsubscript{12} deprivation, association with B\textsubscript{12}-producers can ensure long-term survival whilst also providing a suitable environment to evolve mechanisms to better tolerate B\textsubscript{12} limitation.

Keywords
\textit{Chlamydomonas reinhardtii}, symbiosis, experimental evolution, vitamin B\textsubscript{12}, auxotrophy, algae
Introduction

Over 50% of algal species require an exogenous source of B₁₂ for growth (Croft et al., 2005), yet large areas of the ocean are depleted of this vitamin (Caterina Panzeca et al., 2009; Sanudo-Wilhelmy et al., 2012). Eukaryotic algae cannot synthesise B₁₂, and must instead obtain it from certain B₁₂-producing prokaryotes (Croft et al., 2005). Indeed, whilst dissolved B₁₂ concentrations are positively correlated with bacterioplankton density (Gobler et al., 2007; C. Panzeca et al., 2008), they have been found to negatively correlate with phytoplankton abundance (Ohwada, 1973; Sañudo-Wilhelmy et al., 2006). Furthermore, nutrient amendment experiments suggest that B₁₂ limits phytoplankton growth in many aquatic ecosystems (Bertrand et al., 2007; Browning et al., 2017; Cohen et al., 2017). Despite this, understanding of the physiological and metabolic adaptations that B₁₂-dependent algae employ to cope with B₁₂ deprivation is rather limited.

In many algae, B₁₂ is required as a cofactor for the B₁₂-dependent methionine synthase enzyme (METH) (Helliwell et al., 2011), although some algae encode a B₁₂-independent isoform of this enzyme (METE) and thus do not require B₁₂ for growth. Bertrand et al. (2012), showed that the B₁₂-dependent marine diatom _Thalassiosira pseudonana_, which possesses only METH, responds to B₁₂ scarcity by increasing uptake capacity and altering the expression of enzymes involved in C₁ metabolism. Heal et al. (2019) found that despite these responses, B₁₂ deprivation disrupted the central methionine cycle, transulfuration pathway, and polyamine biosynthesis. _Phaeodactylum tricornutum_, a marine diatom which uses but does not depend on B₁₂ (encoding both METE and METH), responds similarly to _T. pseudonana_ (Bertrand et al., 2012), but can also rely on increasing expression of METE to maintain the production of methionine. Phylogenetic analysis of the METE gene among diatoms shows no simple pattern of gene loss or gain, as indeed is the case across the eukaryotes (Ellis et al., 2017; Helliwell et al., 2013), but there is a clear link between the lack of a functional copy of the METE gene and B₁₂-dependence (Helliwell et al., 2011; Helliwell, 2017).

As with the diatoms, the phylogenetic distribution of METE within the _Volvocales_ (a family of green freshwater algae) points to gene loss on several independent occasions. The genomes of two volvocalean algae, _V. carteri_ and _G. pectorale_, contain METE pseudogenes, indicating that B₁₂ dependence has evolved relatively recently in these species (Helliwell et al., 2015). _Chlamydomonas reinhardtii_ is a related alga that possesses a functional copy of METE and so is B₁₂-independent. Helliwell et al. (2015) generated a METE mutant of _C. reinhardtii_ by experimental evolution in conditions of high vitamin B₁₂ concentration, demonstrating that sustained levels of B₁₂ in the environment can drive METE gene loss. This mutant, which
contained a Gulliver-related transposable element in the 9th exon of the METE gene, was completely reliant on B₁₂ for growth, but in the presence of the vitamin it was able to outcompete its B₁₂-independent progenitor. In the absence of B₁₂, the METE mutant would sometimes revert to B₁₂ independence and resume growth. Reversion was found to be due to excision of the transposon to leave behind a wild-type METE gene sequence, but there was a single case where a 9-bp fragment of the transposon was left behind resulting in a stable B₁₂-dependent strain, hereafter called metE7.

*C. reinhardtii* is a well-researched model organism that has been instrumental in improving our understanding of algal photosynthesis, ciliogenesis, and responses to fluctuating nutrient environments (Dubini et al., 2009; Grossman, 2000; Rochaix, 1995). We wanted to use the metE7 mutant of *C. reinhardtii* to study how recently acquired B₁₂ auxotrophy impacts an organism’s fitness and physiology, and to provide insight into the metabolic challenges that other B₁₂-dependent algae might have faced when they first evolved. In this work, we characterized the responses of metE7 to different vitamin B₁₂ regimes and compared them to the responses of its ancestral B₁₂-independent strain as well as a closely related, naturally B₁₂-dependent alga *Lobomonas rostrata*. The responses of metE7 to B₁₂ deprivation were quantified by measuring changes in gene expression, cellular composition, photosynthetic activity, and viability, and were contrasted against changes under nitrogen deprivation. To assess whether a recently evolved algal B₁₂ auxotroph could improve its survival during B₁₂ deprivation relatively quickly, we subjected metE7 to a further experimental evolution period of several months in limited B₁₂ or coculture with a B₁₂-producing bacterium and characterised the resulting lines.

**Results**

*B₁₂ deprivation causes substantial changes to C1 metabolism in the metE7 mutant*

Methionine synthase plays a central role in the C1 cycle (Fig. 1A), and thus facilitates nucleotide synthesis and production of the universal methyl donor S-adenosylmethionine, which is essential for many biosynthetic and epigenetic processes (Ducker & Rabinowitz, 2017; Lieber & Packer, 2002). Wild-type (WT) *C. reinhardtii* can operate this cycle in the absence of B₁₂ using the methionine synthase variant METE, but metE7 relies solely on the B₁₂-requiring METH isoform. Although our main aim was to characterise the phenotype of a unique experimentally evolved B₁₂-dependent strain, metE7, we also wanted to confirm that the mutation in METE was solely responsible for the B₁₂ dependence of this strain. We therefore generated an independent METE mutant line (metE4) using CRISPR/Cpf1 on a background strain suitable for genetic manipulation (UVM4) (Ferenczi et al., 2017). This mutant has an in-frame stop codon resulting in a truncated METE amino acid sequence and, as predicted, exhibits B₁₂-dependence (Fig.
S1). Suitably convinced of the role of METE in providing B₁₂ independence, we decided to return our focus to the experimentally evolved B₁₂ dependent strain, metE7, which is perhaps more reflective of B₁₂-dependent algae that have arisen naturally.

Both the WT ancestral line and metE7 were precultured in TAP medium in continuous light with adequate (200 ng·l⁻¹) B₁₂ to maintain a low cellular quota of the vitamin. The cells were then pelleted, washed, and transferred to B₁₂-replete (1000 ng·l⁻¹) or B₁₂-deprived (no B₁₂) TAP medium at 5×10⁵ cells/ml and grown for 30 h. Steady-state transcript levels of six enzymes in the C₁ cycle were then investigated by RT-qPCR (Fig. 1B). In the WT, three transcripts (METE, SAH1, and MTHFR) were significantly (p<0.05) upregulated by B₁₂ deprivation, while in metE7 transcripts for all six enzymes (including METH, METM, and SHMT2) increased. Levels of the methionine-cycle metabolites methionine, SAM, and SAH were quantified by HPLC-MS. In the WT, there was no difference in methionine, SAM, or SAH levels between the two conditions (Fig. 1C). However, in metE7 cells under B₁₂ deprivation, methionine levels were raised 6-fold, which was somewhat unexpected given that methionine synthase activity was impeded. SAH levels were also significantly elevated, whereas there was no effect on SAM. Consequently, the SAM:SAH ratio decreased by 10-fold to 3:1 under B₁₂ deprivation. We then studied the dynamics of these changes by measuring metabolites and RNA abundance at several points during 3 days of B₁₂ deprivation and then for 2 days following the addition of 1000 ng·l⁻¹ B₁₂. The transcripts for all six tested C₁-cycle genes increased rapidly in the first 6 h and then plateaued; reintroduction of B₁₂ led to an immediate reduction to near initial amounts (Fig. S2A). Similar profiles were seen for the metabolites SAM and SAH, although the peak occurred later at 24 h (Fig. S2B). Methionine levels were more variable, but nonetheless there was a similar trend of a peak 24 h after removal of B₁₂. More significantly, the SAM:SAH ratio fell sharply from 30 to less than 1 within 24 h. A subsequent gradual increase occurred over the next 2 days, and resupply of B₁₂ increased this ratio further over the following 2 days. The likelihood therefore is that many cellular processes would be impacted in B₁₂-deprived metE7 cells.

**B₁₂ deprivation significantly impacts cell physiology and biochemical composition**

Our data demonstrate a substantial impact of B₁₂ limitation on the expression of C₁ metabolic genes as well as the abundance of C₁ metabolites. To elucidate downstream consequences of perturbed C₁ metabolism, we also characterised broader physiological responses to B₁₂ deprivation. As has been documented previously (Helliwell et al., 2015), growth of metE7 cells was significantly impaired in B₁₂-deprived conditions (Fig. S3A). However, by day 2 the B₁₂-deprived cells had a 36% larger diameter resulting in a 150% increase in volume (Fig. 2A and Fig. S3B), indicating that cell division was more restricted than overall.
growth. Moreover, cell viability, which was assayed by the ability of cells to form colonies when plated on B$_{12}$-replete TAP agar, decreased to below 25% within 4 days of B$_{12}$ limitation (Fig. S3C). This was preceded by a reduction in photosystem II maximum efficiency ($F_{v}/F_{m}$) (Fig. S3D), an often-used indicator of algal stress (Parkhill et al., 2001; White et al., 2011).

The biochemical composition of *C. reinhardtii* cells is altered considerably and similarly under various nutrient deprivations and so we hypothesised that B$_{12}$ limitation would also induce broadly the same responses (Grossman, 2000; Juergens et al., 2016; S. Saroussi et al., 2017). Therefore, metE7 cells were precultured as before in 200 ng·l$^{-1}$ B$_{12}$, then washed and resuspended in TAP with or without B$_{12}$ (1000 ng·l$^{-1}$) and cultured mixotrophically for 4 days. Cultures were visually inspected by microscopy (Fig. 2A) and the amounts of various cellular components were measured on day 2 and 4 (Fig. 2B and Fig. S4). Chlorophyll levels declined considerably under B$_{12}$ deprivation so that by day four the cells had a bleached appearance with an 85% lower concentration than the B$_{12}$-replete cells. Similarly, free fatty acids (FFA), polar lipids, and proteins were at least 50% lower under B$_{12}$-deprivation conditions on day 4. Starch content on the other hand, showed the largest absolute increase from B$_{12}$-replete to B$_{12}$-deprived cells (Fig. S4), and triacylglycerides (TAG) were 10-fold higher in B$_{12}$-deprived cells (Fig. 2B), which effectively balanced the loss of polar lipids and free fatty acids so that overall lipid levels were roughly 8–10% of dry mass in both treatments. To look in more detail, quantification of free amino acids and fatty acid composition of all lipid classes was carried out (Fig. S5). By day 4, most of the amino acids decreased significantly under B$_{12}$ deprivation. Particularly noteworthy are the reduction in methionine, in contrast to its elevation at an earlier timepoint, and the increase in glutamine, the only amino acid that is more abundant in B$_{12}$-deprived cells (Fig. S5A). Overall the degree of fatty acid saturation was higher under B$_{12}$ deprivation, due mainly to an increase in the dominant saturated fatty acids palmitate (16:0) and stearate (18:0) (Fig. S5B), although levels of several unsaturated fatty acids, in particular 16:2, 16:3$^{(7,10,13)}$, 18:1, and 18:2, were also elevated.

*Responses to nitrogen deprivation improve survival under B$_{12}$ deprivation*

Our results demonstrate that B$_{12}$ deprivation of metE7 causes several changes in biochemical composition, including accumulation of TAG and starch and decreases in chlorophyll and protein, akin to those exhibited following nitrogen deprivation of WT *C. reinhardtii* (Cakmak et al., 2012; Park et al., 2015a; Yang et al., 2015). To further investigate this comparison we measured growth, viability, and photosynthetic efficiency under both conditions over a timecourse (Fig. S6). metE7 culture density increased more under B$_{12}$ than nitrogen deprivation (Fig. S6A), but started to decline after day 2, unlike under nitrogen deprivation.
where growth continued more slowly over 4 days. For cell viability, both conditions caused a decline, but while loss of viability continued in B<sub>12</sub>-deprived cells, under nitrogen deprivation the initial loss was followed by recovery (Fig. S6B). Maximum photosynthetic efficiency of photosystem II, however, did not recover under either condition, and its decline was more rapid in nitrogen-deprived cells (Fig. S6C).

The increased viability of metE7 under nitrogen deprivation compared to under B<sub>12</sub> deprivation suggested that either the metabolic role of B<sub>12</sub> would make it intrinsically more difficult to cope without or that the evolutionary naivety of metE7 to B<sub>12</sub> dependence would mean it had little time to evolve protective responses to B<sub>12</sub> limitation. We therefore tested whether responses to nitrogen deprivation could afford some protection against B<sub>12</sub> deprivation. Viability measurements were monitored over several days, and cultures lacking either nitrogen or B<sub>12</sub> behaved as previously (Fig. 3A). However, metE7 cells deprived of both nitrogen and B<sub>12</sub> simultaneously were more like those starved of nitrogen, with an initial decrease in viability followed by recovery to a level significantly higher than in B<sub>12</sub> deprivation alone. As total growth under B<sub>12</sub>- and nitrogen-deprivation conditions was not significantly different from that under B<sub>12</sub> deprivation alone (Fig. S7), this apparent protective mechanism in response to nitrogen deprivation is not simply a result of inhibiting growth and hence avoiding severe B<sub>12</sub> starvation. Instead, it seems likely that nitrogen deprivation would have elicited photoprotective responses, such as increasing non-photochemical quenching in order to avoid the accumulation of damaging reactive oxygen species (ROS) (Erickson et al., 2015; S. Saroussi et al., 2017, 2019; S. I. Saroussi et al., 2016), initiated the quiescence cycle to mitigate the genomic damage caused by ROS (Takeuchi & Benning, 2019), or activated the gametic survival program (Martin & Goodenough, 1975).

To investigate whether the cell death observed under B<sub>12</sub> deprivation of metE7 could be due to ROS, the general ROS-sensitive dye dihydrodichlorofluorescein diacetate was incubated with cells at different timepoints during nutrient deprivation. We found that ROS levels increased under all nutrient-deprived conditions in the first two days but were highest in those cells deprived of B<sub>12</sub> alone (Fig. 3B). This peak coincided with the start of the substantial decline in cell viability (Fig. 3A). The combination of B<sub>12</sub> and nitrogen deprivation reduced ROS levels to similar amounts to those seen in the nitrogen-deprived cells, and so may be a factor behind reduced cell death.

**Natural B<sub>12</sub> auxotroph Lobomonas rostrata fares better under B<sub>12</sub>-limiting conditions than metE7**
Considering that metE7 quickly lost viability in the absence of B\textsubscript{12} while nitrogen starvation invoked protective responses independent of B\textsubscript{12} status, it is possible that as a novel auxotroph, the response of metE7 to B\textsubscript{12} deprivation is simply underdeveloped. To test this, we compared the B\textsubscript{12} physiology of metE7 with *Lobomonas rostrata*, a naturally B\textsubscript{12}-dependent member of the same Volvocaceae family of chlorophyte algae (Provasoli, 1958; Sausen et al., 2018). Cell viability was significantly greater in *L. rostrata* cells compared to the metE7 line after 2–4 days of B\textsubscript{12} deprivation despite also growing to a greater density (Fig. S8). Moreover, a B\textsubscript{12} dose-response experiment, in which the two species were each cultured mixotrophically in a range of B\textsubscript{12} concentrations, revealed that *L. rostrata* reached a higher optical density than metE7 at all B\textsubscript{12} concentrations below 90 ng·l\textsuperscript{-1}, while the inverse was true above 90 ng·l\textsuperscript{-1} (Fig. 4A). This indicates that *L. rostrata* has a lower B\textsubscript{12} requirement than metE7.

In the natural environment the ultimate source of B\textsubscript{12} is from prokaryotes since they are the only known B\textsubscript{12} producers (Warren et al., 2002). In separate studies it was shown that B\textsubscript{12}-dependent growth of *L. rostrata* and metE7 can be supported by the B\textsubscript{12}-synthesising bacterium *Mesorhizobium loti* (Helliwell et al., 2015; Kazamia et al., 2012). We therefore directly compared the growth of metE7 and *L. rostrata* in B\textsubscript{12}-supplemented (100 ng·l\textsuperscript{-1}) axenic culture and in coculture with *M. loti* in media lacking a carbon source (TP) (Fig. 4A). Even though metE7 grew much more quickly and to a higher density than *L. rostrata* under axenic, B\textsubscript{12}-supplemented conditions, it grew less well in coculture with *M. loti* (Fig. 4B), indicating that B\textsubscript{12} provision from the bacterium is less effective at supporting the growth of metE7 than of *L. rostrata*, perhaps simply due to their different B\textsubscript{12} requirements, but possibly due to more sophisticated symbiotic interactions.

*Experimental evolution in coculture improves B\textsubscript{12} use efficiency and resilience to B\textsubscript{12} deprivation*

Together, our data suggest that the newly evolved metE7 line is poorly adapted to coping with B\textsubscript{12} deprivation, but we wanted to determine whether the metE7 line could evolve improved tolerance to B\textsubscript{12} limiting conditions, so we employed an experimental evolution approach (Fig. S9). We designed three distinct conditions, referred to as H, L, and C. Condition H (TAP medium with high (1000 ng·l\textsuperscript{-1}) B\textsubscript{12}) was a continuation of the conditions that had initially generated metE7 (Helliwell et al., 2015). Condition L (TAP medium with low (25 ng·l\textsuperscript{-1}) B\textsubscript{12}) was chosen so that B\textsubscript{12} would limit growth. Condition C (coculture with *M. loti* in TP medium) was a simplification of an environmental microbial community. Eight independent cultures for each condition were established from a single colony and then subcultured once per week over a total period of 10 months. To account for the different growth rates in the three conditions, we
applied the dilution rates of 10,000, 100, and 5 times per week in condition H, L, and C respectively (Fig. S9). After 10 months under selective conditions, all 24 cultures had survived and were then treated with antibiotics to remove the *M. loti* from condition C and to ensure that there were no other contaminating bacteria. We then subcultured all 24 evolved lines alongside eight replicates of the progenitor strain (which had been maintained on TP agar with 1000 ng·l⁻¹ B₁₂ without subculturing) in mixotrophic conditions with TAP + 200 ng·l⁻¹ B₁₂ three times over nine days to ensure they were all acclimated to the same conditions. The behaviours of the algal populations, hereafter referred to as metE7, metE7H, metE7L, and metE7C, were then compared with one another for their responses to different B₁₂ concentrations, B₁₂ deprivation, and growth in coculture with the B₁₂-producer *M. loti*.

Under high levels of B₁₂ (>320 ng·l⁻¹), a similar carrying capacity was reached by the progenitor metE7 strain and the metE7H and metE7C populations, whereas metE7L density was significantly lower than the progenitor (Fig. S10A). When grown across a range of B₁₂ concentrations to determine a dose response, the metE7C populations reached a significantly higher optical density at the lower concentrations of 20 and 40 ng·l⁻¹ B₁₂ than the other lines (Fig. 5A). The concentration of B₁₂ required to produce half the maximum growth (EC₅₀) of metE7C was therefore much lower than the progenitor metE7 or metE7H (Fig. S10B) and this was reflected in the higher B₁₂ use efficiency i.e. the maximal increase in yield (OD₇₃₀) that results from an increase in B₁₂ concentration (Fig. 5B). However, the maximal growth rate of metE7C was significantly lower (Fig. S10C), and it is tempting to conclude that this is a necessary trade-off. We also compared the viability of the experimentally evolved lines during B₁₂ deprivation (Fig. 5C). Fig. 5C shows that although all lines lost viability during B₁₂ deprivation, metE7L and metE7C survived substantially better, with a median survival time more than a day longer (Fig. 5D) than both the progenitor metE7 and metE7H. Finally, we compared the growth of the evolved lines in coculture with *M. loti*, which showed, perhaps unsurprisingly, that the metE7C lines grew better than the others (Fig. 5E), and at the end of the growth period had a significantly higher number of algae supported per bacterium (Fig. 5F).

To elucidate which factors contributed to improved survival during B₁₂ deprivation, we performed a multi-variable physiological analysis (Fig. 6). Sixteen variables or parameters were measured across the 32 metE7 populations and the dataset (Supplemental dataset S1) was visualised in two ways. Fig. 6A displays the first two components of a principal component analysis of the data, which confirmed that the experimental evolution populations tended towards forming separate clusters, with the metE7C populations most diverged from the progenitor metE7, due mainly to higher B₁₂ use efficiency and median survival time, but lower maximal growth rate, carrying capacity, and EC₅₀ for B₁₂. Fig. 6B is a correlation matrix of the
parameters, which reveals those pairs that are most positively or negatively correlated with one another, for example, median survival time was quite highly negatively correlated with Maximal growth rate and positively correlated with B_{12} use efficiency.

A more definitive statistical approach was then used to determine the most important measurements for predicting survival time during B_{12} deprivation: using stepwise minimisation of the Bayesian information criterion of the full additive linear model, the 15 other measurements (all 16 minus median survival time) were reduced to just three. These three measurements, higher B_{12} use efficiency, lower ROS levels, and lower maximal growth rate, can therefore be considered sufficient to explain longer survival time under B_{12} deprivation of the metE7 populations. Using the same method, we also investigated which values best predicted growth in coculture with *M. loti*, using algae:bacteria ratio as a proxy for this. We found that algae:bacteria ratio was also optimally predicted by just three measurements: higher algal B_{12} use efficiency and lower algal maximal growth rate, as for survival time, but also lower algal B_{12} uptake capacity. Together these results indicate that experimental evolution in coculture not only improves growth in coculture but also increases B_{12} use efficiency and survival during B_{12} deprivation.

**Discussion**

In this study, we exploited a novel model system for the evolution of vitamin B_{12} dependence by analysing the physiological and metabolic responses to B_{12} deprivation of an artificially evolved B_{12}-dependent mutant of *C. reinhardtii*. Our analyses demonstrate that B_{12} deprivation has important consequences for C1 metabolism: we observed a significant increase in the transcript abundance of C1-cycle enzymes in both the WT and metE7 strain, and a decrease in the methylation index (SAM:SAH ratio) in metE7 only. Moreover, B_{12} deprivation of metE7 causes a decrease in chlorophyll, protein, and amino acids, and an increase in starch, lipids, and saturated fatty acids, characteristic of limitation responses to macronutrients such as nitrogen (Cakmak et al., 2012; Juergens et al., 2016; Park et al., 2015b; Yang et al., 2015). The rapid loss of viability seen under B_{12} deprivation could be averted if the metE7 cells were also limited for nitrogen, suggesting that it is not the lack of B_{12} per se that causes cell death, but an inability to respond appropriately. Together this suggests a newly evolved B_{12} auxotroph would be poorly adapted to surviving in the natural environment where a B_{12} supply is not guaranteed. However, we found that metE7 can be supported for several months by a B_{12}-producing bacterium, and experimental evolution under these conditions caused improved B_{12} use efficiency and resilience to B_{12} deprivation.
B₁₂ deprivation of metE7 decreased the SAM:SAH ratio 10-fold, mainly due to an accumulation of SAH, reflecting a recent B₁₂ deprivation study of the diatom *T. pseudonana* (Heal et al., 2019), numerous observational studies of B₁₂ deficiency in humans (Guerra-Shinohara et al., 2004; Stabler et al., 2006), and studies of disrupted C₁ metabolism in *A. thaliana* (Meï et al., 2016). As SAH is a competitive inhibitor of methyltransferases (Chiang et al., 1996), this decrease would likely lead to general hypomethylation in metE7. The epigenetic marks methyldeoxyadenosine and methylcytosine are similarly abundant in *C. reinhardtii* and appear to mark active genes and repeat-rich regions respectively, so the consequences of hypomethylation are unclear (Fu et al., 2015; Lopez et al., 2015). The reduced abundance of B₁₂-bound METH under B₁₂ deprivation would hinder methionine synthesis and could cause the observed reduction in protein abundance (Fig. 2B). However, methionine levels increased between 12 and 24 h of B₁₂ deprivation (Fig. S2B), suggesting a reduction in its use, proteolysis, or increased synthesis due to higher METH expression or via alternative pathways such as the S-methylmethionine cycle, which is known to play an important regulatory role in plants (Ranocha et al., 2001).

*METE* transcript abundance showed a much higher dynamic range than METH during B₁₂ deprivation and reintroduction (Fig. S2A), which is reflected by the higher diurnal range of *METE* observed in global transcriptomics and proteomics datasets (Strenkert et al., 2019). However, on average METE is approximately 60-fold more abundant than METH in *C. reinhardtii* (Strenkert et al., 2019). This may be due to a lower maximal catalytic rate of METE, as has been observed in *E. coli* (Gonzalez et al., 1992), or due to its role in the flagella, which contain METE but not METH (Schneider et al., 2008). Under B₁₂-deprivation conditions, the activity of METH would be compromised, yet in both metE7 and the ancestral strains, it was upregulated. This is more similar to the B₁₂-dependent algae *T. pseudonana* and *Tisochrysis lutea*, which also upregulate METH under B₁₂ deprivation (Bertrand et al., 2012; Nef et al., 2019), than the B₁₂-independent *P. tricornutum*, which decreases METH expression (Bertrand et al., 2013). However, in both *T. pseudonana* and *P. tricornutum*, B₁₂ deprivation substantially upregulates C₁-cycle enzymes including homologs of METM, MTHFR, and SAH1 (Bertrand et al., 2012), reflecting our findings and those of Helliwell et al. (2014). Under sulphur- and nitrogen-deprivation conditions, these C₁-cycle genes are downregulated (González-Ballester et al., 2010; Schmollinger et al., 2014), suggesting that their upregulation during B₁₂ deprivation is not a general response to nutrient stress, but a nutrient-specific one, as indeed is the case for *T. lutea* (Nef et al., 2019).

Chlorosis is a common symptom of nutrient deficiency in *C. reinhardtii*, evident in nitrogen-, sulphur-, iron-, and zinc-limiting conditions, and so it is not surprising that B₁₂
deprivation of metE7 caused a substantial decline in total chlorophyll (Fig. 2B) (Juergens et al., 2015; Kropat et al., 2011; Schmollinger et al., 2014). The decrease in total protein content occurred more slowly and was less substantial (50% reduction over four days) than that reported under nitrogen and sulphur deprivation (80% reduction within one day) (Cakmak et al., 2012). During nitrogen and iron starvation in *C. reinhardtii*, membrane lipids decrease drastically concomitant with the increase in TAGs (Siaut et al., 2011; Urzica et al., 2013). This is very much like what we observed for metE7 under B12 deprivation, although here the level of free fatty acids and polar lipids decreased by a roughly similar amount to the increase in TAGs indicating there is little, if any, de novo fatty acid synthesis. In addition, B12 deprivation causes similar shifts in fatty acid composition to nitrogen and iron deprivation, most notably a substantial increase in palmitic acid (16:0) and decrease in polyunsaturated 16:4 fatty acid (Msanne et al., 2012; Urzica et al., 2013). Despite these similarities, B12 deprivation may elicit an increase in TAGs by a different pathway due to disrupted C1 metabolism, as has been observed in several organisms (da Silva et al., 2014; Mei et al., 2016; Visram et al., 2018). This is thought to be due to a reduction in the methylation potential limiting membrane lipid synthesis and hence diverting more lipids towards TAGs (Malanovic et al., 2008; Visram et al., 2018). Therefore, B12 deprivation could provide a complementary approach to other nutrient deprivation experiments in improving our understanding of lipid metabolism in *C. reinhardtii* and other algae.

From an evolutionary perspective, the prevalence of vitamin B12 dependence among algae appears somewhat at odds with the severe fitness penalties that would be incurred were they exposed to limiting dissolved B12 concentrations. This is made more surprising by the fact that the fitness benefit of B12 dependence under laboratory conditions in replete B12, although statistically significant, appears to be minimal (Helliwell et al., 2015). However, relative to optimal axenic laboratory conditions in which the metE7 line evolved, in the environment multiple nutrients may colimit growth, perhaps even eliciting responses that mitigate against B12 deprivation, as we observed here, and B12-producing bacteria may not simply co-occur with algae but also actively engage in mutualistic interactions (Cooper et al., 2019; Croft et al., 2005; Kazamia et al., 2012, 2016). Furthermore, our evidence suggests that selection under coculture conditions led to the newly evolved B12 auxotroph developing increased B12 use efficiency and becoming better adapted to tolerating B12 limitation, which could make this line more robust to the unreliable B12 supply in the natural environment. However, these improvements appeared to come at the expense of maximal growth rate in B12-replete conditions (Fig. S10C), which is not unexpected in light of previous experimental evolution studies in *C. reinhardtii* (Collins & Bell, 2004). As one of the conserved responses of *C. reinhardtii* upon detecting depletion of various nutrients is to decrease cell division, it is possible that slower growth might even be selected for under B12 deprivation. Indeed, a low growth rate was found to be a significant predictor of
greater survival time under B\textsubscript{12} deprivation, alongside low ROS levels and high B\textsubscript{12} use efficiency.

The fact that metE7 survived a 10-month period either with limited artificial supplementation of B\textsubscript{12} or by relying completely on bacterial B\textsubscript{12} provision does suggest that even a newly evolved and poorly adapted B\textsubscript{12} auxotroph would have ample opportunity to adapt further. What adaptations are likely to improve growth and survival under B\textsubscript{12} deprivation are not altogether clear, but it is not unreasonable to assume that exaptation of existing nutrient limitation responses would play a major role. B\textsubscript{12} dependence is certainly a risky evolutionary strategy, and one which may have ended in extinction countless times, but our work suggests that even the simplest of symbioses with B\textsubscript{12}-producing bacteria may be sufficient to ensure the survival and drive the continued evolution of B\textsubscript{12}-dependent algae.

Materials and Methods

Strains

*Mesorhizobium loti* (MAFF 303099) was a gift from Prof. Allan Downie at the John Innes Centre, Norwich, UK. Algal strains used in this study are shown in Table S1 and include *Lobomonas rostrata* (SAG 45/2), as well as several *Chlamydomonas reinhardtii* strains derived from strain 12 of wild type 137c or the cell wall-deficient strain cw15. The stable B\textsubscript{12}-dependent metE7, the unstable B\textsubscript{12}-dependent (S-type) as well as the B\textsubscript{12}-independent revertant line (R-type) all evolved from the strain 12 of wild type 137c (Ancestral) as described by Helliwell et al. (2015). Another B\textsubscript{12}-dependent mutant (metE4) was generated by targeted (CRISPR/Cpf1) knockout of the *METE* gene in the UVM4 strain using the protocol described in Ferenczi et al. (2017; (21). The guide RNA and single-stranded donor oligonucleotide used for homology-directed repair are given in Table S2, alongside sequencing primers used to confirm that the modification resulted in a premature stop codon producing an 89 amino-acid protein rather than the full length sequence of 815 amino acids sequence.

Culture conditions and growth measurements

Algal colonies were maintained quarterly on Tris-acetate phosphate (TAP) (Table S5) + 1000 ng·l\textsuperscript{-1} cyanocobalamin (B\textsubscript{12}) agar (1.5% w/v) in sealed transparent plastic tubes at room temperature and ambient light. Cultures were grown in TAP or Tris min medium under continuous light or a light-dark period of 16-hr light: 8-hr dark cycle, at 100 µE·m\textsuperscript{-2}·s\textsuperscript{-1}, at a temperature of 25⁰C, with rotational shaking at 120 rpm in an incubator (InforsHTMultitron;
Switzerland). For nutrient starvation experiments the pre-culture TAP medium contained 200 ng·l⁻¹ of B₁₂, and when cell densities surpassed 10⁶ cells·ml⁻¹ or an OD730 nm of 0.2, cultures were centrifuged at 2,000 g for 2 minutes, followed by supernatant removal and resuspension of the cell pellet in media. For nitrogen deprivation, ammonium chloride was omitted from the media with no replacement.

Algal cell density and optical density at 730 nm were measured using a Z2 particle count analyser (Beckman Coulter Ltd.) with limits of 2.974–9.001 µm, and a FluoStar Optima (BMG labtech) or Thermo Spectronic UV1 spectrophotometer (ThermoFisher), respectively. Mean cell diameter was also quantified on a Z2 particle analyser (Beckman Coulter Ltd.). Dry mass was measured by filtering 20 ml of culture through pre-dried and weighed grade-5 Whatmann™ filter paper (Sigma-Aldrich WHA1005090), drying at 70°C for 24 hours, followed by further weighing on a Secura mass balance (Sartorius). Algal and bacterial viable cell density were determined by plating serial dilutions on solid media and counting colonies to calculate colony-forming units per ml (CFU·ml⁻¹).

**Measurement of photosynthetic parameters**

200 µl of cultures with an OD730 nm>0.1 were transferred to a 96-well plate which was then incubated at 25°C in the dark for 20 minutes. F₀ was measured prior to, and Fₘ during, a saturating pulse at 6172 µE·m⁻²·s⁻¹. The light intensity was increased to 100 µE·m⁻²·s⁻¹ and the cells allowed to acclimate for 30 seconds prior to another set of fluorescence measurements before and during a saturating pulse. From these fluorescence measurements, the CF imager software calculated non-photochemical quenching (Fₘ/Fₘ'), PSII maximum efficiency (Fᵥ'/Fₘ'), and the coefficient of photochemical quenching (F₉/Fᵥ).

**Measurement of cellular biochemical composition**

Lipids were extracted from the cell pellet from 10 ml of culture using the chloroform/methanol/water method, and triacylglycerides (TAGs), polar lipids and free fatty acids in the total lipid extract and total fatty acid methyl esters (FAMEs) were analysed by GC-FID and GC-MS, as described in Supplemental Materials and Methods supplemental file 1 and Davey et al. (2014) (22). A 1-ml aliquot of algal culture was used for pigment and starch quantification as described in Davey et al. (2014), and a 10-ml aliquot for protein quantification using a Bradford assay and amino acids by HPLC as described in Supplemental Materials and Methods supplemental file 1 and Helliwell et al. (2018) (23).
Reactive oxygen species quantification

2 µl of 1 mM 2’,7’ Dichlorofluorescein diacetate (Sigma-Aldrich) dissolved in DMSO was added to 198 µl of cell culture in a black f-bottom 96-well plate (Greiner bio-one) and incubated at room temperature in the dark for 60 minutes before recording fluorescence at 520 nm after excitation at 485 nm in a FluoStar Optima Spectrophotometer (BMG labtech). Fresh cell culture media devoid of any cells was used as a blank.

Vitamin B$_{12}$ quantification

A 1-ml aliquot of the culture to be tested was boiled for 5 minutes to release B$_{12}$ into solution and then the growth response of a B$_{12}$-dependent strain of _Salmonella typhimurium_ (AR3612) incubated for 16 hours at 37°C in 50% (v/v) 2*M9 media + 50% (v/v) boiled extract was quantified by measuring optical density at 600 nm. B$_{12}$ concentration was calculated by comparing OD600 nm to a standard curve of known B$_{12}$ concentrations using a fitted logistic model. To calculate B$_{12}$ uptake by algal cells, B$_{12}$ was added to an aliquot of algal culture to a concentration of 1000 ng·l$^{-1}$, followed by incubation for 1 hour under previously described growth conditions, then measuring the B$_{12}$ remaining in the media (supernatant of the centrifuged aliquot). B$_{12}$ uptake (Initial B$_{12}$ – remaining B$_{12}$) was divided by OD730 nm of the aliquot to give B$_{12}$ uptake capacity in ng·l$^{-1}$·OD730 nm$^{-1}$.

SAM and SAH quantification

10 ml of samples were centrifuged at 2,000 g for 2 minutes, supernatant removed, and cell pellet lyophilised at <-40°C and <10 pascals for 12—24 h. 300 µl of 10% methanol (v/v) (LC-MS grade) spiked with stable isotope-labelled amino acids (L-amino acid mix, Sigma-Aldrich, Co., St. Louis, MO, USA) was added to each sample. They were vortexed 3 times, every 10 min, before sonicating for 15 min in an iced water bath then centrifuging (16,100 x g) for 15 min at 4°C. Quantitative analysis was performed on 150 µl of supernatant using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. Details of the HPLC-MS are given in supplemental file 1 Supplemental Materials and Methods and Table S3.

Transcript quantification
Total RNA extraction was performed on the cell pellet from 10 ml of algal culture using the RNeasy® Plant Mini Kit (QIAGEN). DNase treatment was carried out using TURBO DNA-free™ kit (Ambion), and cDNA synthesis using SuperScript®III First-Strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. RT-qPCR was performed as described in supplemental file 1Supplemental Materials and Methods and Helliwell et al. (2018; (24), using primers listed in Table S4.

**Artificial Evolution setup**

A culture of metE7 cells was plated on TAP + 1000 ng·l\(^{-1}\) B\(_{12}\) agar, then 8 colonies picked and resuspended in TAP + 200 ng·l\(^{-1}\) B\(_{12}\) in a 96-well plate. Each well was split into 3 wells, each in a different 96-well plate containing 200 µl of a different media: TAP +1000 ng·l\(^{-1}\) B\(_{12}\), TAP +25 ng·l\(^{-1}\) B\(_{12}\), and TP medium. *M. loti* was prepared in a similar manner to metE7, except preculturing was performed in TP + 0.01% glycerol (w/v). *M. loti* was added to the TP culture containing metE7 at a density roughly 20 times greater than the alga. The 96-well plates were incubated at 25°C, under continuous light at 100 µE·m\(^{-2}\)·s\(^{-1}\), on a shaking platform at 120 rpm. Each week the cultures were diluted: those in TAP +1000 ng·l\(^{-1}\) B\(_{12}\) were diluted 10,000-fold, TAP +25 ng·l\(^{-1}\) B\(_{12}\) = 100-fold, and TP = 5-fold. Every three weeks, 10 µl of serial dilutions of each culture was also spotted onto TAP agar + Ampicillin (50 µg·ml\(^{-1}\)) and Kasugamycin (75 µg·ml\(^{-1}\)) and TAP agar + 1000 ng·l\(^{-1}\) B\(_{12}\) to check for B\(_{12}\)-independent *C. reinhardtii*, or bacterial contaminants and to act as a reserve in the case of contamination. If cultures were found to be contaminated, then at the next transfer they were replaced by colonies from the same well that had grown on the TAP agar plates. At four points during the 12-month evolution period, all cultures were transferred to TAP agar plates where they were stored for 2 weeks during an absence from the lab, meaning that the total time in liquid culture was 10 months. See Fig. S9 for an illustration of the experimental evolution setup and the tests of B\(_{12}\) dose-response viability during B\(_{12}\) deprivation and growth in coculture with the B\(_{12}\)-producer *M. loti* that were performed on all the evolved lines.

**Accession Numbers**

Names and gene IDs of genes referred to in the text are given in Supplemental Table S4.

**Supplemental Data**

**Supplemental Figure S1.** Testing the B\(_{12}\) requirement of several *C. reinhardtii* strains.
Supplemental Figure S2. C1 metabolism enzyme transcript and metabolite abundances in metE7 during B_{12} deprivation and resupply.

Supplemental Figure S3. Characteristics of metE7 cells cultured in B_{12}-replete and B_{12}-deprived conditions.

Supplemental Figure S4. Composition of metE7 cells cultured in B_{12}-replete and B_{12}-deprived conditions.

Supplemental Figure S5. Amino acid and fatty acid composition of metE7 cells in B_{12}-replete and -deprived conditions.

Supplemental Figure S6. Growth and survival of metE7 under nitrogen- and B_{12}-deprivation conditions.

Supplemental Figure S7. Growth and survival of metE7 under a combination of nitrogen- and B_{12}-deprivation conditions.

Supplemental Figure S8. Growth and survival of *L. rostrata* and metE7 under B_{12}-deprivation conditions.

Supplemental Figure S9. Diagram for the experimental evolution setup and the experiments used to analyse the evolved lines.

Supplemental Figure S10. Growth parameters of the metE7 progenitor strain, and its experimental evolution descendants.

Supplemental Table S1. Information about *C. reinhardtii* strains used in this study.

Supplemental Table S2. CRISPR/Cpf1 guide RNAs and ssDNA repair templates.

Supplemental Table S3. Optimised values for Mass-spectroscopic analysis of methionine cycle metabolites MRM - Positive Polarity.

Supplemental Table S4. RT-qPCR primer sequences.

Supplemental Table S5. TAP medium composition.

Supplemental Dataset S1.

Supplemental Materials and Methods.

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Competing interests

The authors declare no competing interests.
Figure legends

**Figure 1.** C1 cycle metabolites and transcripts increase during B$_{12}$ deprivation of metE7. (A) Metabolic map of a portion of the C1 cycle centred around METE and METH, with enzyme abbreviations in black, metabolite abbreviations in grey, and arrows depicting enzyme-catalysed reactions. (B) Abundances of six transcripts for enzymes of the C1 cycle measured by RT-qPCR on RNA extracted from the ancestral line and metE7 after 30 h of incubation in mixotrophic conditions with (1000 ng·l$^{-1}$) or without B$_{12}$. (C) Abundances of Met, SAM, and SAH metabolites measured by HPLC-MS on the same samples as above. Metabolite and transcript abundances are expressed as levels in B$_{12}$-deprived conditions relative to B$_{12}$-replete conditions and presented on a log$_{2}$(x) scale. Error bars = SD, n=3–4, 'ns'=not significant, *=p<0.05, **=p<0.01, ***=p<0.001, Welch’s t-test. WT = ancestral B$_{12}$-independent strain, metE7 = experimentally evolved B$_{12}$-dependent line. See also Fig. S2.

**Figure 2.** B$_{12}$ deprivation of metE7 causes cell enlargement and significant changes in biochemical composition. (A) Micrographs taken at 1000x magnification (with scale bars showing 10 µm) of metE7 cells grown in TAP medium with (1000 ng·l$^{-1}$) or without B$_{12}$ over a period of 4 days (B) Biochemical composition of B$_{12}$-deprived cells on day 2 and day 4 of the growth period expressed as mass of those compounds normalised to total cell dry mass and then expressed relative to the amounts in B$_{12}$ replete conditions. Error bars = SD, n = 5. **=p<0.01, ***=p<0.001, Welch’s t-test. See also Fig. S3, Fig. S4, and Fig. S5.

**Figure 3.** metE7 survives better and produces lower levels of reactive oxygen species (ROS) when limited for both N and B$_{12}$ than just B$_{12}$ alone. (A) Percentage of cells that could form colonies (a measure of viability) on nutrient replete agar when removed at different timepoints from various nutrient deprivation conditions (Indicated in panels above the graphs as follows: +B12-N: 1000 ng·l$^{-1}$ B12 and 0 mM NH4Cl, -B12+N: 0 ng·l$^{-1}$ B12 and 7.5 mM NH4Cl, or -B12-N: 0 ng·l$^{-1}$ B12 and 0 mM NH4Cl). (B) ROS measured by dichlorofluorescein diacetate (DCFDA) fluorescence and normalised both on a per cell basis and to the nutrient replete treatment (+B$_{12}$+N). Error bars = SD, n = 3–6. See also Fig. S6 and Fig. S7.
Figure 4. L. rostrata grows better than metE7 in coculture with a B_{12}-producing bacterium, in part due to its lower demand for B_{12}. (A) Precultures of the algae, grown with 200 ng·l^{-1} B_{12}, were washed and inoculated at roughly 100 cells·ml^{-1} then grown mixotrophically (TAP medium in continuous light), with B_{12} concentrations from 0 to 200 ng·l^{-1}. Optical density at 730 nm was measured after 5 and 9 days of growth for metE7 and L. rostrata, respectively. (B) Cultures were grown photoautotrophically (TP media in 16-h light (100 µE·m^{-2}·s^{-1}):8-h dark cycles) in axenic culture (with 100 ng·l^{-1} B_{12}) or coculture (with the B_{12}-producing bacterium M. loti) over a period of 16 days with cell density measurements performed every 1–2 days. For both panel A and B, black = L. rostrata, grey = metE7, error bars = SD, n=4. See also Fig. S8.

Figure 5. Experimental coevolution of metE7 with the bacterium M. loti selects for improved algal growth in coculture, increased B_{12} use efficiency, and better resilience to B_{12} deprivation. (A) Maximum optical density achieved by mixotrophically-grown cultures of experimentally evolved lines of metE7 grown over a period of 12 days in six different concentrations of B_{12}. (B) B_{12} use efficiency of evolved lines calculated using a fitted Monod equation and expressed as the maximum rate of increase in OD_{730} that would result from an increase in B_{12} concentration. (C) Viability (measured as the percentage of cells capable of forming colonies on B_{12}-replete agar) of mixotrophically-grown cultures of experimentally evolved lines of metE7 over a 5-day period cultured in 40 ng·l^{-1} B_{12}. (D) Median survival time of evolved lines after dilution of culture to 40 ng·l^{-1} B_{12} calculated using a fitted Verhulst equation. (E) Algal cell density of photoautotrophically grown cocultures of experimentally evolved lines of metE7 with M. loti over a 9-day period. (F) Ratio of algae to bacteria on the final day (day 9) of growth in coculture. metE7H = metE7 evolved in TAP + 1,000 ng·l^{-1} B_{12} for 10 months; metE7L = metE7 evolved in TAP media + 25 ng·l^{-1} B_{12}; metE7C = metE7 evolved in Tris minimal medium in coculture with the B_{12}-producing bacterium M. loti. Mean of 7–8 independently evolved lines are shown. Error bars = 95% confidence interval, letters above error bars indicate statistical groupings provided by Tukey’s range test, which was performed following a significant ANOVA result. See also Fig. S9 and Fig. S10.

Figure 6. Analysis of a range of measurements made or parameters calculated from the metE7 progenitor strain and its experimental evolution descendants. (A) Plot of the first two principal components derived from PCA applied to these measured variables and calculated parameters. Each point represents an evolved line or replicate of the progenitor strain and is given a colour and shape according to experimental evolution condition, which is specified in the
key in the top left corner. Ellipses are created to show the standard deviation of the eight lines belonging to these experimental evolution groups. (B) Correlation matrix of all the measured parameters in which white circles represent positive correlation, black circles represent negative correlation, and the size of the circle represents the magnitude of the $R^2$ value. $B_{12}$ use efficiency, carrying capacity, EC$_{50}$ for $B_{12}$, and max growth rate were calculated using all timepoints from the $B_{12}$ dose response experiment (Figure 5A). Median survival time was calculated using all measurements of viability during $B_{12}$ deprivation (Fig. 5C). Algae:Bacteria ratio was taken from the final day (day 9) of coculturing the strains with the bacterium *M. loti* (Fig. 5E). All other variables [$B_{12}$ uptake ($B_{12}$ absorbed in one h per unit OD), max PSII efficiency ($F_v/F_m$), CFU density (Colony forming units/mL), OD 730nm (Optical density of cultures at 730nm), cell density (Total cells/ml), NPQ (Non-photochemical quenching), ROS (Reactive oxygen species per unit OD), $F_q/F_v$ (coefficient of photochemical quenching), protein (Protein concentration per unit OD), and lipid (Lipid concentration per unit OD)] were the averages of all available measurements taken during $B_{12}$ deprivation.

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**Figure 1.** C1 cycle metabolites and transcripts increase during B12 deprivation of metE7. (A) Metabolic map of a portion of the C1 cycle centred around METE and METH, with enzyme abbreviations in black, metabolite abbreviations in grey, and arrows depicting enzyme-catalysed reactions. (B) Abundance of six transcripts for enzymes of the C1 cycle measured by RT-qPCR on RNA extracted from the ancestral line and metE7 after 30 hours of incubation in mixotrophic conditions with (1000 ng/l) or without B12. (C) Abundance of Met, SAM, and SAH metabolites measured by HPLC-MS on the same samples as above. Metabolite and transcript abundances are expressed as levels in B12-deprived conditions relative to B12-replete conditions and presented on a log2 scale. Error bars = sd, n=3-4, ‘ns’=not significant, *=p<0.05, **=p<0.01, ***=p<0.001, Welch’s t test. WT = ancestral B12-independent strain, metE7 = experimentally evolved B12-dependent line. See also figure S2.
Figure 2. B₁₂ deprivation of metE7 causes cell enlargement and significant changes in macromolecular composition. (A) Microscope photographs taken at 1000x magnification of metE7 cells grown in TAP medium in B₁₂ replete (1000 ng l⁻¹) or B₁₂ deprived (0 ng l⁻¹) conditions over a period of 4 days (B) Macromolecular composition of B₁₂-deprived cells on day 2 and day 4 of the growth period expressed as mass of those compounds normalised to total cell dry mass and then expressed relative to the amounts in B₁₂ replete conditions. Error bars = sd, n = 5. **=p<0.01, ***=p<0.001, Welch’s t test.
Figure 3. *metE7* survives better and produces lower levels of reactive oxygen species (ROS) when limited for both N and B12 than just B12 alone. (A) Percentage of cells that could form colonies (a measure of viability) on nutrient replete agar when removed at different timepoints from nutrient deprivation conditions (Indicated in panels above the graphs). (B) Reactive oxygen species (ROS) measured by dichlorofluorescein diacetate (DCFDA) fluorescence and normalised both on a per cell basis and to the nutrient replete treatment (+B12+N). Error bars = sd, n = 3-6.
Figure 4. *L. rostrata* grows better than *metE7* in coculture with a B₁₂-producing bacterium, in part due to its lower demand for B₁₂. (A) Cultures were grown mixotrophically (TAP medium in continuous light), B₁₂ concentrations ranged from 0 to 200 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·mл⁻¹. Culture density was measured as optical density at 730 nm after 5 days of growth for the *C. reinhardtii* strains and 9 days for *L. rostrata*. (B) Cultures were grown photoautotrophically (Tris minimal media in 16h:8h light:dark cycles) in axenic culture (with 100 ng·l⁻¹ B₁₂) or coculture (with the B₁₂-producing bacterium *M. loti*) over a period of 16 days with measurements of cell density every 1-2 days. For both panel A and B, black = *L. rostrata*, grey = *metE7*, error bars = sd, n=4.
Figure 5. Experimental coevolution of metE7 with the bacterium M. loti selects for improved algal growth in coculture, increased B₁₂ use efficiency and better resilience to B₁₂ deprivation. (A) Maximum optical density achieved by mixotrophically-grown cultures of experimentally evolved lines of metE7 grown over a period of 12 days in six different concentrations of B₁₂. (B) B₁₂ use efficiency of evolved lines calculated using a fitted Monod equation and expressed as the maximum rate of increase in OD₇₅₀ that would result from an increase in B₁₂ concentration. (C) Viability (measured as the percentage of cells capable of forming colonies on B₁₂-replete agar) of mixotrophically-grown cultures of experimentally evolved lines of metE7 over a 5-day period cultured in 40 ng·L⁻¹ B₁₂. (D) Median survival time of evolved lines after dilution of culture to 40 ng·L⁻¹ B₁₂, calculated using a fitted Verhulst equation. (E) Algal cell density of photoautotrophically-grown cocultures of experimentally evolved lines of metE7 with M. loti over a 9-day period. (F) Ratio of algae to bacteria on the final day (day 9) of growth in coculture. metE7H = metE7 evolved in TAP + 1000 ng·L⁻¹ B₁₂, 10 months, metE7L = metE7 evolved in TAP media + 25 ng·L⁻¹ B₁₂, metE7C = metE7 evolved in Tris minimal medium in coculture with the B₁₂-producing bacterium M. loti. Error bars = 95% confidence interval, n = 7-8, letters above error bars indicate statistical groupings provided by Tukey’s test, which was performed following a significant ANOVA result.
Figure 6. Analysis of a range of measurements made or parameters calculated from the metE7 progenitor strain and its experimental evolution descendants. (A) Plot of the first two principal components derived from PCA applied to these measured variables and calculated parameters. Each point represents an evolved line or replicate of the progenitor strain and is given a colour and shape according to the key in the top left corner. Ellipses are created to show the standard deviation of the eight lines belonging to these experimental evolution groups. (B) Correlation matrix of all the measured parameters in which white circles represent positive correlation, black circles represent negative correlation, and the size of the circle represents the magnitude of the R² value. B₁₂ use efficiency, Carrying capacity, EC₅₀ for B₁₂, and Max growth rate were calculated using all timepoints from the B₁₂ dose response experiment (Figure 5A). Median survival time was calculated using all measurements of viability during B₁₂ deprivation (Fig 5C). Algae:Bacteria ratio was taken from the final day (day 9) of coculturing the strains with the bacterium M. lotti (Fig 5E). All other variables (B₁₂ uptake (B₁₂ absorbed in one hour per unit OD), Max PSII efficiency (Fv/Fm), CFU density (Colon forming units/mL), OD 730nm (Optical density of cultures at 730nm), Cell density (Total cells/mL), NPQ (Non-photochemical quenching), ROS (Reactive oxygen species per unit OD), Fq/Fv (coefficient of photochemical quenching), Protein (Protein concentration per unit OD), Lipid (Lipid concentration per unit OD)) were the averages of all available measurements taken during B₁₂ deprivation.
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