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ORIGINAL ARTICLE

Fundamental shift in vitamin B_{12} eco-physiology of a model alga demonstrated by experimental evolution

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A widespread and complex distribution of vitamin requirements exists over the entire tree of life, with many species having evolved vitamin dependence, both within and between different lineages. Vitamin availability has been proposed to drive selection for vitamin dependence, in a process that links an organism's metabolism to the environment, but this has never been demonstrated directly. Moreover, understanding the physiological processes and evolutionary dynamics that influence metabolic demand for these important micronutrients has significant implications in terms of nutrient acquisition and, in microbial organisms, can affect community composition and metabolic exchange between coexisting species. Here we investigate the origins of vitamin dependence, using an experimental evolution approach with the vitamin B_{12}-independent model green alga *Chlamydomonas reinhardtii*. In fewer than 500 generations of growth in the presence of vitamin B_{12}, we observe the evolution of a B_{12}-dependent clone that rapidly displaces its ancestor. Genetic characterization of this line reveals a type-II Gulliver-related transposable element integrated into the B_{12}-independent methionine synthase gene (*METE*), knocking out gene function and fundamentally altering the physiology of the alga.

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Introduction

All organisms must balance the cost of maintaining metabolic independence with the risk of restricting their niche by depending on environmental sources of enzyme cofactors. These cofactors perform essential metabolic functions and, when supplied externally, are known as vitamins. Animals obtain vitamins from their diet and are thus described as vitamin auxotrophs. Some organisms avoid the need for external sources of vitamins, because they synthesize the cofactors themselves. However, vitamin biosynthesis can be metabolically expensive, and as these compounds are required in only trace quantities, outsourcing production could be selected for if an exogenous vitamin supply is available. The loss of vitamin synthesis has happened frequently in both prokaryotes and eukaryotes (Helliwell *et al.*, 2013), suggesting that the conditions for evolutionary shifts in vitamin requirements commonly occur in space and time. One well-known example of this is vitamin C auxotrophy, which arose independently in primates, guinea pigs, teleost fish and certain bat species as the result of loss of the final enzyme in the biosynthetic pathway, L-gulonolactone oxidase (Nishikimi *et al.*, 1994; Drouin *et al.*, 2011). As the lineages that can no longer synthesize this vitamin have a vitamin C-rich diet, it has been hypothesized that diet may have led to the evolution of this trait (Drouin *et al.*, 2011).

Vitamin dependence is not, however, confined to animal taxa (Helliwell *et al.*, 2013). For instance, the requirement for biotin (vitamin B_7) varies between strains of the yeast *Saccharomyces cerevisiae.* Genomic evidence has revealed a partial pathway for biosynthesis of this vitamin in the strain *S. cerevisiae* S288c, suggesting that the ability to synthesize this cofactor has been lost recently (Hall and Dietrich, 2007). Among algae—taxonomically diverse photosynthetic eukaryotes—vitamin auxotrophy is also a highly variable trait. Over 50% of species surveyed require vitamin B_{12} (cobalamin), approximately 21% B_1 (thiamine), and 5% B_7 (biotin) (Croft *et al.*, 2006), and the distribution of requirement does not follow phylogenetic lines. Unlike other B vitamins, vitamin B_{12} is synthesized

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only by prokaryotes (Warren *et al*., 2002). In aquatic ecosystems, ambient concentrations of B_{12} are extremely low (Sañudo-Wilhelmy *et al*., 2012), and it has been proposed that the availability of this factor may exert significant constraints on the distribution, taxonomic composition and primary productivity of algal communities (Gobler *et al*., 2007; Sañudo-Wilhelmy *et al*., 2012; Bertrand *et al*., 2012a). However, the prevalence of algal vitamin B_{12} requirers in nature implies that there is a readily available/common niche for auxotrophic algae to occupy. Current understanding suggests that B_{12} requirers may obtain a source of vitamin B_{12} through: (i) direct interactions with heterotrophic bacteria (Croft *et al*., 2005; Wagner-Döbler *et al*., 2010; Kazamia *et al*, 2012) and/or (ii) uptake from the dissolved vitamin pool, in patches of elevated microbial activity—that is, non-specific interactions with prokaryote producers (Karl, 2002; Azam and Malfatti, 2007). Based on genome analyses, prokaryotic taxa implicated in cobalamin synthesis include members of the Alphaproteobacteria, Gammaproteobacteria, Cyanobacteria and Bacteroidetes (Sañudo-Wilhelmy *et al*., 2014). A more recent study also revealed a globally significant role for the Archaea (*Thaumarchaeota*) in vitamin B_{12} production in aquatic ecosystems (Doxey *et al*., 2015).

Insights into the molecular basis underlying the vitamin requirements of algae have also been gained using available genome sequences. Unlike for other vitamins, where possession of the biosynthetic pathway means an organism does not require an external supply of the compound, vitamin B_{12} independence is conferred by the presence of an enzyme that does not need a cobalamin cofactor (Croft *et al*., 2005, 2006). Three B_{12}-requiring enzymes are known in eukaryotes: (i) methylmalonyl-CoA mutase, used for odd chain-fatty-acid metabolism, (ii) type II ribonucleotide reductase involved in deoxyribose biosynthesis, and (iii) methionine synthase (METH), which catalyses the biosynthesis of methionine (Marsh, 1999). A B_{12}-independent form of methionine synthase (METE) is found in land plants and fungi, and therefore these organisms do not require vitamin B_{12}. A survey of algal genomes showed that algal B_{12} independence correlates with the presence of a functional copy of *METE* (Croft *et al*., 2005; Helliwell *et al*., 2011; Bertrand and Allen 2012b). The model green alga *Chlamydomonas reinhardtii* does not require vitamin B_{12} and possesses both isoforms of methionine synthase, whereas *METE* has been lost in other closely related B_{12}-dependent species (Helliwell *et al*., 2011).

Determining and testing the selective pressures contributing to the evolution of vitamin dependence is a key component in understanding the evolution of a species niche and its biotic interactions with co-occurring species. Although comparative analyses can show which environmental conditions correlate with the evolution of vitamin dependencies, only experimentation can test definitively whether particular drivers, such as a shift in diet/environment, are sufficient to cause such major metabolic changes. A reliable and abundant external source of B_{12} may lead to the deterioration of *METE* through relaxed selection (Helliwell *et al*., 2011), whereby the negative regulatory effect of B_{12} on *METE* expression could facilitate this process (Helliwell *et al*., 2013, 2014). Here we adopt an experimental evolution approach using the fast growing alga *C. reinhardtii* to study the processes shaping the metabolic demand for vitamin B_{12}. We focus on identifying the genetic changes involved, as previous work has suggested that the presence/absence of a single gene *METE* is a sufficient predictor of B_{12} auxotrophy in algae (Croft *et al*., 2005; Helliwell *et al*., 2011). Linking environmental conditions to evolutionary changes in basic metabolism in phytoplankton is vital to understand better ecosystem function and biogeochemical cycling in dynamic aquatic environments.

Materials and methods

Selection experiment

Selection was carried out in 24-well plates containing 2 ml of TAP medium (Gorman and Levine, 1965) at 25 °C in continuous light ($20\,\mu mol\,m^{-2}\,sec^{-1}$) with shaking (140 r.p.m.). Forty-six independent populations were founded from a single colony of the ancestral line (AL) *C. reinhardtii* strain 12, derived from wild-type strain 137c. Cells were transferred every Monday, Wednesday and Friday, with growth periods approximately 51, 53 and 64 h, respectively. Optical density (OD_{730}) was measured after every transfer, which determined the subsequent transfer volume to obtain ~8000 cells per inoculum. As such, cells never exceeded a cell density of $\sim 3 \times 10^6$ cells ml^{-1}. Stock-points were taken after 13, 25, 40, 50, 60 and 70 transfers and maintained on 2% TAP agar in 24-well plates in the dark.

Pure culture growth rates

Pure culture growth assays were measured in 24-well plates in the presence of vitamin B_{12} ($1000\,ng\,l^{-1}$) in the same growth chamber and conditions as used for the selection experiment (described above). Ten independent S-type (B_{12}-dependent), H-type (B_{12}-independent) and R-type (B_{12}-independent, derived from S-type clones following loss of the transposon from *METE*) clones from population $E8^+$ at transfer T70, alongside 10 AL clones were isolated from single colonies grown on 2% TAP agar and allowed to recover for 3–6 days. Prior to the growth assay, cultures were acclimated to the growth assay conditions (with $1000\,ng\,l^{-1}$ B_{12} supplementation) for 4 days and then diluted to a cell density of 4000 cells ml^{-1} (that is, an 8000-cell

inoculum). The number of cells ml^{-1} was subsequently measured every 12 h over a 96-h time period using the Duel Threshold Beckman Coulter (Z2) Particle Counter and Size Analyzer (Brea, CA, USA) with a 70-μm diameter aperture, counting between 3 μm (Tl) and 9 μm (Tu). Values given are means of 10 independent replicates.

Molecular methods
DNA/RNA were extracted, and PCR/reverse transcriptase-PCR experiments were performed as described by Helliwell *et al.* (2011). Primers used are listed in Supplementary Table S1.

Southern blotting
Extracted DNA, 1.5 μg from each sample, was digested with *Nae*I and *Bam*HI (NEB, Hichin, UK), separated by agarose-gel electrophoresis and then transferred to Hybond-N+ (GE-Healthcare, Chalfont St Giles, UK) membranes. A 339-bp probe (Supplementary Table S1) was amplified using PCR and labelled with [α-^{32} P]dCTP using Ready-to-Go DNA-labelling beads (GE-Healthcare). The blots were prehybridized overnight at 65 °C in Church buffer (Church and Gilbert, 1984). The probe was denatured by heating at 100° C for 10 min and added to the hybridization tubes. Hybridization was carried out overnight at 65 °C. Filters were washed at 65 °C in increasingly stringent buffers (2 × sodium chloride/sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) to 0.2 × SSC, 0.1% SDS) until counts were ~1000 c.p.m.

Western blotting
Total protein was extracted, and western blotting experiments were performed as described by Helliwell *et al.* (2014). To verify adequate transfer and equal loading, the membrane was stained in Ponceau stain (0.2% (*w*/*v*) Ponceau-S, 3% (*w*/*v*) TCA) (Romero-Calvo *et al.*, 2010).

Results

Rapid evolution of a vitamin B_{12}-dependent line of C. reinhardtii
To investigate whether an exogenous supply of vitamin B_{12} could lead to auxotrophy, we established an evolution experiment where 46 independent populations of *C. reinhardtii*, were founded from a single clone (the AL). Half the populations were grown without B_{12} on TAP medium (in Materials and methods section) and the other half with 1000 ng l^{-1} vitamin B_{12}, an amount that exceeds the growth requirements of B_{12}-requiring algae (Croft *et al.*, 2005). The populations were subcultured into fresh medium at regular intervals, with the maximum cell density reaching ~3×10^6 cells ml^{-1}. Populations were scored for B_{12} dependence every 10 transfers (T). At T60 (~600 generations), one of the populations supplemented with B_{12} (evolved line, E8$^+$) had impaired ability to grow without the vitamin. When E8$^+$ cells were plated on solid media so that colonies could grow from single cells, in the absence of B_{12} two colony morphologies were evident: healthy (H-type) normal-sized colonies, and smaller (S-type) colonies impaired in growth (Figure 1a); on B_{12}-containing medium all colonies appeared normal-sized. Growth assays in liquid culture revealed that cells isolated from H-type colonies were vitamin B_{12} independent, while S-type cells were dependent on the vitamin for growth in liquid culture during a 72-h cultivation window (Figure 1b). We found no evidence of S-type cells in any of the other replicate populations, when cells were plated out on TAP media in the absence of B_{12}.

Selective sweep of the novel B_{12}-dependent clone
Stocks of independent populations were collected throughout the experiment at T13, 25, 40, 50, 60 and 70, and stored on solid medium. To identify the point at which the S-type cells arose, we grew each stock-point for the E8$^+$ population in liquid medium with or without B_{12}. Growth in the presence of B_{12} was comparable between stocks (Figures 1c and d). In contrast, on medium without the vitamin, the B_{12}-dependent phenotype was more pronounced in the E8$^+$ population with increasing transfers (Figures 1c and d). Plate assays to quantify the percentage of cells giving rise to S-type colonies on medium without B_{12} showed S-type cells increased in frequency within the population from 1.6% to 99.7% over 30 transfers (T40–T70) (Figure 1e). To define the level of B_{12} sufficient to produce this response, a 'replay' experiment was conducted. We returned to stock-point T50 (where S-type cells comprised <30% of the population) and repeated the selective regime, at a range of concentrations of B_{12}. After 10 transfers with ⩾200 ng l^{-1} (0.2 μM), the B_{12}-dependent cells rose in frequency within the population (Figure 1f). Indeed, a B_{12}-dose response confirms that S-type cells can grow unimpaired at this concentration (Supplementary Figure S1).

A transposition event underlies the B_{12}-dependent phenotype
To characterize the genetic cause of the novel B_{12}-dependent phenotype, we conducted a PCR-based analysis of the *METE* gene in S- and H-type clones. This approach revealed a size polymorphism between the different clone types, in the region corresponding to the ninth exon of the gene (Figure 2a). Sequencing and BLAST analysis revealed that a 238-bp class-II ('cut-and-paste') *Gulliver*-related transposable element (GR-TE) had integrated into *METE* in S-type cells (Figure 2a, Supplementary Figures S2a and b). GR-TEs have

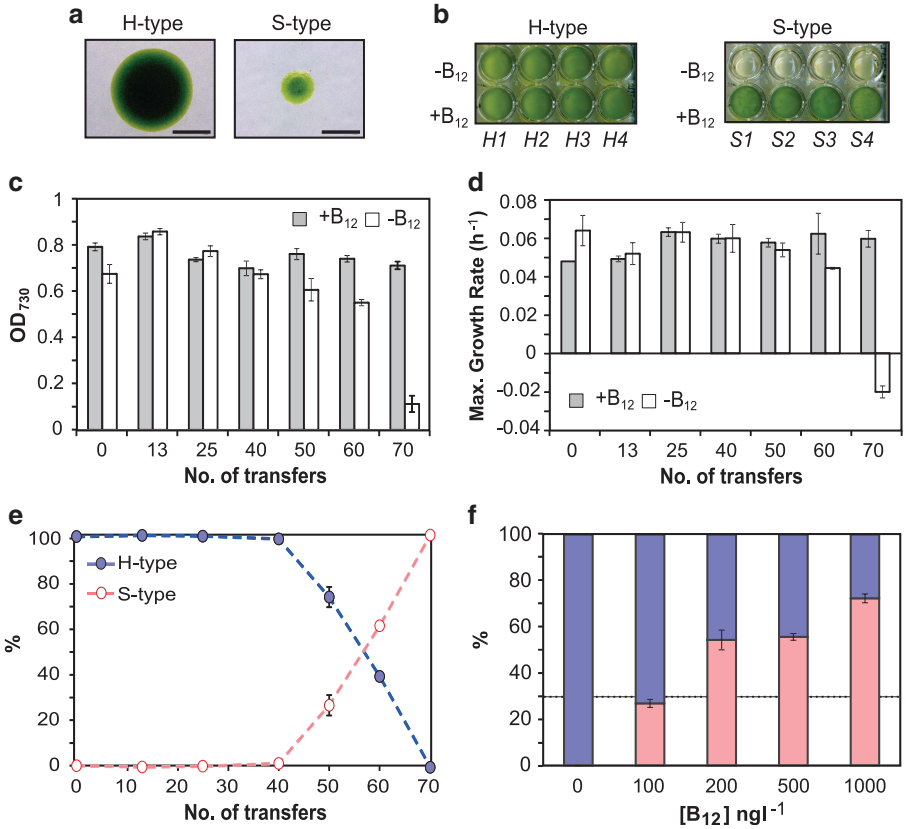

Figure 1 The evolution of vitamin B_{12} dependence in *C. reinhardtii*. (**a**) $E8^+$ cells plated onto solid medium $-B_{12}$ give rise to two colony morphologies: healthy (H-type) colonies, and smaller (S-type) colonies (as visualized under a dissecting microscope), scale bar: 1 mm. (**b**) Growth of four independent H- and S-type colonies $+B_{12}$ (1000 ng l^{-1}) and $-B_{12}$ after 72 h (mean ± s.e.m.) $n=3$. Mean optical density $(OD)_{730}$ values for H- and S-type clones at this time point were: 0.78 ± 0.08 s.e.m. ($+B_{12}$) and 0.78 ± 0.04 ($-B_{12}$) and 0.64 ± 0.009 ($+B_{12}$) and 0.04 ± 0.02 ($-B_{12}$), respectively. (**c**) OD_{730} of stock-points cultures on liquid medium with (1000 ng l^{-1}; grey) and without B_{12} after 72 h (mean ± s.e.m.) $n=3$ and (**d**) maximal growth rate, μ (h^{-1}) of stock-points cultures on liquid medium with (1000 ng l^{-1}; grey) and without B_{12} as calculated from panel (**c**) (mean ± s.e.m.) $n=3$. (**e**) Percentage of S- vs H-type colonies within the population at independent stock-points (mean ± s.e.m.) $n=3$. (**f**) Percentage of S- (red) and H-type colonies (blue) after replaying selection from T50 (where S-type cells represent <30% of the population, broken black line) for 10 transfers at different concentrations of B_{12} (mean ± s.e.m.) $n=3$.

been described previously in *C. reinhardtii* (Kim *et al.*, 2005, 2006). Such elements belong to a family of >200 small, non-autonomous TEs and feature characteristic 15-bp imperfect terminal-inverted repeats that are also found in a larger transposon (~12 kb) known as *Gulliver*, which is thought to activate mobilization of the GR-TE elements (Ferris, 1989; Kim *et al.*, 2006). The transposition event described here causes an 8-bp duplication of the target-site in the gene (Figure 2a), characteristic of *Gulliver* elements (Ferris, 1989). Insertion of the GR-TE was into a highly conserved region of the protein and resulted in an in-frame stop codon that would be likely to cause premature termination of translation (Gonzalez *et al.*, 1992; Pejchal and Ludwig, 2005) (Supplementary Figure S3). Indeed, western blotting analysis using a polyclonal antibody against *C. reinhardtii* METE (Schneider *et al.*, 2008) detected a band of 86.5 kDa in AL cells but no cross-reacting polypeptide in an S-type clone from the $E8^+$ population (Figure 2b). Nonetheless, the *METE* transcript remained expressed (at 0 and 20 ng l^{-1} B_{12}), and repressed by B_{12} (1000 ng l^{-1}), as is characteristic for wild-type *C. reinhardtii METE* (Figure 2c; Croft *et al.*, 2005; Helliwell *et al.*, 2011, 2014).

A Southern blotting analysis of genomic DNA prepared from each of the stock-points was carried out, using a probe (338 bp) to an internal region of *METE* (Figure 2d). This probe hybridized to a band of the expected size (1430 bp) in the AL and in all but the last stock-points (Figure 2e). However, a second, larger band appears at T50 (~500 generations), which corresponds to the B_{12}-dependent phenotype, likely to be the arrival of the TE. Both the large and small *METE* bands are evident between T50 and T60, until T70, where only the large band is detectable. We interpret these data to confirm that a B_{12}-dependent phenotype of *C. reinhardtii* arose between T40 and T50, through transposition of a GR-TE into *METE*, correlating with growth experiments (Figures 1d and e). These B_{12}-dependent cells remained in co-culture with their B_{12}-independent predecessors for a further 20 transfers (<200

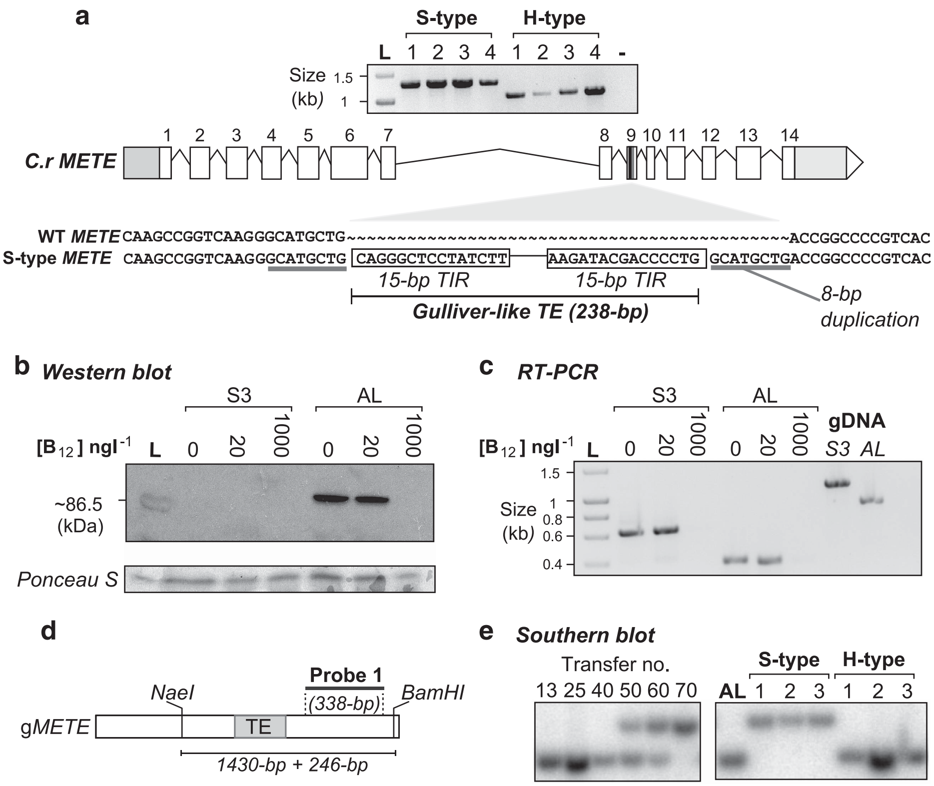

Figure 2 Identification of a *Gulliver*-related transposable element (GR-TE) in the *METE* gene of $E8^+$ S-type cells. (**a**) PCR on genomic DNA of four independent S- and H-type clones using primer pair F2b/R3b (amplifying a 1-kb region between 4.4 kb and 5.4 kb from the start codon) reveals an unexpectedly large product for S-type clones (expected product size for wild-type (WT) *METE*: 1003 bp). A BLAST search using the sequence from the S-type product revealed a strong (E-value: $8e^{-67}$) hit for *C. reinhardtii METE* (Supplementary Figure S2a). Another hit (E-value: $2e^{-87}$) 238 bp in size was identified as a class-II GR-TE (Kim *et al*., 2005, 2006; Supplementary Figure S2b). The schematic diagram shows an alignment between *C. reinhardtii* WT *METE* in this region compared with the 'S-type' product sequence. A target-site duplication of *METE* (grey underline) flanks a 15-bp terminal-inverted-repeat (boxed). (**b**) Western blotting analysis on total protein of $E8^+$ and AL cells using a polyclonal antibody against *C. reinhardtii* METE ($\sim$86.5 kDa; Schneider *et al*., 2008; L: Ladder). To verify adequate transfer and equal loading, the membrane was stained in Ponceau stain (Ponceau S) (**c**) Reverse transcriptase-PCR reveals that *METE* is expressed and regulated by B_{12} in $E8^+$. Expected products using primers Transcript_F1/R1: AL gDNA: 902 bp (+246 bp with TE + 8-bp *METE* repeat, that is, 1148 bp), cDNA: 371 bp (+246 bp, that is, 617 bp). (**d**) Schematic diagram of probe used for Southern blotting. (**e**) Southern blotting analysis using the *METE* probe (probe 1) on genomic samples for stock-points and independent S- and H-type clones.

generations), until eventually the B_{12}-dependent clones dominated the population (T70, <700 generations). Samples prepared from individual S- and H-type clones (Figure 1b) show only the larger and smaller products, respectively (Figure 2e).

Phenotypic plasticity in response to exogenous levels of vitamin B_{12}

Reversion of mutant phenotypes by transposon excision is well documented, especially in conditions of physiological stress (McClintock, 1948; Maumus *et al*., 2009). We sought to investigate the occurrence of reversion in the evolved $E8^+$ S-type cells in B_{12}-deplete conditions. Eight days after plating on solid medium, S-type colonies were seen on plates lacking B_{12} (Supplementary Figure S4). However, after a further 3 days, darker bodies of cells appeared within the S-type colonies on the plates without B_{12} (Figure 3a; Supplementary Figure S4). As they grew after colonies on the control plate with B_{12} were already visible, we reasoned that they were likely to be revertants. Sequencing revealed complete excision of the transposon from the *METE* gene in such cells. We also screened 11 S-type colonies that showed no evidence of phenotypic reversion after 15 days on B_{12}-deplete medium (Supplementary Figure S4). All 11 clones were confirmed to be vitamin B_{12} dependent, and using PCR with primers spanning the GR-TE the majority were also shown to have GR-TE (Figure 3b). However, one B_{12}-dependent clone (clone no. 7) generated a PCR product with the size expected for wild-type *METE* (Figure 3b). Sequencing revealed that the GR-TE was absent except for a 9-bp footprint sequence (CACCATGCT), the latter 6 bp of which is a remnant of the *METE* repeat (Figure 2a; Figure 3c). This in-frame insertion leads to inclusion of three extra amino acids in a conserved region of the *METE* gene (Supplementary Figure S3) resulting in a stable vitamin B_{12}-dependent mutant.

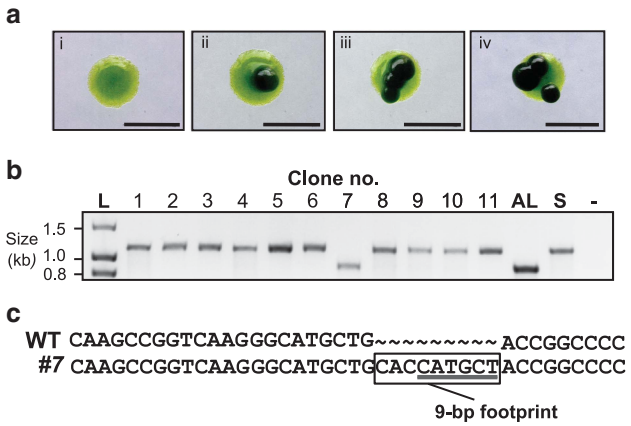

Figure 3 Characterization of mutant phenotype revertants and isolation of a stable *METE* insertion mutant (**a**) A non-reverting colony (i) alongside three independent revertant colonies (ii–iv) visualized under a dissecting microscope, after 11 days on solid medium $-B_{12}$. (**b**) PCR screen for the presence of GR-TE insertion in *METE* gene of clones using primers spanning GR-TE insertion site (METE_revert F1/R1). Clone no. 7 is vitamin B_{12} dependent yet lacks the GR-TE (expected product sizes: wild-type *METE*− 913 bp, and *METE* with GR-TE insertion 913 bp + 246 bp = 1159 bp). Sequencing revealed a 9-bp footprint (CAC-CATGCT) in this clone (**c**) the latter 6 bp of which (underlined grey) is a remnant of the *METE* repeat.

Comparison of growth rates of S, H, R and AL clones in pure culture

The selective sweep, which we observed in several independent experiments (including different B_{12} treatments), suggests that S-type cells have a growth advantage compared with their B_{12}-independent counterparts in B_{12}-replete conditions. Theoretical calculations (Table 1) illustrate that only a very minor increase in specific growth rate (~4%) is required to cause the rise from 30% to 71% on hypothetical 'strain B' within 10 transfers, similar to the population shifts we observe over this timescale with S-type cells in the replay experiment (Figure 1f). To investigate whether a growth advantage is detectable, a growth assay with pure cultures of 10 independently isolated S-type, H-type and AL clones was carried out. We also included within this analysis 10 independent R-type clones (that is, revertants derived from 10 different S-type colonies and thus representing independent reversion events) to investigate the link between fitness and *METE* presence/absence. We detected a ~9% higher maximal growth rate (h^{-1}) of S-type compared with H-type clones (Figures 4a and b); however, the difference was not statistically different using a Student's *t* test with a *P*-value of $\leqslant 0.05$. We did, however, observe a statistical difference in growth rate between S- and R-type cells (two-tailed Student's *t*-test $P \leqslant 0.05$, $n = 10$). The mean growth rates for H- and R-type cells were virtually identical (0.146 ± 0.008 s.e.m. and 0.144 ± 0.006). Moreover, all evolved lines (S-, H- and R-type) exhibited a faster maximal growth rate under the selective regime compared with the AL ($P \leqslant 0.001$, $n = 10$).

Table 1 Theoretical calculation of population shifts between two algal strains in co-culture after 24 days (10 transfers), assuming initial populations of 70% A: 30% B

Strain A μ (h^{-1})	*Strain B* μ (h^{-1})	*Strain B divisions per day*	*Strain B % population (24 days)*
0.075	0.075	2.60	30
0.075	0.076	2.63	44
0.075	0.077	2.67	58
0.075	0.078	2.70	71

The calculations assume a constant specific growth rate (μ) of $0.075\,h^{-1}$ in Strain A and are designed to mimic the conditions of the 'selective sweep' experiment described in Figure 1f. The data demonstrate that only a minimal increase in specific growth rate in Strain B is required to observe a dramatic shift in the proportions of the respective populations over 24 days.

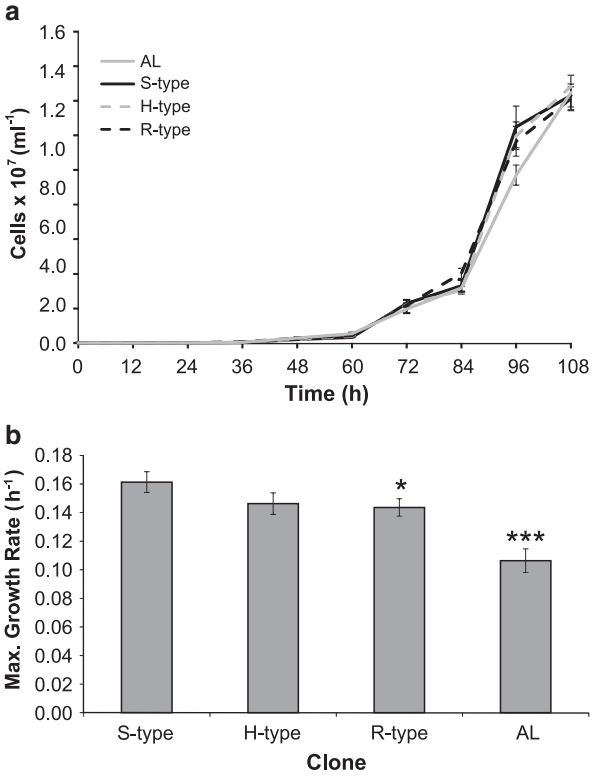

Figure 4 Characterization of growth of S-type, H-type, R-type and AL cells (**a**). Growth over time of S-type, H-type, R-type and AL clones in the presence of vitamin B_{12} ($1000\,ng\,l^{-1}$) (mean ± s.e.m.) $n = 10$. (**b**) Mean maximal growth rate, μ (h^{-1}) of S-type, H-type, R-type and AL clones as calculated from panel (a). $^*P \leqslant 0.05$, $^{**}P \leqslant 0.001$ compared with the S-type clones (two-tailed Student's *t*-test) (mean ± s.e.m.) $n = 10$.

Vitamin B_{12}-dependent growth is rescued by B_{12}-synthesizing bacteria

Vitamin B_{12} biosynthesis is confined to prokaryotes (Croft *et al.*, 2005). The irreversible loss of *METE*, therefore, not only forces the evolution of vitamin auxotrophy but also an absolute dependency on a bacterial supply of the vitamin. Algal acquisition of vitamin B_{12} through direct mutualism with bacteria

has been demonstrated previously by our laboratory (Kazamia *et al*., 2012), in which *Lobomonas rostrata*, a known B_{12} auxotroph, and a bacterial partner, *Mesorhizobium loti*, can grow stably for an indefinite period in co-culture in the absence of vitamin B_{12} or fixed carbon. This system has also been described mathematically (Grant *et al*., 2014). To test whether a similar exchange is able to support the growth of the newly evolved line, we set up co-cultures of the non-stable S-type line with one of the three B_{12}-synthesizing rhizobial species of bacteria (*M. loti* (strain MAFF 303099) *Rhizobium leguminosarum* (RL3841) and *Sinorhizobium meliloti* (RM 1021)) in TAP medium lacking B_{12}. Using chlorophyll concentration as a proxy for algal growth, we found that for the first 5 days there was no growth of the alga, except when exogenous B_{12} was present in the medium (Figure 5a). However, after 5 days all inocula grew well, even the control with no B_{12}/bacterial supplementation. We interpreted that this was a result of B_{12}-independent revertants rising to dominance within the population. Using PCR with primers spanning the GR-TE, a larger product in the $+B_{12}$ treatment was identified (Figure 5b) indicating the presence of the GR-TE in the *METE* gene. However, for the $-B_{12}$, and *M. loti* treatments the product was smaller, confirming excision of the transposon in these cultures. Interestingly, in the other co-cultures two products were amplified, revealing a mixed population of revertant and non-revertant clones (Figure 5b). The proportion of the two bands varied depending on which bacterial species was present, suggesting that different bacteria can support the alga to different levels and thus may dictate the frequency of B_{12}-dependent vs independent algal clones within a population. We repeated this experiment with the stable B_{12}-requiring clone no. 7. All three bacteria were able to support the mutant in the absence of B_{12}, with no growth observed in the $-B_{12}$ treatment (Figure 5c). Moreover, the algal–bacterial co-culture reached a lower carrying capacity compared with the $+B_{12}$ treatment indicating a degree of regulation, as seen with the *L. rostrata*/*M. loti* co-culture (Kazamia *et al*., 2012; Grant *et al*., 2014). A similar result was observed in medium lacking an organic carbon source, so bacterial growth is in turn dependent on algal photosynthate (Supplementary Figure S5).

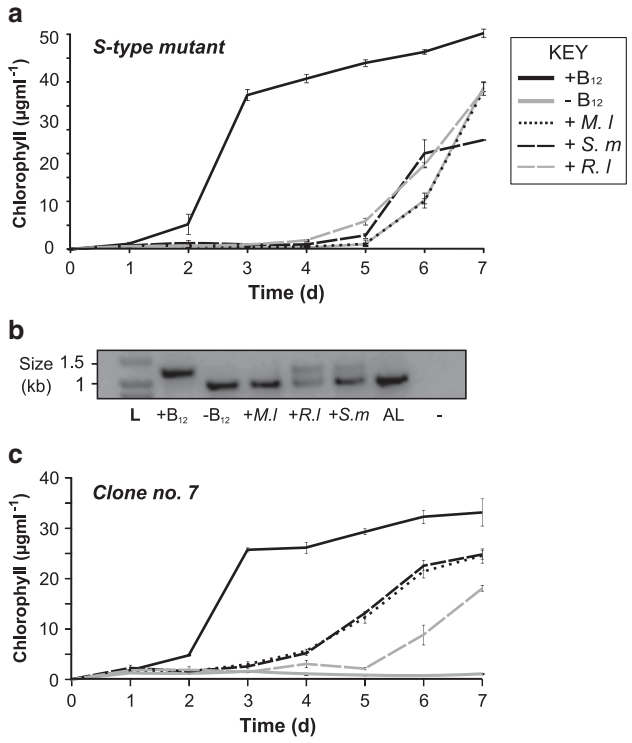

Figure 5 Vitamin B_{12} dependence is rescued by three B_{12}-synthesizing rhizobial species of bacteria. (**a**) Growth of S-type mutant (unstable) in different B_{12} regimes, including: (i) $+B_{12}$ ($1000\,ng\,l^{-1}$), (ii) $-B_{12}$, (iii) *Mesorhizobium loti*, (iv) *Sinorhizobium meliloti* and (v) *Rhizobium leguminosarum*. The latter three treatments were grown in the absence of B_{12} in TAP medium (mean ± s.e.m.) $n = 3$. (**b**) PCR with *METE* primers spanning the GR-TE from DNA extracted from the different conditions at day 7. (**c**) Growth of stable-*METE*-insertion mutant clone no. 7 in B_{12} regimes described in panel (a). This experiment was carried out in TAP medium (mean ± s.e.m.) $n = 3$.

Discussion

The evolution of vitamin dependence has been a recurrent event across the tree of life, with important implications for the basic physiology and ecology of all organisms. The processes underlying how species become dependent on an external source of these organic micronutrients are inherently difficult to test empirically. In this study, we explored directly whether a key factor hypothesized to drive the evolution of vitamin auxotrophy was able to do so. By adopting an experimental evolution approach, we found direct support for the hypothesis that an exogenous supply of vitamin B_{12} can lead to the evolution of B_{12} dependence (Figure 1). Additionally, we were able to define in detail the genetic mechanism (transposition), population dynamics (including phenotypic reversibility) and the environmental context in which this evolutionary event occurred. By establishing the genetic basis for the change in phenotype, we were able to pinpoint the precise timing of the change in genotype and characterize temporally the rise to dominance of the novel clone within the population.

Experimental evolution has been used widely as a powerful approach for understanding microbial evolution—exploiting the fast generation time and large population size of these organisms (Elena and Lenski, 2003). It allows fundamental evolutionary principles to be tested directly, and with greater rigour than alternative approaches, such as specific

genome manipulation. Moreover, this technique allows detection of subtle fitness differences that would otherwise be overlooked via standard growth assays (Collins, 2011). *C. reinhardtii* has the lowest spontaneous mutation rate described for any eukaryote (Ness *et al.*, 2012), and yet previous artificial selection experiments with *C. reinhardtii* have observed major evolutionary novelties (likely encompassing multiple gene alterations) such as loss of regulation in the carbon-concentrating mechanism (Collins and Bell, 2004) and evolution of a two-stage life cycle (Ratcliff *et al.*, 2013), after 1000 and 312 generations, respectively. Nonetheless, the underlying genetic components of these phenotypes were not determined, so the contributions of epigenetics, point mutations, transposition events and other genetic changes to adaptive phenotypes remain unknown. To our knowledge, this is the first study characterizing transposition in an experimentally evolved algal population. Indeed, although TEs have been studied extensively in animals, plants and fungi, little is known about their significance in algal evolution. Transposons have, however, been identified in the genomes of several algal species (Armbrust *et al.*, 2004; Bowler *et al.*, 2008; Cock *et al.*, 2010; Read *et al.* 2013), and nutrient stress (nitrate limitation) activated transposition has been observed in the marine diatom *Phaeodactylum tricornutum* (Maumus *et al.*, 2009), which has also been observed with our system. Moreover, differential insertion patterns among natural isolates of diatom species from different geographic locations have been observed (Maumus *et al.*, 2009). Together these findings suggest that TEs may have an important role in naturally evolving algal populations. An exciting area of future research will be to elucidate the impact of TEs on genome evolution of individual members of complex microbial communities, in particular understanding the frequency of transposition events and whether certain gene classes are more prone to disruption.

The fact that the *METE* gene loss in $E8^+$ that we observed was due to transposition (Figure 2) has further significance, as the re-excision of the transposon allows reversion to B_{12} independence in response to the absence of environmental B_{12} (Figure 3). This temporary 'get out of jail free card' could thus facilitate evolutionary escape from a B_{12}-dependent lifestyle before *METE* further deteriorates (Helliwell *et al.*, 2011). If similar processes happened in other algal lineages, this may explain the differences in B_{12} requirements observed between closely related strains by allowing for rapid and reversible evolution in environments where levels of B_{12} may fluctuate. The observed selective sweep of the novel evolved line $E8^+$ within the population shows that this clone has a selective advantage compared with its ancestor. However, we must consider the possibility that genetic changes other than that to the *METE* gene have contributed to this fitness advantage. Whole genome analyses will be important in the future to pinpoint whether/what other genome modifications may have occurred. Nonetheless, as multiple independent isolates exhibiting reversion of the *METE* transposition event have a reduced growth rate relative to S-type cells (Figure 4), the selective advantage appears to be associated specifically with the loss of *METE.*

Vitamin B_{12} auxotrophy is found in half of over 300 species surveyed (Croft *et al.*, 2005; Tang *et al.*, 2010), and evidence suggests that B_{12}-dependent metabolism is beneficial in certain scenarios, if B_{12} is readily available. For instance, METH has a catalytic efficiency 100 times greater (Gonzalez *et al.*, 1992) and exhibits enhanced thermal tolerance, in comparison to METE (Xie *et al.*, 2013). Moreover, theoretical calculations estimate that utilization of METH in *P. tricornutum* is more resource efficient than B_{12}-independent metabolism, as the use of METE was calculated to require 30 ± 9 times more nitrogen and 42 ± 5 times more zinc than METH (Bertrand *et al.* 2013). B_{12}-dependent growth that favours the use of METH could therefore offer an advantage when Zn/N are limited. However, as *METE* expression is repressed in the presence of B_{12} (Croft *et al.*, 2005; Helliwell *et al.*, 2011; Bertrand *et al.*, 2012a; Bertrand and Allen, 2012b; Bertrand *et al.*, 2013; Helliwell *et al.*, 2014) how fitness maybe conferred from inactivating a gene that is already switched off remains a conundrum. One possibility is that, in habitats where levels of vitamin B_{12} fluctuate, algae that have both forms of the enzyme may benefit from maintaining a low level of the METE protein, to facilitate rapid response to environmental fluctuations of B_{12} levels. Indeed, some METE transcript/protein can be detected under B_{12}-replete conditions (Xie *et al.*, 2013; Helliwell *et al.*, 2014). However, as the levels are so low, it is unclear whether complete loss of METE would confer a metabolic saving. It is possible that METE function, even at low protein abundance, may exert an as yet unidentified energetic cost beyond simply the composition of the protein.

Whatever the explanation for the observed selective advantage of the S-type line, this study validates the hypothesis that B_{12} availability in the environment can lead to the taxonomically variable presence and absence of *METE.* In this context, it is relevant to consider levels of B_{12} occurring in natural aquatic environments. Recent measurements have revealed vitamin B_{12} depletion in large areas of coastal ocean, and the vitamin is typically absent from the euphotic zone (Sañudo-Wilhelmy *et al.*, 2012). Moreover, levels of B_{12} are reportedly $<10\,\mathrm{ng\,l^{-1}}$ ($\sim 10\,\mathrm{pM}$) in some freshwater habitats (Kurata, 1986). However, as this molecule will likely be rapidly consumed as it becomes available within the water column, measurements of standing stock concentrations alone might not accurately reflect B_{12} availability. Moreover, vitamin levels will vary to

some extent on the microscale, with discrete vitamin patches arising due to localized microbial activity and/or the presence of particulate matter (Azam and Malfatti 2007; Stocker, 2012; Yawata *et al.*, 2014). Interestingly, a recent study found that microscale nutrient heterogeneity could drive ecological differentiation in nutrient-acquisition strategies in marine bacteria (Yawata *et al.*, 2014). This raises interesting eco-evolutionary considerations with regards to algal vitamin-acquisition strategies and *METE* presence/absence. A comprehensive comparison of the geographic distribution of vitamin B_{12} auxotrophs vs non-requirers in aquatic environments in relation to B_{12} levels has not yet been attempted. However, it is known that B_{12} auxotrophs such as the picoeukaryote *Ostreococcus tauri* are represented in environments, where ambient concentrations of vitamins are extremely low (Sañudo-Wilhelmy *et al.*, 2012). Evolutionary adaptations enabling B_{12} auxotrophs to be successful competitors in B_{12}-deprived regions could include becoming specialized at nutrient patch exploitation—being able to migrate rapidly to new nutrient sources upon a temporal change in the nutrient landscape for instance. Or alternatively, these organisms may meet their vitamin demands though the establishment and maintenance of direct symbiotic interactions with other microbes (Croft *et al.*, 2005; Wagner-Döbler *et al.*, 2010; Kazamia *et al.*, 2012). As algae in possession of both METE and METH may use B_{12} if it is available, loss of *METE* could be a plausible mechanism to cause sympatric populations to embark on different evolutionary trajectories, driving the evolution of symbiotic interactions and/or other specialist nutrient-acquisition strategies. A challenging question that remains to be answered is to what extent these different strategies are represented in the natural world.

Conflict of Interest

The authors declare no conflict of interest.

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