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

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**RUNNING TITLE:** Rapid evolution of vitamin $B_{12}$ dependence in *Chlamydomonas*

**Subject Category:** Evolutionary genetics

**ABSTRACT**

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 (GR-TE) integrated into the $B_{12}$-independent methionine synthase gene (*METE*), knocking out gene function and critically altering the physiology of the alga.

KEYWORDS: Algae/ Experimental Evolution /DNA Transposon/ /Gene loss/ /Vitamin $B_{12}$ /auxotrophy

## 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 co-factors. These co-factors perform essential metabolic functions, and when supplied externally, are known as vitamins. Animals obtain vitamins in 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, out-sourcing production could be selected for if an exogenous vitamin supply is available. The loss of vitamin synthesis has happened frequently across eukaryote diversity (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). Since 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 $B_7$) is a variable trait 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 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. Approximately 50%, 22% and 5% of species surveyed require vitamin $B_{12}$ (cobalamin), $B_1$ (thiamine) and $B_7$ (biotin), respectively (Croft et al, 2006), and the

distribution of requirement does not follow phylogenetic lines. Unlike other B vitamins, vitamin $B_{12}$ is synthesised only by prokaryotes (Warren et al, 2002). In aquatic ecosystems, ambient concentrations of $B_{12}$ are extremely low (Sanudo-Wilhelmy et al, 2012) and it has been proposed that availability of this factor may exert significant constraints on the distribution, taxonomic composition and primary productivity of algal communities (Gobler et al, 2007; Bertrand et al, 2012a; Sanudo-Wilhelmy et al, 2012). 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 $B_{12}$ 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 – i.e. non-specific interactions with prokaryote producers (Karl et al, 2002; Azam et al, 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, 2014).

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; Croft et al, 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 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 et al, 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. While 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; Helliwell et al, 2014). Here, we adopt an experimental evolution approach using *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 $^{0}$C in continuous light (20 μmol/m$^2$/sec) with shaking (140 rpm). Forty six independent populations were founded from a single colony of the ancestral line (AL) *C. reinhardtii* strain 12, derived from WT strain 137c. Cells were transferred every Monday, Wednesday and Friday, with growth periods approximately 51, 53, and 64 hours respectively. Optical density ($OD_{730}$) was measured every transfer, which determined the subsequent transfer volume to obtain ~8000 cells/inoculum. As such, cells never exceeded a cell density of ~3 × $10^6$ cells/mL. Stock-points were taken after 13, 25, 40, 50, 60, and 70 transfers respectively, 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) 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 ancestral line (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 $B_{12}$ supplementation) for 4 days, then diluted to a cell density of 4000 cells/mL (i.e. an 8000 cell inoculum). The number of cells/mL was subsequently measured every 12 hours over a 96 hour time period using the Duel Threshold Beckman Coulter (Z2) Particle Counter and Size Analyser 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/RT-PCR experiments were performed as described by (Helliwell et al, 2011) (Supplementary Table S1).

## Southern blotting

Extracted DNA was digested with *Nae*I and *Bam*HI (NEB, UK). A total of 1.5 µg was loaded and separated by agarose-gel electrophoresis, and transferred to Hybond-N+ (GE-Healthcare) 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 pre-hybridized overnight at 65°C in Church buffer (Church and Gilbert, 1984). The probe was denatured by 10 min boiling, 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% SDS to 0.2× SSC, 0.1% SDS) until counts were ~1000 cpm.

## Western blotting

Total protein was extracted, and Western blot experiments 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 $B_{12}$-depenndent 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 the fast growing green alga, *Chlamydomonas reinhardtii*, were founded from a single clone (the ancestral line,

AL). Half the populations were grown without $B_{12}$ on TAP medium (Methods), and the other half with 1000 ng/L vitamin $B_{12,}$ an amount that exceeds the growth requirements of $B_{12}$-requiring algae (Croft et al, 2005). The populations were sub-cultured into fresh medium at regular intervals, with the maximum cell density reaching ~$3 \times 10^6$ cells/mL. 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 medium 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 cells isolated from H-type colonies were vitamin $B_{12}$ independent, whilst S-type cells were dependent on the vitamin for growth in liquid culture during a 72 hour 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, 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 (Figure 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 (Figure 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 to 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 (0.2 μM) and above, 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-type and H-type clones. This approach revealed a size polymorphism between the different clone types, in the region corresponding to the $9^{th}$ 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 Figure S2a and b). GR-TEs have 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 (TIRs) 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 blot 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 $B_{12}$), and repressed by $B_{12}$ (1000 ng/L), as is characteristic for WT *C. reinhardtii METE* (Figure 2c) (Croft et al, 2005; Helliwell et al, 2011; Helliwell et al, 2014).

A Southern blot 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-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-T50, through transposition of a GR-TE into *METE*, correlating with growth experiments (Figure 1d and e). These $B_{12}$-dependent cells remained in co-culture with their $B_{12}$-independent predecessors for a further 20 transfers (<200 generations), until eventually the $B_{12}$-dependent clones dominated the population (T70, <700 generations). Samples prepared from individual S and H-type clones (Figure1b) 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 three days, darker bodies of cells appeared within the S-type colonies on the plates without $B_{12}$ (Figure 3a, Supplementary Figure S4). Since 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 were also shown to have GR-TE (Figure 3b). However, one $B_{12}$-dependent clone (clone #7) generated a PCR product with the size expected for WT *METE* (Figure 3b). Sequencing revealed 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 3 extra amino acids that disrupt a conserved region of the *METE* gene (Supplementary Figure S3) resulting in a stable vitamin $B_{12}$-dependent mutant.

**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 to 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 (i.e. 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 to H-type clones (Figure 4a and b), however the difference was not statistically different using a Student's t test with a p value of $\leq 0.05$. We did however observe a statistical difference in growth rate between S- and R- type cells (two-tailed Student's t test $P \leq 0.05$, $n$=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 to the AL ($P \leq 0.001$, $n$=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 if 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 three $B_{12}$-synthesising 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 five 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 #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 to 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 dependant on algal photosynthate (Figure S5).

## 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 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 characterise 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 characterising 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, 2014), and nutrient stress (nitrate limitation) activated transposition has been observed in the marine diatom *P. tricornutum* (Maumus et al, 2009), which has also been observed with our system. Moreover, differential insertion patterns amongst natural isolates of diatom species from different geographic locations have been observed (Maumus et al, 2009). Together these findings suggest that TEs may play 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, since 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 to 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 155 species 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 utilisation of METH in *P. tricornutum* is more resource efficient than $B_{12}$-independent metabolism, as 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 if 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, 2012;

Bertrand et al, 2013; Helliwell et al, 2014) how fitness maybe conferred from inactivating a gene that is already switched off remains unclear. 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 (Helliwell et al, 2014; Xie et al, 2014). However, since 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 (Sanudo-Wilhelmy et al, 2012). Moreover, levels of $B_{12}$ are reportedly less than 10 ng/L (~10 pM) in some freshwater habitats (Kurata, 1986). However, since 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 localised microbial activity, and/or the presence of particulate matter (Azam et al, 2007; Stocker et al, 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 versus 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 oligotrophic environments, where ambient concentrations of vitamins are extremely low (Sanudo-Wilhelmy et al, 2012). Evolutionary adaptations enabling $B_{12}$ auxotrophs to be successful competitors in $B_{12}$ deprived regions could include becoming specialised 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). Since 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.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

Supplementary information is available at ISMEJ's website

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## FIGURES LEGENDS

**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 visualised under a dissecting microscope), scale bar: 1 mm. (**b**) Growth of four independent H- and S-type colonies plus (1000 ng/L) and minus $B_{12}$ after 72 hours (mean ± s. e. m) *n*=3. Mean $OD_{730}$ values for H- and S- type clones at this time point were: 0.78 ± 0.08 s.e.m. ($+B_{12}$), 0.78± 0.04 ($-B_{12}$), and 0.64 ± 0.009 ($+B_{12}$), 0.04± 0.02 ($-B_{12}$) respectively. (**c**) $OD_{730}$ of stock-points cultures on liquid medium with (1000 ng/L; grey) and without $B_{12}$ after 72 hours (mean ± s. e. m) *n*=3 and, (**d**) Maximal growth rate ($h^{-1}$) of stock-points cultures on liquid medium with (1000 ng/L; grey) and without $B_{12}$ as calculated from **c** (mean ± s. e. m) *n*=3. (**e**)**.** Percentage of S-type 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.

**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 4.4-5.4 kb from the start codon) reveals an unexpectedly large product for S-type clones (expected product size for 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 *Gulliver*-related transposable element (GR-TE) (Kim et al, 2005; Kim et al, 2006) (Supplementary Figure S2b). The schematic diagram shows an alignment between *C. reinhardtii* WT *METE* in this region compared to the 'S-type' product sequence. A target-site duplication of *METE* (grey

underline) flanks a 15-bp terminal-inverted-repeat (boxed). (**b**) Western blot analysis on total protein of $E8^+$ and AL cells using a polyclonal antibody against *C. reinhardtii* METE (~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**) RT-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 with TE + 8 bp *METE* repeat, i.e. 1148 bp), cDNA: 371 bp (+ 246 bp i.e. 617 bp). **(d**) Schematic diagram of Southern blot strategy (**e**) Southern blot analysis using the *METE* probe (probe 1) on genomic samples for stock-points, and independent S- and H-type clones.

**Figure 3. Characterisation 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 visualised 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: WT *METE*- 913 base, and *METE* with GR-TE insertion 913 + 246 = 1159 bp). Sequencing revealed a 9 base footprint (CACCATGCT) in this clone (**c**) the latter 6 bp of which (underlined grey) is a remnant of the *METE* repeat.

**Figure 4. Characterisation 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) (mean ± s. e. m) $n$=10. **c.** Mean maximal growth rate ($h^{-1}$) of S-type, H-type, R-type and AL clones as calculated from **a**. *P ≤ 0.05, **P ≤ 0.001 compared with the S-type clones (two-tailed Student's t test) (mean ± s. e. m) $n$=10.

**Figure 5. Vitamin $B_{12}$ dependence is rescued by three $B_{12}$-synthesising rhizobial species of bacteria.** (**a**) Growth of S-type mutant in different $B_{12}$ regimes including: i. + $B_{12}$ (1000 ng/L), 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 #7 in $B_{12}$ regimes described in (a). This experiment was carried out in TAP medium (mean ± s. e. m) *n*=3.