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Chlamydomonas reinhardtii thermal tolerance enhancement mediated by a mutualistic interaction with vitamin B₁₂-producing bacteria

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Temperature is one of the most important environmental factors affecting the growth and survival of microorganisms and in light of current global patterns is of particular interest. Here, we highlight studies revealing how vitamin B₁₂ (cobalamin)-producing bacteria increase the fitness of the unicellular alga *Chlamydomonas reinhardtii* following an increase in environmental temperature. Heat stress represses *C. reinhardtii* cobalamin-independent methionine synthase (*METE*) gene expression coinciding with a reduction in *METE*-mediated methionine synthase activity, chlorosis and cell death during heat stress. However, in the presence of cobalamin-producing bacteria or exogenous cobalamin amendments *C. reinhardtii* cobalamin-dependent methionine synthase *METH*-mediated methionine biosynthesis is functional at temperatures that result in *C. reinhardtii* death in the absence of cobalamin. Artificial microRNA silencing of *C. reinhardtii* *METH* expression leads to nearly complete loss of cobalamin-mediated enhancement of thermal tolerance. This suggests that methionine biosynthesis is an essential cellular mechanism for adaptation by *C. reinhardtii* to thermal stress. Increased fitness advantage of *METH* under environmentally stressful conditions could explain the selective pressure for retaining the *METH* gene in algae and the apparent independent loss of the *METE* gene in various algal species. Our results show that how an organism acclimates to a change in its abiotic environment depends critically on co-occurring species, the nature of that interaction, and how those species interactions evolve.

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Introduction

There has been increasing interest in the interactions between bacteria, algae and their environment, and how those interactions influence alga morphogenesis, nutrient acquisition and community composition as well as bacterial growth and biogeochemical cycles (for example, Croft *et al.*, 2005; Matsuo *et al.*, 2005; Matz *et al.*, 2008; Amin *et al.*, 2009; Barott *et al.*, 2011; Seyedsayamdost *et al.*, 2011). Understanding how these interactions influence the adaption of bacteria and algae to the abiotic environment is important for understanding the physiological processes and evolutionary outcomes of those species as nearly all organisms co-occur with other species. In the present study, we

identified how a beneficial interaction between certain bacterial species and the alga *Chlamydomonas reinhardtii* influences the ability of *C. reinhardtii* to survive a change in its abiotic environment that is lethal to *C. reinhardtii* in isolation.

C. reinhardtii, a unicellular eukaryotic microalga found in soil and aquatic environments, grows with CO₂ and light, and is considered mesophilic (20–32 °C optimum). An abrupt transfer to a temperature above the typical growth range (for example, 39 °C) results in heat shock, triggering adaptive responses that may or may not allow resumption of growth or survival at the new temperature (Schroda, 2004). Prolonged exposure to incompatible temperatures results in a cessation of metabolism, chlorosis and ultimately cell death. Acclimation to high temperature relies on basal mechanisms to initiate physiological adjustments that permit expression of adaptive mechanisms required for prolonged survival at those and higher temperatures. Similar to other eukaryotes, heat shock transcription factor and heat shock proteins

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that facilitate proper protein folding and turnover are highly induced upon heat treatment in *C. reinhardtii* (Schulz-Raffelt *et al.*, 2007).

Many algal species require exogenous cobalamin (vitamin B₁₂), thiamine (vitamin B₁) or biotin (vitamin B₇) for growth (Croft *et al.*, 2006). As cobalamin is produced only by prokaryotes (Roth *et al.*, 1996; Warren *et al.*, 2002), cobalamin could be acquired by scavenging it from the environment or through a mutualistic or commensal association with cobalamin-producing bacteria (Croft *et al.*, 2005, 2006; Kazamia *et al.*, 2012). Cobalamin is a Co²⁺-containing, modified tetrapyrrole that functions as a cofactor for enzymes involved in C1 and other metabolic reactions (Roth *et al.*, 1996). In many eukaryotic algae (Croft *et al.*, 2005; Helliwell *et al.*, 2011), cobalamin requirements arise primarily from its use by methionine synthase, responsible for synthesizing methionine and tetrahydrofolate from homocysteine and 5-methyl-tetrahydrofolate (Roth *et al.*, 1996; Warren *et al.*, 2002). *C. reinhardtii* has only one known cobalamin-dependent enzyme, methionine synthase (METH), and the accessory proteins methionine synthase reductase and AdoCbl synthase required for S-adenosylhomocysteine reduction and adenosylcobalamin synthesis, respectively (Croft *et al.*, 2005; Helliwell *et al.*, 2011). Yet, *C. reinhardtii* also has a cobalamin-independent methionine synthase (METE) (Croft *et al.*, 2005; Helliwell *et al.*, 2011) that accomplishes the same reaction as METH albeit likely with lower efficiency (Gonzalez *et al.*, 1992). Aside from avoiding dependency on cobalamin for methionine synthesis and the greater ecological flexibility it provides in environments with scarce cobalamin availability, little is known of the advantages or disadvantages of one isoform of methionine synthase over the other in contributing to the ecological success of *C. reinhardtii* or other algae in natural habitats.

In the course of another study, we observed that *C. reinhardtii* thermal tolerance was enhanced when cultures were contaminated with bacteria, which led to a series of experiments to elucidate the mechanism of this enhancement. Our data show that certain cobalamin-producing bacterial species when co-cultivated with *C. reinhardtii* enhance algal thermal tolerance, and that cobalamin-dependent methionine biosynthesis has a critical role in thermal tolerance enhancement. These findings will have implications for better understanding ecosystem functioning as marine algae contribute substantially to primary productivity (Field *et al.*, 1998; Falkowski *et al.*, 2004), and are therefore important factors in global carbon cycling. Consequently, it might be appropriate to include cobalamin availability and cobalamin-producing bacteria and their production and consumption dynamics in efforts to predict and model marine or terrestrial primary productivity as temperatures rise.

Materials and methods

C. reinhardtii and bacterial strains and growth conditions

Wild-type *C. reinhardtii* strains 21gr (cc1690) and 137c (cc125) were obtained from the Chlamydomonas Resource Center (<http://chlamycollection.org/>) and were grown phototrophically under continuous illumination (120 $\mu\text{E m}^{-2} \text{sec}^{-1}$) in minimal salts medium or on agar (1.5%) solidified plates (Geraghty *et al.*, 1990) in a 5% CO₂ atmosphere. Bacterial species were grown on TY (Beringer 1974) or rhizobial minimal medium (Broughton *et al.*, 1986) at 25 or 30 °C. *Sinorhizobium meliloti* wild-type 1021 and its Tn5 transposon insertion mutants *cobD*[−], *bluB*[−] and *metH*[−] (Campbell *et al.*, 2006; Taga *et al.*, 2007) were generously provided by Michiko Taga (University of California-Berkeley). The cobalamin-deficient *cobD*[−] mutant was grown in TY media supplemented with 2 mg l^{−1} cyanocobalamin. Algal growth media were supplemented with cyanocobalamin (Cbl), methionine (Met), thiamine, biotin or folate at the indicated concentrations. *S. meliloti* growth media were supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin or 20 $\mu\text{g ml}^{-1}$ gentamycin. Culture supernatant was obtained by centrifugation of a 3-day-old stationary phase *S. meliloti* culture grown in rhizobial minimal medium, and decanting the culture supernatant, filter sterilizing it, drying it in a speed-vac and re-suspending it in water. The identity of air-borne bacterial contaminants of *Chlamydomonas* cultures that exhibited thermal tolerance enhancing properties was based on sequencing of the 16S rRNA gene (see Supplementary Information for details).

Thermal tolerance assay

Our thermal tolerance plate assays consisted of growing *Chlamydomonas* for 0–3 days at 25 °C in the presence or absence of bacteria, or cobalamin or methionine amendments to the culture media before transferring plates (upshift) to higher temperatures. Enhancement of thermal tolerance is defined as a delay in chlorosis at normally lethal temperatures and was assessed 5 days post temperature upshift. *Chlamydomonas* liquid cultures were adjusted to 1.5×10^6 cell per ml with fresh medium, based on direct microscopy counts. *Sinorhizobium* were washed twice and re-suspended from plate-grown cultures in a 0.85% saline solution, and serially diluted in a saline solution before mixing equal volumes of bacteria and *C. reinhardtii* cell suspensions to generate varying inoculum ratios; cell densities were verified by determining colony forming units. These mixtures were inoculated onto plates as co-cultures comprised of the same number of *Chlamydomonas* cells (5000, unless stated otherwise), but the abundance of *S. meliloti* cells varied. To minimize cyanocobalamin contamination from the medium, the *S. meliloti cobD*[−] mutant was

sub-cultured for 1.5 days in TY broth before preparing cells for co-inoculation as described above. Alternatively, *C. reinhardtii* was streaked onto minimal salts medium plates before inoculating selected areas with bacteria by streaking. For determining viable cell numbers, co-cultures were removed from the plate and serially diluted before plating aliquots onto minimal salts medium or TY plates for enumeration of *Chlamydomonas* or *Sinorhizobium*, respectively. Cobalamin production was determined using a modified version of a previously described bioassay (Raux *et al.*, 1996) and epifluorescence microscopy imaging of co-cultures are outlined in Supplementary Information.

Methionine synthase activity assays

Chlamydomonas cells were lysed by sonication in 0.1 M KPO₄ buffer containing 10 mM DTT, pH 7.2 and 0.1 mM phenylmethylsulfonyl fluoride, and the mixture was centrifuged at 14 000 g for 60 min at 4 °C. The supernatant provided a crude enzyme prep for methionine synthase activity assays using the method described previously (Drummond *et al.*, 1995), with modification. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA) and 0.1–0.2 mg ml⁻¹ BSA-equivalents of crude enzyme prep was used in each assay. Assay substrates include 0.5 mM L-homocysteine (Fisher Scientific, Pittsburgh, PA, USA) and 0.16 mM 5-methyl-tetrahydrofolate monoglutamate (Schircks Laboratories, Jona, Switzerland) for cobalamin-dependent METH or 0.16 mM 5-methyl-tetrahydrofolate triglutamate (Schircks Laboratories) for cobalamin-independent METE activity assays. Cyanocobalamin was provided at a concentration of 0.05 µM for METH activity assays. Assays were performed at 37 °C for 45 min and quenched by adding a 5-N HCl/60% formic acid solution. As the product tetrahydrofolate is unstable in acidic solutions, the reaction mixture was heated at 80 °C for 10 min to form methenyltetrahydrofolate. After centrifugation at 14 000 g for 5 min, the absorbance (350 nm) of methenyltetrahydrofolate in the reaction mixture was measured. Assay mixtures devoid of crude enzyme prep or substrates were used as blanks.

Gene silencing of METH in *C. reinhardtii*

Artificial miRNA oligonucleotides were designed using the Web MicroRNA Designer platform (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>). The MetHmi-1F and MetHmi-1R oligonucleotides (Supplementary Table S1) targeting *C. reinhardtii* METH coding sequence (439–448 bp downstream of the start nucleotide base) were annealed and ligated into *Spe*I-digested pChlamiRNA3int as described previously (Molnar *et al.*, 2009) to generate the artificial microRNA (amiRNA) vector pChlamiRNA3int-METH for transformation with *C. reinhardtii*

strain CC125. Transformants were selected on TAP (Gorman and Levine, 1965) solid medium containing 15 µg ml⁻¹ paromomycin. The mRNA levels of METH were analyzed using qRT-PCR with the primers listed in Supplementary Table S1. The knockdown lines with reduced METH mRNA levels were then examined for their ability to delay chlorosis at elevated temperatures in the absence or presence of cyanocobalamin, methionine or *S. meliloti* 1021. Procedures for RNA isolation for RT and qRT-PCR are described in Supplementary Information.

Results

Enhancement of thermal tolerance in *C. reinhardtii* by bacteria

In the course of another study, we observed that *C. reinhardtii* exhibited delayed chlorosis at normally lethal temperatures when contaminated with bacteria. This observation lead us to examine what features of this bacterial-*Chlamydomonas* interaction facilitated increased fitness at elevated temperatures. The contaminating bacterial isolates and others available in our collection were co-cultured with *C. reinhardtii* to assess whether enhancement of thermal tolerance (a.k.a. delayed chlorosis) was species specific using the assay described in the Materials and methods and the footnote to Table 1. Only bacteria of the α -proteobacterial lineage strongly decreased *C. reinhardtii* chlorosis at elevated temperatures (Table 1, Supplementary Figure S1).

As *S. meliloti* 1021 (recently renamed *Ensifer meliloti*) most consistently enhanced *Chlamydomonas* thermal tolerance and this strain has been shown to benefit from its interaction with algae (Kazamia *et al.*, 2012 and unpublished data), we used this strain to further establish the ability of bacterial interactions to delay algal chlorosis and increase survival at elevated temperatures. In these co-cultures *S. meliloti* forms aggregates between and in close association with *C. reinhardtii* cells and is not internalized by *C. reinhardtii*, based on epifluorescence microscopy (Supplementary Figure S2). Our visual assessment of *Chlamydomonas* chlorosis and survival at 42 °C was validated by *C. reinhardtii* 21gr chlorophyll content and viability of the co-cultures which was greatly influenced by the *S. meliloti* inoculum size (Figures 1a,d and e). Shifts in the relative abundances of *C. reinhardtii* and *S. meliloti* occurred quickly (Figure 1b) leading to a 5–9-fold greater abundance of *S. meliloti* by 8 DAI (Figure 1c) in the absence of heat stress. *C. reinhardtii* death in the 42 °C treatment does not appear to provide sufficient resources to promote *S. meliloti* growth or survival beyond the levels observed before the temperature upshift (compare Figure 1b with Figure 1d). In contrast, co-cultures with *S. meliloti* in the absence of thermal stress had

Table 1 Specificity of bacterial enhancement of *Chlamydomonas* thermal tolerance^a

Species	Phylogenetic class (family)	Thermal tolerance ^b	Aerobic vitamin B ₁₂ synthesis ^c
<i>Aeromicrobium tamlense</i> ^d	Actinobacteria	±	+
<i>Brevibacillus panacihumi</i> ^d	Firmicutes	+	+
<i>Mesorhizobium sp.</i> ^d	α-Proteobacteria (Phyllobacteriaceae)	2+	+
<i>Mesorhizobium plurifurum</i> ^d	α-Proteobacteria (Phyllobacteriaceae)	2+	+
<i>Roseomonas mucosa</i> ^d	α-Proteobacteria (Acetobacteriaceae)	2+	+
<i>Sinorhizobium meliloti</i> 1021	α-Proteobacteria (Rhizobiaceae)	2+	+
<i>S. meliloti</i> Mn04-gfp	α-Proteobacteria (Rhizobiaceae)	2+	+
<i>Alcaligenes faecalis</i>	β-Proteobacteria (Alcaligenaceae)	–	+
<i>Burkholderia cepacia</i> ATCC 25416	β-Proteobacteria (Burkholderiaceae)	±	+
<i>Pseudomonas aeruginosa</i> PA01	γ-Proteobacteria (Pseudomonadaceae)	–	+
<i>Pseudomonas putida</i> mt2	γ-Proteobacteria (Pseudomonadaceae)	–	+
<i>Enterobacter cloacae</i> JL1157	γ-Proteobacteria (Enterobacteriaceae)	–	–
<i>Serratia marcescens</i>	γ-Proteobacteria (Enterobacteriaceae)	–	–
<i>Salmonella enterica typhimurium</i> LT2 ^e	γ-Proteobacteria (Enterobacteriaceae)	±	–

^a*C. reinhardtii* and bacterial co-cultures were grown at 25 °C for 3 days before shifting to 42 °C, a temperature that typically causes chlorosis and presumably cell death after 3–5 additional days of incubation. Controls were kept at 25 °C.

^bThermal tolerance was assessed visually as illustrated in Supplementary Figure S1. Thermal tolerance enhancing effect is defined as no (–), weak (±), moderate (+) or robust (2+).

^cVitamin B₁₂ production was verified in aerobic cultures using a bioassay described in the Materials and methods and Supplementary Information file.

^dBacterium isolated from contaminated *Chlamydomonas* cultures and identified by sequencing a near full-length PCR amplicon of the 16S rRNA gene.

^eVitamin B₁₂ synthesized anaerobically (Jeter et al., 1984).

no effect on *C. reinhardtii*, based on chlorophyll content (Figures 1a and e) and survival (Figure 1c), even with large initial *S. meliloti* inoculum sizes. Ethanol-killed *S. meliloti* inoculated in a 100-fold excess of *C. reinhardtii* did not confer thermal protection although highly concentrated sterilized culture supernatants did (Figure 1f), indicating that *S. meliloti* secretes a metabolite(s) or the component(s) can be released upon cell death.

Enhancement of *C. reinhardtii* thermal tolerance by vitamin B₁₂ or methionine

Many algal species require cobalamin, thiamine or biotin for growth (Croft et al., 2006), and given that only some bacteria or Archaea species produce cobalamin this resource is possibly acquired through an algal–bacterial symbiosis (Croft et al., 2005; Helliwell et al., 2011). *C. reinhardtii* does not require cobalamin for growth as it has genes for both cobalamin-dependent (*METH*) and -independent (*METE*) methionine synthase (Croft et al., 2005; Helliwell et al., 2011). Yet, it's conceivable one or more of these vitamins could affect algal growth under high temperature. Of the vitamins or related compounds examined, only cyanocobalamin (Table 2 and Figure 2a) significantly delayed *C. reinhardtii* chlorosis at elevated temperatures, with a minimum requirement of 100 ng l^{–1} (74 pMol) (Figure 2a). As cobalamin is the methyl-donating cofactor of methionine synthase *METH*, which catalyzes the synthesis of methionine and tetrahydrofolate from homocysteine and methyltetrahydrofolate, we included methionine and folate as well. The requirement for near mM methionine concentrations (Table 2 and Figure 2) to effectively

enhance *C. reinhardtii* thermal tolerance likely reflects the fortuitous uptake by transporters not specific to methionine as described earlier (Kirk and Kirk, 1978). Folate had no effect on thermal tolerance. Finally, cobalamin or methionine rescue of *C. reinhardtii* heat sensitivity is not restricted to specific strains, but was observed in a variety of wild-type and mutant strains (Supplementary Table S2) albeit the extent of tolerance varied among strains.

Chlamydomonas thermal tolerance was influenced by when cells were exposed to cobalamin or methionine (Figure 2b) and by the length of the acclimation period (Figure 2c) before a temperature upshift in a cell density-dependent manner (Figures 2b and c). In the absence of any pre-acclimation at 25 °C before a temperature upshift, planktonic cultures grown in the presence of cobalamin or methionine required higher cell densities to exhibit, albeit weak, enhanced thermal tolerance (Figure 2b). In a cell-dependent manner, the longer the acclimation time at 25 °C the better the thermal tolerance (Figure 2c). In contrast, methionine-mediated enhancement of thermal tolerance occurred only after a prolonged (3-day) acclimation period before the temperature upshift (Figure 2c). Cyanocobalamin and to a lesser extent methionine could alleviate heat sensitivity up to 44 °C, but had no effect at higher temperatures (Supplementary Table S3). Cyanocobalamin rescue of chlorosis and heat sensitivity did not rely on large temperature upshifts (for example, 25–42 °C), as we observed similar thermal tolerance enhancement with temperature upshifts from 38 to 42 °C, although chlorosis occurred more slowly in the absence of cyanocobalamin presumably owing to heat-induced metabolic adjustments or reduced metabolic activity of cells

Figure 1 Enhancement of *C. reinhardtii* thermal tolerance by *S. meliloti* co-cultivation. (a) *S. meliloti* 1021 enhances *C. reinhardtii* 21gr survival at lethal temperatures in a cell density-dependent manner using a co-culture thermal tolerance assay described in the Materials and methods and the footnote to Table 1. Photographs show the extent of chlorosis 5 days after the temperature upshift. (b–d) Viability of *C. reinhardtii* 21gr and *S. meliloti* 1021 in co-cultures incubated at 25 °C 3 (b) and 8 (c) days after inoculation (DAI) and at 42 °C for 5 days (d) after the temperature upshift (8 DAI). (e) Chlorophyll content of *C. reinhardtii*–*S. meliloti* co-cultures 8 DAI. (f) Co-inoculation of *C. reinhardtii* 21gr with dead *S. meliloti* (Sm) cells (1:100 Cr 21gr:Sm 1021), un-concentrated 12-fold (1X) and concentrated 12-fold (12X) culture medium (M) or *S. meliloti* culture supernatant (S). Values are the mean $\pm$ s.e.m. of three independent experiments each comprised of three replications. A separate one-way ANOVA was performed for *C. reinhardtii* 21gr and *S. meliloti* 1021 at each temperature and sampling time. Bars with different letters are significantly different based on a Duncan's test ($P \leq 0.05$).

Table 2 Enhancement of *C. reinhardtii* 21gr thermal tolerance by exogenous supplements^a

	Thermal tolerance enhancing effect	Minimum enhancing concentration (mg l ⁻¹)	Maximum concentration tested (mg l ⁻¹)
Cyanocobalamin	2 +	0.0001	5
Biotin	–	–	0.5
Thiamine	–	–	0.16
Methionine	±	20	2000
Folate	–	–	0.04
Methionine + folate	1 +	20 + 0.04	200 + 0.04
Cyanocobalamin + methionine	2 +	0.0001 + 20	1 + 2000

Thermal tolerance enhancing (TE) effect is defined as no (–), weak (±), moderate (+), or robust (2+).

^aMedia were amended with various concentrations of supplements to identify those that were effective in enhancing *Chlamydomonas* thermal tolerance. Cultures were grown on solid media at 25 °C for 3 days before shifting to 42 °C for 5 days after which results were recorded.

grown at higher temperatures. Collectively, these data suggest that better thermal tolerance at high cell densities may be a consequence of earlier entry into a stationary-phase physiological state.

The ability of cyanocobalamin and, to a lesser extent, methionine to enhance *C. reinhardtii* heat tolerance did not rely on surface growth as similar effects were attained in liquid culture (Figure 2d,

Figure 2 Enhancement of *C. reinhardtii* thermal tolerance by cyanocobalamin and methionine. (a) Thermal tolerance enhancing effect of *C. reinhardtii* 21gr on solid media un-amended (MM) or amended with cyanocobalamin (Cbl), methionine (Met) or both cyanocobalamin (1 mg l⁻¹) and methionine (0.2 mg ml⁻¹), based on the thermal tolerance assay. Photographs were taken 8 DAI. Thermal tolerance enhancing effect (TE effect) is defined in Table 1. (b) Thermal tolerance of *C. reinhardtii* 21gr grown in the presence of cyanocobalamin (1 mg l⁻¹) or methionine (0.2 mg ml⁻¹) and in the absence of a 3-day acclimation period before the temperature upshift. Solid media was un-amended or amended with cyanocobalamin (1 mg l⁻¹) or methionine (0.2 mg ml⁻¹). Photographs were taken 5 DAI. (c) Influence of acclimation time before the temperature upshift on thermal tolerance in the absence or presence of cyanocobalamin (1 mg l⁻¹) or methionine (0.2 mg ml⁻¹) for 1–3 day before the temperature upshift. Photographs were taken 5 days after the temperature upshift. (d) Survival of *C. reinhardtii* 21gr in liquid culture without or with cyanocobalamin (1 mg l⁻¹), methionine (0.2 mg ml⁻¹) or both amendments without (top) or with (bottom) 42 °C temperature upshift. Values are the mean ± s.e.m. of three independent experiments each comprised of five replications.

Supplementary Table S4), although viability of planktonic cells more quickly diminishes in the absence of cyanocobalamin or methionine following a temperature upshift (Figure 2d) compared with surface-grown cells (Figures 2a–c). A consequence of reduced viability at 42 °C in the absence of cyanocobalamin or methionine is a dramatic reduction in biomass with cyanocobalamin treatments (Supplementary Table S4), indicative of scavenging cellular resources to maintain viability.

S. meliloti produced adenosylcobalamin enhances *C. reinhardtii* thermal tolerance

The preceding data indicated that cyanocobalamin mimics the effect of bacterial-mediated delay in chlorosis and prolonged survival of *C. reinhardtii* at lethal temperatures. To determine if bacterial-mediated thermal tolerance was due to cobalamin production, we selected the *S. meliloti* cobalamin-deficient *bluB*⁻ and *cobD*⁻ mutants to test this

possibility. *BluB* synthesizes 5,6-dimethylbenzimidazole, the lower ligand of cobalamin while *CobD* is required for corrin ring synthesis (Campbell *et al.*, 2006; Taga and Walker 2010). We also included a methionine auxotroph (*meth*⁻ mutant) (Campbell *et al.*, 2006; Taga and Walker, 2010). As expected, both the *bluB*⁻ and *cobD*⁻ mutants were dramatically impaired in delaying chlorosis or promoting *C. reinhardtii* survival (Figure 3). Differential survival of the *bluB*⁻ and *cobD*⁻ mutants is likely due to the presence of trace amounts of 5,6-dimethylbenzimidazole in the agar (Campbell *et al.*, 2006), or *C. reinhardtii* exudates that facilitate *bluB*⁻ mutant survival (Figure 3c), but neither strain produces sufficient adenosylcobalamin to promote *C. reinhardtii* heat tolerance. Despite the poor survival of the *meth*⁻ mutant in the absence of exogenous methionine (Figure 3c), sufficient amounts of cobalamin was produced to facilitate survival of *Chlamydomonas* at moderate levels. Collectively, these results suggest that cobalamin

Figure 3 Adenosylcobalamin biosynthesis by *S. meliloti* confers thermal tolerance to *C. reinhardtii*. (a) Chlorosis of *C. reinhardtii* 21gr when cultivated with *S. meliloti* strain 1021 and various mutants deficient in adenosylcobalamin lower ligand (*bluB*⁻) or corrin ring (*cobD*⁻) synthesis, *methH*⁻ in the thermal tolerance assay. Viability at 25 °C (b) and 42 °C (c) was determined by plate counts 8 DAL. The Cr21gr:Sm 1021 inoculum ratio was 1:100 in all comparisons. Values represent the mean $\pm$ s.e.m. of two independent experiments, each comprised of three replications. A separate ANOVA was performed for *C. reinhardtii* 21gr and *S. meliloti* 1021 and bars with different letters are significantly different based on a Duncan's test ($P \leq 0.05$).

has a critical role in bacterial-mediated enhancement of *C. reinhardtii* thermal tolerance. Compared with growth at 25 °C, survival of *cobD*⁻ and *methH*⁻ mutants alone or in co-culture with *C. reinhardtii* were dramatically reduced at 42 °C, implying that

adenosylcobalamin and methionine production is also important for heat tolerance of *S. meliloti*.

This observation led us to investigate whether the inability of certain bacteria to enhance *C. reinhardtii* thermal tolerance was due to their inability to produce cobalamin. Many bacterial strains capable of aerobic cobalamin production were unable to enhance *Chlamydomonas* thermal tolerance (Table 1). *Salmonella* has served as a model for cobalamin synthesis (Raux *et al.*, 1996; Roth *et al.*, 1996; Warren *et al.*, 2002), but only produces it under anaerobic conditions and it is unclear whether the other Enterobacteriaceae we tested also synthesize cobalamin anaerobically.

Regulation of *C. reinhardtii* *METE* and *METH* by temperature or bacteria

As exogenous cyanocobalamin and methionine rescues *C. reinhardtii* thermal sensitivity it suggests that either the expression or activity of the cobalamin-independent methionine synthase (*METE*) is heat-sensitive, whereas cobalamin-dependent (*METH*) is not. We used quantitative RT-PCR to determine whether temperature, cyanocobalamin or *S. meliloti* 1021 co-culture affected *METE* and *METH* expression. At a non-lethal 25 °C temperature, *C. reinhardtii* *METE* is highly expressed (Figure 4a) but in the presence of cyanocobalamin *METE* is strongly repressed, whereas *METH* expression is unaffected, which is consistent with other studies (Croft *et al.*, 2005; Helliwell *et al.*, 2011). Importantly, following a 1-h heat shock at 42 °C, *METE* expression is reduced to <10% of the control but *METH* expression decreases to about 30% of the control (Figure 4a). As expected the heat shock treatment dramatically induces expression of the heat shock protein gene *HSP90A* (Schulz-Raffelt *et al.*, 2007) (Supplementary Figure S3).

We also assessed *C. reinhardtii* *METE* and *METH* expression during its interaction with *S. meliloti* wild-type and the *bluB*⁻ mutant 2 days after a temperature upshift. At 25 °C, no significant differences in *C. reinhardtii* *METE* expression could be detected without or with *S. meliloti* wild-type or *bluB*⁻ mutant co-cultivation (Figure 4b). After an upshift to 38 °C, a temperature near the maximum *C. reinhardtii* growth temperature, *METE* expression was greatly reduced in the presence of *S. meliloti* wild-type and the *bluB*⁻ mutant, although the decrease was greater in the presence of the wild-type than the *bluB*⁻ mutant. There was almost no *METE* detected following a 42 °C upshift (Figure 4b). In contrast, following a 38 or 42 °C temperature upshift, *METH* expression was not significantly affected by the presence or absence of *S. meliloti* wild-type and the *bluB*⁻ mutant (Figure 4b). These findings support conclusions inferred in another study (Kazamia *et al.*, 2012) that *C. reinhardtii* *METE* expression can be repressed as a consequence of its interaction with a cobalamin-producing bacterial

Figure 4 Quantitative real-time PCR of *C. reinhardtii* *METE* and *METH* mRNA. (a) 21gr cells were exposed to a 1-h 42 °C heat shock or maintained at 25 °C in the absence (MM) or presence of cyanocobalamin (Cbl; 1 mg l⁻¹), methionine (Met; 0.2 mg ml⁻¹) or both amendments (Cbl + Met). (b) *C. reinhardtii* 21gr grown on solid media without or with *S. meliloti* wild-type 1021 or *bluB*⁻ mutant. The 21gr:Sm inoculation ratio was ~1:100. Co-cultures were exposed to a temperature upshift for 2 days before isolating RNA. The constitutively expressed *CBLP* gene (Schloss 1990) was used as the reference gene and values are normalized relative to 21gr cells grown at 25 °C in MM medium. Bars are the mean ± s.d. of two independent experiments. A separate ANOVA was performed for *METE* and *METH* and bars with different letters are statistically different based on Duncan's test ($P \leq 0.05$).

Figure 5 Effect of temperature on *C. reinhardtii* methionine synthase activity. *METE* and *METH* activity of *C. reinhardtii* cells grown in the absence (MM) or presence of cyanocobalamin (Cbl; 1 mg l⁻¹), methionine (Met; 0.2 mg ml⁻¹) or both amendments (Cbl + Met). Cultures were grown for 3 days at 25 °C before a 42 °C temperature upshift, except the controls were maintained at 25 °C. Crude cell extracts were used in the methionine synthase activity assays and all assays were conducted at 37 °C for 45 min, as described in the Materials and methods. Both assays measure the rate of methenyltetrahydrofolate (CH⁺-THF) production. Data are the mean ± s.e.m. ($n = 3$).

species. The difference between these two studies is the requirement for a higher, non-lethal temperature (Figure 4b) and the supplementation of a bacterial carbon source in the study by Kazamia *et al.* (2012).

Our methionine synthase activity assays reflect the pool of enzymes isolated from *C. reinhardtii*

cultures exposed to a temperature upshift for varying lengths of time (Figure 5). In both the METH and METE activity assays, the production of tetrahydrofolate was measured spectrophotometrically following its conversion to the more stable methenyltetrahydrofolate. METE activity was greatly

reduced in the presence of a heat stress or cyanocobalamin, whereas during heat stress METH activity in the presence of cyanocobalamin was maintained at higher levels than METE (Figure 5). When not grown in the presence of cyanocobalamin and heat stress, METE activity was high, but heat stress caused a precipitous decline in activity. Following the temperature upshift, the METH activity of cyanocobalamin-treated *C. reinhardtii* decreased over time although these levels were substantially greater than METE activity (Figure 5); these levels may suffice to maintain adequate methionine pools to prolong survival.

Gene silencing in *C. reinhardtii*

To further our functional analysis of the role of METH in *C. reinhardtii* thermal tolerance, we took advantage of an amiRNA system to knock down METH expression. For this purpose a plasmid (pChlamiRNA3int-MetH) carrying the strong constitutive *PSAD* promoter (Molnar *et al.*, 2009) was used to direct expression of an antisense cDNA amiRNA oligonucleotide targeting the 5'-end of METH mRNA. Attempts to reduce METH expression in strain 21gr transformed with this vector were unsuccessful based on screening 60 clones by qRT-PCR and in the chlorosis assay. Five (Hi-1, -2, -4, -12, -21) of the 65 paromycin-resistant transformants of wild-type CC125 screened for reduction in METH expression by quantitative RT-PCR in 3-day-old cultures cultivated at a non-lethal (25 °C) temperature exhibited 10–50% of the METH expression of the empty vector control (Figure 6a). Furthermore, a reduction in METH expression in these five lines was highly correlated with a more rapid onset of chlorosis after temperature upshift even in the presence of cyanocobalamin, methionine or *S. meliloti* 1021 (Figure 6b). Low levels of METH expression in the five amiRNA lines had no apparent effect on growth and survival at 25 °C in the absence of cobalamin (data not shown). Collectively these data provide strong evidence for the requirement of METH activity for the ability of cobalamin-producing bacteria to enhance *C. reinhardtii* thermal tolerance.

Discussion

Our findings indicate that interactions with cobalamin-producing bacteria can have profound effects on *C. reinhardtii* fitness as it responds to changes in environmental temperature and that methionine biosynthesis has a central role in thermal tolerance. This highlights the importance of the presence of co-occurring species in the acclimation of an organism to changes in its abiotic environment. Although concentrated culture supernatants delaying *C. reinhardtii* chlorosis at high temperature (Figure 1f) implies cobalamin could be scavenged from dead

Figure 6 Phenotypes of *C. reinhardtii* lines expressing artificial microRNA (amiRNA) targeting METH mRNA. Quantitative real-time PCR (a) analysis of METH mRNA in METH-amiRNA expressing lines (Hi). Values are normalized relative to wild-type CC125 cells containing an empty pChlamiRNA3int vector (cc125-int3) in 3-day-old broth cultures grown at 25 °C. Bars are the mean $\pm$ s.e.m. ($n=3$). Bars with different letters are statistically different based on a Duncan's test ($P \leq 0.05$). Chlorosis phenotypes (b) of METH-amiRNA expressing cell lines following a temperature upshift in the presence of cyanocobalamin (Cbl; 1 mg l⁻¹), methionine (Met; 0.2 mg ml⁻¹) or *S. meliloti* 1021 (Sm1021; *Chlamydomonas*: *S. meliloti* 1021 inoculum ratio of 1:100). Photographs were taken 8 DAI.

bacteria (Droop, 2007), several lines of evidence suggest otherwise. First, not all cobalamin-producing bacteria are capable of enhancing *C. reinhardtii* thermal tolerance (Table 1) and second, cobalamin needs to be present for a sufficient length of time before the temperature upshift to be functional (Figures 2b and c). In addition, cobalamin production is regulated by environmental conditions such as oxygen in a species-dependent manner (Jeter *et al.*, 1984). Why some bacteria capable of aerobic cobalamin production enhance *C. reinhardtii* thermal tolerance and others do not is unclear, but conceivably it's a consequence of differences in bacterial thermal tolerance or ability to utilize *C. reinhardtii* resources that support bacterial growth under elevated temperatures. Bacteria we examined that strongly enhance *C. reinhardtii* thermal tolerance are members of the α -proteobacterial class, which includes the Rhizobiales order. This is consistent with other reports (Hold *et al.*, 2001; Sapp *et al.*, 2007) of specific bacterial lineages, including α -proteobacteria, being associated with various algal species in natural environments. These associations may reflect a certain degree of specificity, possibly a consequence of the suitability of photosynthate algae provide to heterotrophic bacteria (Kazamia *et al.*, 2012), that is, there may be a degree of specificity in this chemical dependency.

The ecology of bacterial–algal interactions is not well understood, but recent studies have highlighted how each partner can influence the physiology of the other. For example, bacterial promotion of algal assimilation of iron in exchange for photosynthate in marine systems (Amin *et al.*, 2009) and *C. reinhardtii* secretion of substances mimicking the activity of *N*-acyl-L-homoserine lactone signal molecules used by *S. meliloti* and other soil bacteria for quorum-sensing regulation of gene expression (Teplitski *et al.*, 2004; Rajamani *et al.*, 2008). None of the *S. meliloti* proteins differently regulated by *C. reinhardtii* mimics were involved in adenosylcobalamin biosynthesis (Teplitski *et al.*, 2004). Repression of *C. reinhardtii* *METE* expression at non-lethal elevated temperatures (Figure 4b) by adenosylcobalamin-producing *S. meliloti* provides direct evidence that algae gene expression can be influenced by cobalamin-producing bacteria. At present, it is unclear whether the greater *METE* repression at higher temperatures is a consequence of more adenosylcobalamin production by *S. meliloti*, more adenosylcobalamin release into the environment by *S. meliloti* cell death or more efficient uptake by *C. reinhardtii* cells. Diatoms increase cobalamin acquisition, reduce cobalamin demand or mitigate cellular damage caused by reduced methionine biosynthesis capabilities under low cobalamin conditions (Bertrand *et al.*, 2012). At present, we do not know whether similar strategies are employed by *C. reinhardtii* or other chlorophyta to alleviate cobalamin scarcity under elevated temperatures or whether they stimulate closer associations with cobalamin-producing bacteria. The minimum cobalamin concentration (74 pMol) required for enhancing *C. reinhardtii* thermal tolerance is greater than the concentration reported in the few studies examining natural cobalamin concentrations in aquatic and terrestrial environments (Schneider and Stroinski, 1987; Panzeca *et al.*, 2009; Sañudo-Wilhelmy *et al.*, 2012), and greater than that required to support growth of algal vitamin B₁₂ auxotrophs (Croft *et al.*, 2005; Kazamia *et al.*, 2012). Thus, in environments depleted in cobalamin, such as those along the southern California-Baja California coastal ocean (Sañudo-Wilhelmy *et al.*, 2012), alga cobalamin auxotrophs would be scarce or algae would be more efficient in cobalamin acquisition or rely more on closer associations with cobalamin-producing bacteria.

Methionine is central to *C. reinhardtii* thermal tolerance, given that as little as a 50% reduction in *METH* expression incapacitates cobalamin-mediated enhancement of thermal tolerance (Figure 6). Methionine is not only required for protein synthesis but also for methylation reactions and methyl cycling, while methionine deficiencies can lead to accumulation of homocysteine to toxic levels. Heat repression of *C. reinhardtii* *METE* gene expression could explain decreases in *METE* pools during heat stress (Figures 4 and 5). In contrast

expression of *METH* was less affected by heat shock, (Figure 4) which likely contributes to the presence of, albeit small, pool sizes of *METH* during heat stress (Figure 5) over prolonged heat exposure. The effect of heat on *C. reinhardtii* *METE* gene expression and *METE* pools is consistent with those reported for other organisms, including *C. reinhardtii* (Muhlhaus *et al.*, 2011); it is conceivable that *METH* is also heat-labile although to a lesser extent than *METE*. In *E. coli*, MetE protein is susceptible to heat-induced aggregation and degradation (Mogk *et al.*, 1999), which stimulates the heat shock responses. Thus, *C. reinhardtii* methionine pools and biosynthesis proteins may be central to growth rate control at elevated temperatures and in activating the heat shock response (Ron and Davis, 1971; Ron 1975; Gur *et al.*, 2002) as well as for maintaining *de novo* protein biosynthesis capabilities required for photosynthetic machinery acclimation to higher temperatures (Tanaka *et al.*, 2000).

The fitness advantage to *C. reinhardtii* of the cobalamin-dependent (*METH*) over the cobalamin-independent (*METE*) methionine synthase might help explain the retention of *METH* gene and the apparent independent loss of *METE* gene in various algal species. A recent survey of cobalamin-dependent enzymes in 15 sequenced algal genomes representative of the Chlorophyta, Chromalveolata and Rhodophyta highlighted the nearly ubiquitous presence of the *METH* gene, whereas the *METE* gene was detected in only 50% of the genomes (Helliwell *et al.*, 2011). Organisms with both *METE* and *METH* are clearly more flexible in environments that routinely or periodically lack cobalamin availability, but the greater thermal tolerance properties of *METH* provides for greater plasticity to address changes in environmental temperature despite the cobalamin chemical dependency.

Our findings also have implications for understanding ecosystem responses to global temperature changes. Following an increase in environmental temperature, we could anticipate changes in microbial community composition, including more cobalamin-producers, more efficient cobalamin-scavengers or variants with physiological adaptations circumventing methionine limitations or with more heat tolerant cobalamin-independent methionine synthases (*METE*). As a consequence of shifts in microbial community structure or metabolic activities, primary productivity, a key component of the global carbon cycle, could be disrupted in the absence of sufficient abundance of cobalamin or cobalamin-producing bacteria. Thus, it might be appropriate to include cobalamin availability and cobalamin-producing bacteria and their production and consumption dynamics in efforts to predict and model marine or terrestrial primary productivity as temperatures rise. Our studies also suggest that optimal algal productivity in engineered systems could be more consistently achieved by relying on cobalamin-dependent (*METH*) rather than

cobalamin-independent (METE) methionine synthase in systems that experience operating temperatures that cause heat stress to algae, thereby decreasing productivity. Consequently, production systems could be operated at higher temperatures in the presence of cobalamin, thereby reducing cooling costs.

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