Ostreococcus tauri is cobalamin-independent

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Abstract

The marine microalga *Ostreococcus* is considered to depend on the methionine synthase METH and its methylated cobalamin cofactor for methionine synthesis. Here I describe minimal media lacking both cobalt and cobalamin yet suitable for clonal growth of *Ostreococcus tauri*. Because *Ostreococcus* lacks the methylcobalamin-independent methionine synthase METE, *Ostreococcus* growth without cobalamin is unexplained.

Keywords

Ostreococcus; METE; METH; minimal medium; gellan gum; *METE* selection

The minimal marine unicellular alga *Ostreococcus* has three clades, each with sequenced representatives: A, B, and C as well as clade D, *Ostreococcus mediterraneus* (Subirana et al., 2013). The model of methionine biosynthesis in *Ostreococcus* states that METH [methylcobalamin-dependent] is the sole methionine synthase and is based on the identification of the gene encoding METH in all three clades. In addition, the gene encoding METE [5-methyltetrahydrofolate-dependent; cobalamin-independent] appears missing for all three clades, leading to the prediction of cobalamin-dependent growth (Helliwell et al., 2011). These observations suggest that if *Ostreococcus* cells were transformed with synthetic DNA designed to express METE, transformants would utilize the endogenous METH substrate 5-methyltetrahydofolate to synthesize methionine and could be selected in the absence of cobalamin.

Materials and Methods

Ostreococcus cell cultures were obtained from the Roscoff Culture Collection, Roscoff, France, and the Provasoli-Guillard National Center for Marine Algae and Microbiota, East Boothbay, Maine, USA. Panels of candidates to be evaluated for DNA transformation were assembled. The panel for clade A consisted of NCMA2972 (Palenik et al., 2007), RCC754, RCC755, and RCC756. The panel for clade C included RCC614 and RCC745 (Derelle et al., 2006). The

members of the panel for clade D were RCC789, RCC1107, RCC1119, and RCC1121. *Cyanidioschyzon merolae* NIES1332 was obtained from the National Institute for Environmental Studies, Tsukuba, Japan.

Ostreococcus cells were cultured in suspension by rocking under continuous blue illumination in minimal medium at room temperature. Minimal medium is artificial seawater (McDonald et al., 2010) supplemented with 0.9 mM $NaNO_3$, 100 µM $Na_2EDTA•2H_2O$, 10 µM $NaH_2PO_4•H_2O$, 10 µM FeNaEDTA, 0.9 µM $MnCl_2•4H_2O$, 300 nM thiamine•HCl, 100 nM Na_2SeO_3, 80 nM $ZnSO_4•7H_2O$, 30 nM $Na_2MoO_4•2H_2O$, 10 nM $CuSO_4•5H_2O$, and 400 pM cyanocobalamin. This list of supplements is simplified from that formulated by Keller (Keller et al., 1987), most notably lacking biotin, $CoSO_4•7H_2O$, NH_4Cl, and tris(hydroxymethyl)aminomethane. In addition, the selenite concentration is 10 nM in Keller medium.

For polymerase chain reaction DNA amplification, template DNA was prepared with Quick-gDNA™ from Zymo Research, PCR primer pairs and gBlocks® were synthesized by Integrated DNA Technologies, and targets were amplified with the Q5 DNA polymerase from New England BioLabs.

Results

Using DNA from RCC614 and RCC745 as template and two primer pairs for each of the two targets *METH* and *URA5/3*, polymerase chain reaction DNA amplification yielded in each case DNA fragments of the predicted size, and authenticated cell culture identity as *Ostreococcus* after long-term serial culture in minimal medium. For NCMA2972, a primer pair also targeting *URA5/3* was used (Table 1).

Minimal medium with cobalamin supported extended serial growth for each cell culture in each clade. In addition, minimal medium containing cobalamin and solidified with 0.125% gellan gum (Sigma-Aldrich) supported green colony growth (Figure 1) for each cell culture in each panel, with the exception of NCMA2972.

As a transformation host, RCC614 was chosen. Mock transformation experiments, without synthetic DNA, were designed to quantitate and evaluate background growth on solidified minimal medium lacking cobalamin. RCC614 suspension cells were cultured in minimal medium with 400 pM cobalamin. 10^6 cells (100 µl) were mixed with 50 µl CutSmart® restriction enzyme buffer [50 mM potassium acetate-10 mM magnesium acetate-20 mM tris(hydroxymethyl)aminomethane acetate (pH 7.9)-100 µg/ml bovine serum albumin; New England BioLabs], layered onto 100 µl protoplast transformation buffer [100 mM $CaCl_2$-200 mM mannitol-40% PEG4000; Yoo et al., 2007], briefly vortexed, incubated for 5 minutes at room temperature, diluted into 10 ml of minimal medium containing 20 pM cobalamin, and cultured for 24 hours. Cells were then aerosolized onto minimal media lacking cobalamin solidified with 0.125% gellan gum at a density of 10^5 cells per 90 mm plate (20 ml solid media per plate). After 10 days at room temperature, approximately 10 to 20 green colonies per plate emerged at normal growth rates. Twelve cobalamin-independent clones were picked into minimal medium without cobalamin and cultured; all clones remained cobalamin-independent after more than 10 serial passages (1/200 dilution/passage). Each cobalamin-independent clone

was authenticated as RCC614 by polymerase chain reaction DNA amplification using the primer pair for *URA5/3* listed in Table 1 which yields a 3207 bp diagnostic fragment (Figure 2).

Because minimal medium lacks cobalt and because these cobalamin-independent cultures are derived from single colonies (clonal), it is unlikely a microbe contaminating these cobalamin-independent *Ostreococcus* cultures is synthesizing cobalamin. It is concluded that cobalamin-independent *Ostreococcus* clones are obtained at a frequency of approximately 0.01% when selected on gellan gum-solidified minimal medium without cobalamin.

Discussion

How does *Ostreococcus* RCC614 synthesize methionine in the absence of cobalamin when it does not appear to have the gene encoding the cobalamin-independent methionine synthase METE? One possibility is that METH has a METE-like function. The dynamic and flexible tetradomain architecture Hcy~Fol~Cob~AdoMet of METH assumes different domain arrangements in its catalytic cycle (Matthews, 2009; Evans et al., 2004), acting as an assembly line for methyl group transfer and methionine biosynthesis. In the absence of cobalamin, there may exist a relative arrangement of the Hcy barrel and the Fol barrel which would position the Zn(II)-activated sulfur of homocysteine bound to the Hcy barrel near the methyl group of methyltetrahydofolate bound to the neighboring Fol barrel and facilitate direct, cobalamin-independent methyl group transfer. In this arrangement the linker between Hcy and Fol unpacks and the Fol domain pivots toward the Hcy domain, such that the substrate-bearing faces of the two barrels approach each other, thereby reducing the distance between the thiolate and the methyl group. The METH structure switches to function as a METE, bypassing the cobalamin shuttle.

If disruption or deletion/replacement of *METH* in cobalamin-independent *Ostreococcus tauri* results in methionine auxotrophy, then METH must have a METE function; other cryptic, unidentified METE-like loci conferring cobalamin-independence are eliminated. Transformation of these *Ostreococcus metH* cells with a *OtaMETH* DNA fragment or with either the *OtaMETH-CmeMETE-OtaMETH* cassette or the *OtaUra5/3-CmeMETE-OtaUra5/3* cassette (Supplementary file 1) would restore methionine prototrophy in medium lacking cobalamin.

Both RCC614 and *Ostreococcus* RCC809 (Grigoriev, 2011; clade B) lack the gene encoding the algal-specific cobalamin acquisition protein 1, whereas NCMA2972 has it (Bertrand et al., 2012). Is cobalamin transported into the cell for clades C and B? If not, is METH a cobalamin apoenzyme in clades C and B and a cobalamin holoenzyme in clade A?

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Table 1

List of PCR primer pairs, DNA sequences, and DNA product sizes

for RCC614 and RCC745 (*Ostreococcus tauri*):

METH 5'.f	GGGGCAGATTTTGTACATTAGCAGTAA	1068 bp
METH 5'.r	AAAACCTAGAGTATGTGCGATGGACATGGGGTCGACGAACTCCTTGTAAGTC	
METH 3'.f	CGCGCCGAATATGGGAAAGAGTAGAAGCAGCGTATTTACTTAGTCTATTTCTTTTTAAAAG	1024 bp
METH 3'.r	CGCAGTTACCGGATGAGGATG	
URA5/3.f	TATCGCATGCGCTTTGCATG	3689 bp
URA5/3.r	TGAAAGTGGAGAAGGTTCCGGACT	
URA5/3.f	CAGCGGCCTGCAGGAAATGAGAAG	3207 bp
URA5/3.r	CTCGAGCTTTTGAACCAACTCGACGGTTT	

for NCMA2972 (*Ostreococcus lucimarinus*):

URA5/3.f	GTCTAGAGCTTGATTTCCACGTCAACC	4039 bp
URA5/3.r	GAGGAATTGCACATGTTGCAGGA	

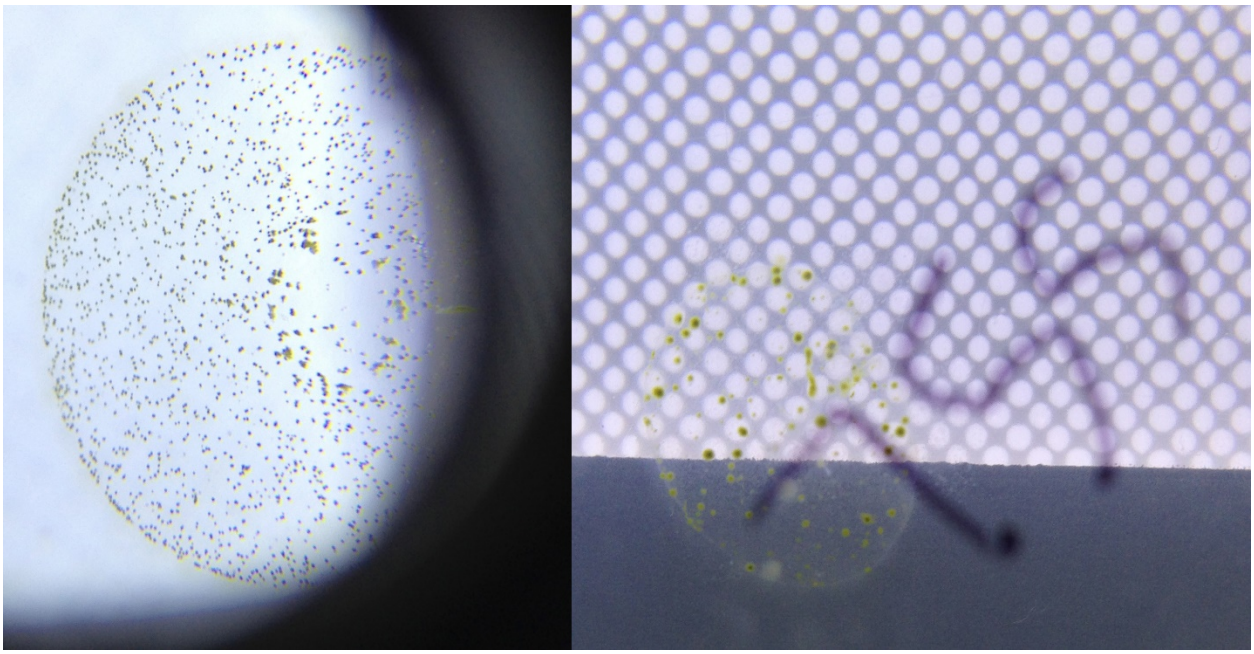

Figure 1. Colony growth of *Ostreococcus* on minimal medium with cobalamin solidified with 0.125% gellan gum.

(left) green minicolonies after 5-7 days [10X magnification].

(right) green colonies of RCC755 after 14 days. The larger colonies are 0.1 mm to 0.2 mm in diameter.

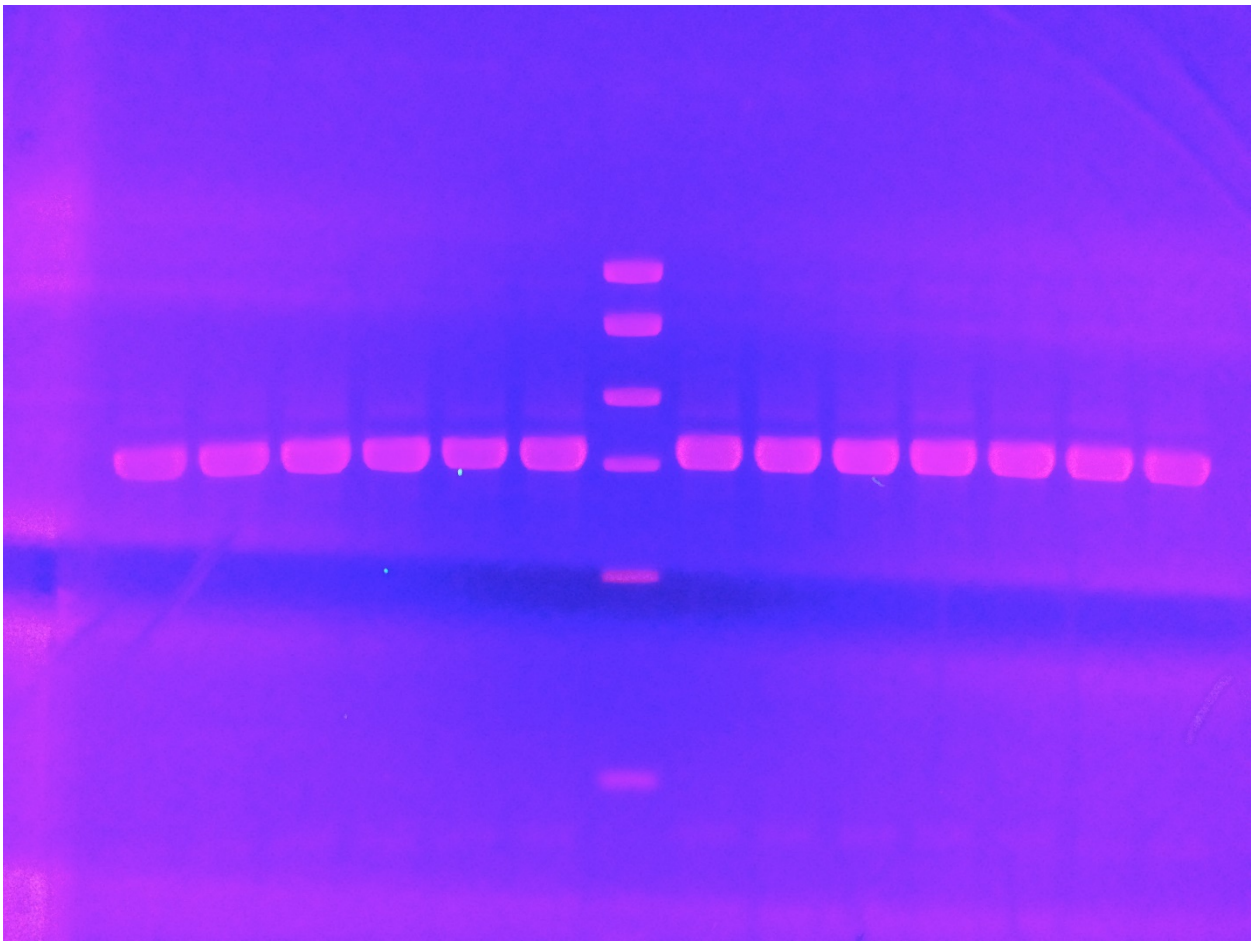

Figure 2. PCR authentication of cobalamin-independent clones as *Ostreococcus tauri* RCC614.

Lanes 1-6 and 9-14 correspond to cobalamin-independent clones isolated on gellan gum-solidified minimal medium lacking cobalamin. Lane 8 is RCC614. Lane 7 is the Mass DNA ladder from New England BioLabs. The diagnostic 3207 bp DNA fragment is synthesized with a primer pair targeting the *URA5/3* locus (Table 1).

Supplementary file 1

OtaMETH-CmeMETE-OtaMETH

Using the 1068 bp *METH* 5' DNA fragment, the 1024 bp *METH* 3' DNA fragment, and the 2366 bp DNA fragment containing *METE* from *Cyanidioschyzon merolae* (Table 1), a 4304 bp cassette *OtaMETH-CmeMETE-OtaMETH* was assembled using the PCR-overlap method (Davidson et al., 2002) with primers *METH* 5'.f and *METH* 3'.r (Table 1), and inserted into the *Sma*I site of pUC19. This cassette, upon integration via homologous recombination (Lozano et al., 2014) into the *Ostreococcus* genome, specifies a replacement of *OtaMETH* with *CmeMETE* (*metH* genotype).

OtaUra5/3-CmeMETE-OtaUra5/3

A second cassette designed to express *Cyanidioschyzon merolae* METE, *OtaUra5/3-CmeMETE-OtaUra5/3*, was Gibson-assembled (Gibson, 2011; New England BioLabs) with four DNA fragments. The first fragment was the 2301 bp PCR product encoding *CmeMETE* (Table 1). The 5' gBlock® contains 188 bp of upstream *Ura5/3 Ostreococcus* sequence and the 3' gBlock® contains 213 bp of downstream *Ura5/3 Ostreococcus* sequence and are below. The fourth fragment was *Eco*53KI-linearized pUC19. When integrated, this cassette specifies a replacement of *OtaURA5/3* with *CmeMETE*, resulting in 5-fluoroorotic acid resistance and uracil auxotrophy (*ura5/3* genotype).

gBlocks® used to synthesize *OtaUra5/3-CmeMETE-OtaUra5/3*

19|745*URA5/3*[188]|1332*METE*

CCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGATTCTCGTACTGTAGAT
CTTTTAGATCTGTGTACGTGTTCTTCGGGTGTGCTGTGCGTCGAGTGCGTCTCTGCTG
AGTCATGAGTCGTCCATGAATGCGTGGGTCATGACTCCACGAGTGCAGGCACTCCAA
CGAGGTCCCGGCGGCGTCCCGCGCGGTGTCGCCACAGAGACGCGCGCGCGCGAGTC
ATGTCCATCGCACATACTCTAGGTTTTCCTCGCTTTGGCG

1332*METE*|745*URA5/3*[213]|19

CGCAGTGGCCAAGGCTATGCGCGCCGAATATGGGAAAGAGTAGAGACATTTTCAAT
AATAATTTGAGCAGATTTCGCGCTACCTATTCGAAACATTACGGCATAACATTCGCT
ATTTTTCATTCAAGCGTAAGAAATGAGCGGTGTGCGCTTCCAGAGGTGCGCCGCTCC
ACGCGTGGAATCGACGGCGCTGTGCGCTCGAGAGATCCAAATCCTCGTCATGTTCTT
ACAATCGCTAATCTGACGTTTGCGAACTCGGTACCCGGGGATCCTCTAGAGTCGACC
TGCAGGCAT