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Complementation of Cobalamin Auxotrophy in *Synechococcus* sp. Strain PCC 7002 and Validation of a Putative Cobalamin Riboswitch *In Vivo*

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ABSTRACT

The euryhaline cyanobacterium *Synechococcus* sp. strain PCC 7002 has an obligate requirement for exogenous vitamin B_{12} (cobalamin), but little is known about the roles of this compound in cyanobacteria. Bioinformatic analyses suggest that only the terminal enzyme in methionine biosynthesis, methionine synthase, requires cobalamin as a coenzyme in *Synechococcus* sp. strain PCC 7002. Methionine synthase (MetH) catalyzes the transfer of a methyl group from N^5-methyl-5,6,7,8-tetrahydrofolate to L-homocysteine during L-methionine synthesis and uses methylcobalamin as an intermediate methyl donor. Numerous bacteria and plants alternatively employ a cobalamin-independent methionine synthase isozyme, MetE, that catalyzes the same methyl transfer reaction as MetH but uses N^5-methyl-5,6,7,8-tetrahydrofolate directly as the methyl donor. The cobalamin auxotrophy of *Synechococcus* sp. strain PCC 7002 was complemented by using the *metE* gene from the closely related cyanobacterium *Synechococcus* sp. strain PCC 73109, which possesses genes for both methionine synthases. This result suggests that methionine biosynthesis is probably the sole use of cobalamin in *Synechococcus* sp. strain PCC 7002. Furthermore, a cobalamin-repressible gene expression system was developed in *Synechococcus* sp. strain PCC 7002 that was used to validate the presence of a cobalamin riboswitch in the promoter region of *metE* from *Synechococcus* sp. strain PCC 73109. This riboswitch acts as a cobalamin-dependent transcriptional attenuator for *metE* in that organism.

IMPORTANCE

***Synechococcus* sp. strain PCC 7002 is a cobalamin auxotroph because, like eukaryotic marine algae, it uses a cobalamin-dependent methionine synthase (MetH) for the final step of L-methionine biosynthesis but cannot synthesize cobalamin *de novo*. Heterologous expression of *metE*, encoding cobalamin-independent methionine synthase, from *Synechococcus* sp. strain PCC 73109, relieved this auxotrophy and enabled the construction of a truly autotrophic *Synechococcus* sp. strain PCC 7002 more suitable for large-scale industrial applications. Characterization of a cobalamin riboswitch expands the genetic toolbox for *Synechococcus* sp. strain PCC 7002 by providing a cobalamin-repressible expression system.**

Synechococcus sp. strain PCC 7002 is a euryhaline, unicellular cyanobacterium that tolerates high light intensities and a wide range of sodium chloride concentrations (1, 2). This organism has one of the highest growth rates known among cyanobacteria (3), has a fully sequenced genome, and is naturally transformable (2, 4). A versatile system for genetic complementation and overexpression has been developed for this organism (5). Despite generally being considered to be photoautotrophic, *Synechococcus* sp. strain PCC 7002 has an obligate requirement for exogenous cobalamin (6), a large and structurally complex cobalt-chelating tetrapyrrole compound. Although cobalamin can only be synthesized *de novo* by some eubacteria and archaea, it is widely used as a coenzyme by many organisms, including eukaryotes, which cannot synthesize cobalamin and must obtain it from the environment (7, 8).

Similar to *Synechococcus* sp. strain PCC 7002, various cyanobacteria encompassing the genera *Dermocarpa*, *Synechocystis*, and *Pleurocapsa*, as well as other *Synechococcus* spp., also share this absolute requirement for exogenous cobalamin (9). In some prokaryotes, cobalamin acts as an essential coenzyme during the anaerobic fermentation of small molecules for growth, but it can also have roles in methionine synthesis and ribonucleotide reduction, functions that are common in aerobic organisms (8). *Synechococcus* sp. strain PCC 7002 performs oxygenic photosynthesis and can also perform aerobic respiration in the dark to provide energy for its metabolic processes (10). When aerobic respiration depletes the available oxygen, these photoautotrophs resort to fermentation to produce ATP for cellular processes (10). Only one anno-

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tated gene in *Synechococcus* sp. strain PCC 7002, that for cobalamin-dependent methionine synthase (*metH*), encodes an enzyme known to require cobalamin as a coenzyme.

Methionine synthases catalyze the transfer of a methyl group from N^5-methyl-5,6,7,8-tetrahydrofolate to L-homocysteine in the final step in the biosynthesis of L-methionine (11). Two unrelated isozymes catalyze this methyl transfer: cobalamin-dependent methionine synthase (MetH, EC 2.1.1.13) and cobalamin-independent methionine synthase (MetE, EC 2.1.1.14). The distribution of the genes for these two enzymes varies widely. In plants, *metE* is common because these organisms do not synthesize cobalamin, but in mammals, *metH* is usually present because they can take up cobalamin from their food sources or acquire cobalamin from their intestinal microbiome. In bacteria and archaea, the distribution of *metE* and *metH* is less predictable, and although only *metH* is present in the *Synechococcus* sp. strain PCC 7002 genome, many eubacteria and some cyanobacteria have genes for both isozymes (12). Unlike the cyanobacterium *Synechocystis* sp. strain PCC 6803, which also solely utilizes MetH for methionine synthesis but does not require exogenous cobalamin as a growth factor (13, 14), *Synechococcus* sp. strain PCC 7002 is not capable of *de novo* cobalamin biosynthesis and thus is naturally a cobalamin auxotroph (6).

Bacteria with both *metH* and *metE* genes can take advantage of switching between the two isozymes to acclimate to changes in the supply of exogenous cobalamin (15). This regulation is usually modulated by cobalamin riboswitches (16). Riboswitches are highly structured elements in the 5′ untranslated leader regions of mRNAs that allow control of that gene by a metabolite, such as cobalamin, by inducing conformational changes in the RNA that result in attenuation at either the transcriptional or the translational level (17). Cobalamin riboswitches are widespread in prokaryotes (18) and are capable of sensing different vitamers of cobalamin with various affinities (19). The use of cobalamin riboswitches to regulate methionine synthase is not unique. Cobalamin is also known to regulate the *btu* genes responsible for its active transport (20), and the *cob* operon responsible for its *de novo* biosynthesis (21). Cobalamin riboswitches that control the transcription of the class Ia ribonucleotide reductase genes, *nrdABS*, are also known (e.g., in *Streptomyces coelicolor* [22]).

Because *Synechococcus* sp. strain PCC 7002 previously could not be studied in the complete absence of cobalamin, the effects of cobalamin limitation were little studied in this cyanobacterium until now. In this study, we show that the cobalamin auxotrophy of *Synechococcus* sp. strain PCC 7002 can be alleviated by complementation with the *metE* gene from a closely related cyanobacterium. The facile deletion of the native *metH* gene from *metE*-complemented *Synechococcus* sp. strain PCC 7002 with no evidence of any deleterious phenotype implies that cobalamin might solely be needed for the functioning of this single enzyme. Furthermore, a cobalamin riboswitch that potentially acts as a transcriptional attenuator was identified and functionally verified in the *metE* promoter of *Synechococcus* sp. strain PCC 73109. This riboswitch was used to create a cobalamin-repressible gene expression system in *Synechococcus* sp. strain PCC 7002.

MATERIALS AND METHODS

Strains, culture conditions and transformation procedure. All of the cyanobacterial strains utilized in this study were obtained from the Pasteur Culture Collection, Institut Pasteur, Paris, France (9). A summary of the strains constructed and the plasmids utilized in this work can be found in Table 1. Wild-type (WT) *Synechococcus* sp. strain PCC 7002 was grown in medium A supplemented with 1 mg of $NaNO_3$ ml^{-1} (designated medium A^+), which additionally contains 4 μg of cobalamin (as cyanocobalamin) $liter^{-1}$ (4, 23, 24). In order to evaluate if selected strains could grow in the absence of cobalamin, medium A^+ was made without cobalamin (medium A^+ B_{12}^-). WT *Synechococcus* sp. strain PCC 7002, *Synechococcus* sp. strain PCC 73109, and *Synechococcus* sp. strain PCC 7002 mutants (described below) were grown photoautotrophically in 20-mm culture tubes containing medium A^+ or A^+ B_{12}^- at 38°C with continuous cool white fluorescent illumination at 250 μmol of photons m^{-2} s^{-1} and sparging with 1% (vol/vol) CO_2 in air, otherwise referred to as "standard conditions" (2). For mutant strains, the following antibiotic concentrations were supplied when appropriate: 50 μg of spectinomycin ml^{-1}; 100 μg of kanamycin ml^{-1}; 20 μg of erythromycin ml^{-1}; and 20 μg of gentamicin ml^{-1}. Transformation of *Synechococcus* sp. strain PCC 7002 was performed as described previously (5).

PCR amplification, digestions, and ligations. The PCR primers utilized in this study are listed in Table S1 in the supplemental material. The PCR amplifications in this work were performed with Phusion High-Fidelity DNA polymerase (catalog number M0530S) from New England BioLabs Inc. (Ipswich, MA). Purification of the PCR products prior to digestion was performed as described previously (5). Genomic DNA was isolated as described previously (25). Digested fragments were purified by electrophoresis on 0.8% (wt/vol) agarose gels with the EZ-10 Spin Column DNA Extraction kit from Bio Basic Inc. (catalog number BS353). Ligations were carried out with T4 DNA ligase in an equimolar reaction mixture (volume of 20 μl) for 8 h at 16°C. Recombinant DNA procedures were performed with chemically competent *Escherichia coli* strain TOP10F′ cells (Invitrogen, ThermoFisher Scientific, Waltham, MA).

Construction of *metE*-complemented *Synechococcus* sp. strain PCC 7002. The BLASTP program (26) was utilized to identify the cobalamin-independent methionine synthase (MetE) gene (http://www.ncbi.nlm.nih.gov/protein/975878911) in *Synechococcus* sp. strain PCC 73109 by comparing it to other cyanobacterial *metE* genes (see Fig. S1 in the supplemental material). The *metE* gene of *Synechococcus* sp. strain PCC 73109 was amplified from isolated genomic DNA with primers metEF and metER (see Table S1 in the supplemental material). The *metE* amplicon was cloned into the endogenous plasmid-based pAQ1Ex_P_{cpcBA} expression system (5) under the control of the strong constitutive phycocyanin promoter (P_{cpcBA}) of *Synechocystis* sp. strain PCC 6803 (Fig. 1A) to generate plasmid pAQ1Ex-P_{cpcBA}[*metE*]. This plasmid contains an *aadA* cassette from pSRA81 (27, 28) that confers spectinomycin resistance and an N-terminal His_{10} tag between the NcoI and NdeI sites. pAQ1Ex-P_{cpcBA}[*metE*] was linearized by ScaI digestion and transformed as described previously (5) into WT *Synechococcus* sp. strain PCC 7002 to generate strain AAP001 (Table 1). Spectinomycin resistance was used for selection of the mutant strain. MetE synthesis was verified by immunoblotting of whole-cell extracts using commercial rabbit anti-His_6 epitope tag antibodies with conjugated horseradish peroxidase (Rockland Antibodies & Assays, Gilbertsville, PA; product code 600-403-382). Immunoblotting was performed as previously described (29).

Deletion of the native *metH* gene from *metE*-complemented *Synechococcus* sp. strain PCC 7002. Upstream and downstream fragments of the *metH* gene (GenBank locus tag SYNPCC7002_A2466) were obtained by PCR with *Synechococcus* sp. strain PCC 7002 genomic DNA as the template (see Fig. S2 in the supplemental material). The upstream fragment was amplified with primers dmetHA_F and dmetHA_R, and the downstream fragment was amplified with primers dmetHB_F and dmetHB_R (see Table S1 in the supplemental material). The *ermC* erythromycin resistance cassette from plasmid pRL409 (30, 31) was obtained through sequential digestion with BamHI and EcoRI and was ligated with the *metH* upstream and downstream fragments. The reaction product was used as the template for nested PCR with nested primers Nest_metHF and Nest_metHR (see Table S1). The nested PCR product was used to trans-

TABLE 1 Bacterial strains and plasmids used in this study

Plasmid or strain	Relevant characteristics	Reference(s) or source
Plasmids		
pRL409	Positive-selection cloning vector, *ermC* (Em^r)	30, 31
pSRA81	pUC19 containing *aadA* from pHP45Ω lacking transcription terminators (Sm^r Sp^r)	27, 28
pRL161	Positive-selection cloning vector, *aphAII* (Km^r)	30
pAQ1Ex	pGEM-7zf pMB1 vector backbone with *Synechococcus* sp. strain PCC 7002 pAQ1 flanking sites, Sp^r	5
pAQ1Ex-P_{cpcBA}[*metE*]	pAQ1Ex *metE*-expressing vector under control of P_{cpcBA}, Sp^r	This study
pAQ1Ex-P_{psbA2}[*metE*]	pAQ1Ex *metE*-expressing vector under control of P_{psbA2}, Sp^r	This study
pAQ3Ex	pGEM-7zf pMB1 vector backbone with *Synechococcus* sp. strain PCC 7002 pAQ3 endogenous plasmid flanking sites, Km^r	5
pAQ3Ex-P_{metE}[*yfp*]	pAQ3Ex *yfp*-expressing vector under control of P_{metE}, Km^r	This study
pAQ3Ex-P_{fused}[*yfp*]	pAQ3Ex *yfp*-expressing vector under control of Φ(P_{cpcBA}-P_{metE}), Km^r	This study
pAQ3Ex-$P_{metE\text{-}1}$[*yfp*]	pAQ3Ex *yfp*-expressing vector with mutated P_{metE} ($P_{metE\text{-}1}$), Km^r	This study
Strains		
Synechococcus sp. strain PCC 73109		9
Synechococcus sp. strain PCC 7002		9
Modified forms of *Synechococcus* sp. strain PCC 7002		
AAP001	pAQ1Ex-P_{cpcBA}[*metE*]	This study
AAP002	pAQ1Ex-P_{cpcBA}[*metE*] Δ*metH*::*ermC*	This study
AAP003	pAQ1Ex-P_{cpcBA}[*metE*]/pAQ3Ex-P_{metE}[*yfp*]	This study
AAP004	pAQ1Ex-P_{psbA2}[*metE*]	This study
AAP005	pAQ1Ex-P_{psbA2}[*metE*]/pAQ3Ex-P_{fused}[*yfp*]	This study
AAP006	pAQ1Ex-P_{cpcBA}[*metE*]/pAQ3Ex-P_{metE}[*yfp*] Δ*metH*::*ermC*	This study
AAP007	pAQ1Ex-P_{cpcBA}[*metE*]/pAQ3Ex-$P_{metE\text{-}1}$[*yfp*] Δ*metH*::*ermC*	
E. coli Top10F′	F′ [*lacI*^{q} Tn*10*(Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen

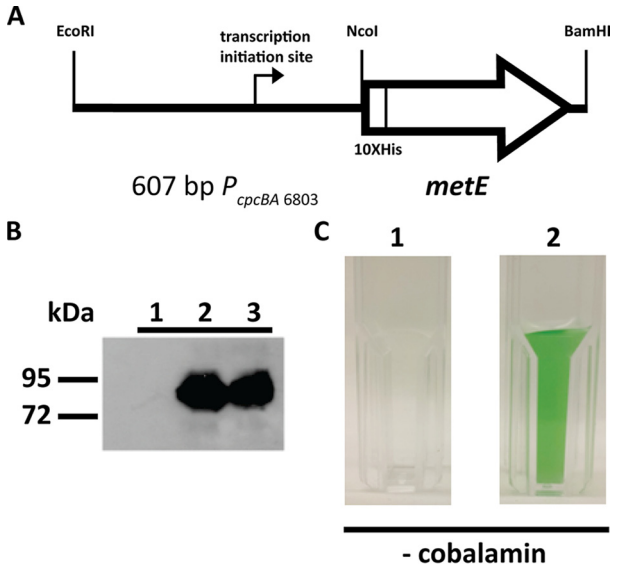

FIG 1 The *metE* gene of *Synechococcus* sp. strain PCC 73109 can complement the cobalamin auxotrophy of *Synechococcus* sp. strain PCC 7002. (A) Representation of pAQ1Ex-P_{cpcBA}[*metE*]. (B) Immunoblot assay of whole-cell extracts with rabbit anti-His_6 antibodies to detect N-His_{10}.MetE. MetE synthesis in strain AAP001 in the presence (lane 2) or absence (lane 3) of cobalamin is shown. No MetE was detected in WT *Synechococcus* sp. strain PCC 7002 (lane 1). (C) Strain AAP001 (panel 2) grows in medium A^+ B_{12}^- lacking cyanocobalamin, whereas a WT strain does not (panel 1).

form strain AAP001 to obtain strain AAP002. Segregation of the *metH* and Δ*metH*::*ermC* alleles was performed on plates prepared with medium A^+ B_{12}^- supplemented with erythromycin and spectinomycin. The segregation status was assessed by PCR with primers Nest_metHF and Nest_metHR (see Table S1).

Studies of *metE*-complemented *Synechococcus* sp. strain PCC 7002 growth. Strain AAP002 was grown in parallel with WT *Synechococcus* sp. strain PCC 7002. Starter cultures of the strains were grown for 2 to 3 days under standard conditions on medium A^+ or A^+ B_{12}^- without the use of antibiotics for the WT and mutant strains. Aliquots were subsequently transferred to 20-mm culture tubes in triplicate. Samples were diluted to an optical density at 730 nm (OD_{730}) of 0.05 with medium, and the OD_{730} was recorded every 2 h for the first 18 h to follow growth during exponential phase; additional measurements were made until 110 h.

Prediction of a cobalamin riboswitch in the promoter region of *metE*. The previously identified B_{12} riboswitch regulons in cyanobacterial genomes were extracted from the RegPrecise database (http://regprecise.lbl.gov) (32). The comparative genomic analysis of *metE* genes in *Synechococcus* sp. genomes was performed with the SEED genomic platform (http://pubseed.theseed.org), which supports the encoding and projection of a metabolic subsystem across prokaryotic genomes (33). Identification of orthologs in closely related genomes and genome neighborhood analysis were performed with the MicrobesOnline tool (http://www.microbesonline.org) (34). Computational identification of B_{12} riboswitches was performed with the RibEx Riboswitch Explorer tool (35). The RNA secondary structures of the B_{12} riboswitches and potential antiterminators and antisequestors were predicted with Zuker's algorithm of free-energy minimization (36) implemented in the Mfold program (http://www.bioinfo.rpi.edu/applications/mfold/). Multiple-sequence alignments of DNA regions upstream of the *metE* gene were constructed with ClustalX (37).

Construction of a cobalamin-regulated expression system. *Synechococcus* sp. strain PCC 73109 genomic DNA was used as the template for the amplification of a 612-bp *metE* promoter region containing the putative cobalamin riboswitch with primers PmetE73109F and PmetE73109R (see Table S1 in the supplemental material). The amplified *metE* promoter (P_{metE}) was cloned into the endogenous plasmid-based expression system pAQ3Ex_P_{cpcBA}[*yfp*] (Km^r) (R. Alvey and D. Bryant, unpublished results), a variant version of the pAQ1Ex_P_{cpcBA} expression platform (5), with flanking sites adapted for homologous recombination with the native pAQ3 plasmid. This construct is identified as pAQ3Ex-P_{metE}[*yfp*] (Table 1). pAQ3Ex_P_{cpcBA}[*yfp*] (Km^r) contains the gene for enhanced yellow fluorescent protein (YFP), the strong phycocyanin promoter, P_{cpcBA}, of *Synechocystis* sp. strain PCC 6803, and the *aphAII* kanamycin resistance cassette from pRL161 (Table 1) (30). Both pAQ3Ex-P_{cpcBA}[*yfp*] (Km^r) and P_{metE} were digested with SacII and NcoI, and resulting fragments were electrophoretically and column purified prior to the ligation reaction. Transformed *E. coli* cells were grown on Luria-Bertani medium and plates supplemented with 30 mg of kanamycin ml^{-1}. Plasmid pAQ3Ex-P_{metE}[*yfp*] was linearized with ScaI and transformed into strain AAP001 grown in medium A^+ B_{12}^-. Transformants were selected on plates prepared with medium A^+ B_{12}^- supplemented with kanamycin and spectinomycin, and a resultant transformant was selected and designated strain AAP003 (Table 1), which is also identified as the YFP fluorescence reporter system.

***In vivo* evaluation of cobalamin-regulated *yfp* expression.** A starter culture of strain AAP003 was grown under standard conditions in medium A^+ B_{12}^- supplemented with kanamycin and spectinomycin to an OD_{730} of 1.0. A WT *Synechococcus* sp. strain PCC 7002 starter culture was grown in medium A^+ under standard conditions. Subcultures of the mutant strain were inoculated at an OD_{730} of 0.05 and grown under standard conditions in the presence (A^+ B_{12}^+) or absence (A^+ B_{12}^-) of cobalamin to an OD_{730} of 1.0. The YFP fluorescence amplitude per OD_{730} unit was determined with an SLM-Aminco 8100C fluorometer modernized for computerized data acquisition by On-Line Instrument Systems (Bogart, GA) (5). Cultures were standardized to an OD_{730} of 1.0 for data acquisition, and the excitation wavelength was 488 nm. The emission wavelength was scanned from 500 to 600 nm, and the emission maximum of YFP at 527 nm was monitored.

Construction of a strong promoter regulated by cobalamin. By using an introduced NheI site, the *metE* promoter region from *Synechococcus* sp. strain PCC 73109, which contains the putative cobalamin riboswitch element, was transcriptionally fused downstream from the strong *cpcBA* promoter of *Synechocystis* sp. strain PCC 6803 (38). Primers cpcBA6803_fusF and cpcBA6803_fusR (see Table S1 in the supplemental material) were used to amplify the *cpcBA* promoter. An intrinsic SacII restriction site near the 5′ end of the P_{cpcBA} amplified region was used for further cloning purposes. Primers metE73109_fusF and metE73109_fusR (see Table S1) were used with genomic DNA from *Synechococcus* sp. strain PCC 73109 as the template to amplify the *metE* promoter with the cobalamin riboswitch elements. Digested fragments were gel purified and then ligated. Nested PCR with primers cpcBA6803_fusF and metE73109_fusR (see Table S1) was performed with the ligation mixture as the template, and the nested PCR product was purified and digested. Plasmid pAQ3Ex-P_{cpcBA}[*yfp*] was digested with SacII and NcoI to replace the existing P_{cpcBA} promoter with the fused promoter construct to generate plasmid pAQ3Ex-P_{fused}[*yfp*]. *E. coli* strains containing pAQ3Ex-P_{fused}[*yfp*] were grown in Luria-Bertani liquid or solid medium supplemented with 30 mg of kanamycin ml^{-1}. To avoid the possibility of homologous recombination between the P_{cpcBA} promoter controlling *metE* expression and the homologous sequence of P_{cpcBA} in the fused promoter, the *psbA2* promoter from *Synechocystis* sp. strain PCC 6803 (5) was used to drive *metE* expression. Primers psbA26803_F and psbA28603_R (see Table S1) were used to amplify P_{psbA2} from isolated *Synechocystis* sp. strain PCC 6803 genomic DNA, which was then introduced into the pAQ1Ex plasmid as described above to obtain plasmid pAQ1Ex-P_{psbA2}[*metE*]. Strain AAP004 was constructed from WT *Synechococcus* sp. strain PCC 7002 by introducing plasmid pAQ1Ex-P_{psbA2}[*metE*] (Table 1; results not shown). Strain AAP005 was then constructed by transforming strain AAP004 with plasmid pAQ3Ex-P_{fused}[*yfp*] that had been linearized with ScaI. Strain AAP005 contains the enhanced YFP fluorescence reporter system. YFP levels in cells containing the fused promoter construct were fluorometrically evaluated *in vivo* as described above.

Evaluation of the putative cobalamin riboswitch in the *metE* leader sequence. Plasmid pAQ3-P_{metE}[*yfp*] was transformed into strain AAP002 to obtain strain AAP006 as a WT control for the assay to study mutations in the P_{metE} cobalamin riboswitch mutant. A variant promoter, $P_{metE\text{-}1}$, was synthesized (GenScript, Piscataway, NJ) by introducing a cytosine-to-thymine transition double mutation (CC to TT in the DNA; CC to UU in the RNA) at the P1 helix-B_{12} box interface of the putative *metE* cobalamin riboswitch. This construct was transcriptionally fused to YFP in pAQ3Ex to obtain plasmid pAQ3-$P_{metE\text{-}1}$[*yfp*], and the resulting construction was transformed into strain AAP002 to obtain strain AAP007. The *in vivo* fluorescence amplitudes of YFP controlled by P_{metE} and $P_{metE\text{-}1}$ in strains AAP006 and AAP007 were measured as described above.

Total RNA extraction. *Synechococcus* sp. strain PCC 73109 cells that had been grown continuously in white light were inoculated into three tubes containing fresh medium A^+ or A^+ B_{12}^- at an OD_{730} of 0.05 and incubated under standard growth conditions. When cells grew to an OD_{730} of ~0.7, 20-ml aliquots of culture were collected from all three tubes and combined. The combined culture sample was centrifuged for 5 min at 5000 × *g* at 4°C, and the resulting cell pellet was quickly frozen with liquid nitrogen and stored at −80°C until processing. For RNA isolation, cell pellets were suspended in 50 mM Tris-HCl, pH 8.0. An equal volume of glass beads was mixed with the cell suspension, which was then subjected to a brief bead beating (4,200 rpm for 10 s) with a Mini-Beadbeater (Biospec Products, Bartlesville, OK). Total RNA was extracted with phenol and further purified with the High Pure RNA Isolation kit (Roche, Indianapolis, IN) as described previously (2).

RT-PCR. Reverse transcription (RT)-PCR analysis of total RNA isolated from WT *Synechococcus* sp. strain PCC 73109 was performed with a MyTaq One-Step RT-PCR kit (Bioline USA Inc., Taunton, MA). The concentration of RNA was determined by NanoDrop measurements (ThermoFisher Scientific, Waltham, MA). The absence of DNA in RNA samples was verified by RT-PCR assays without reverse transcriptase. Primers RT_metE_F and RT_metE_R (see Table S1 in the supplemental material) were designed to amplify specifically a region of the *metE* gene of *Synechococcus* sp. strain PCC 73109. Primers RT_16S_F and RT_16S_R (see Table S1) were designed to amplify a region of the 16S rRNA gene as a control. The amplicons from the RT-PCR analysis were analyzed by agarose gel electrophoresis.

Accession numbers. The genome sequence of *Synechococcus* sp. strain PCC 73109 is available in GenBank under accession numbers CP013998 to CP014003.

RESULTS

***Synechococcus* sp. strain PCC 73109 contains both *metE* and *metH* genes.** *Synechococcus* sp. strain PCC 73109 is a marine cyanobacterium that is very closely related to *Synechococcus* sp. strain PCC 7002 (39). This cyanobacterium can grow in the presence or absence of exogenous vitamin B_{12} (cyanocobalamin) (9; Z. Liu, R. Alvey, Z. Li, and D. A. Bryant, unpublished results). With the BLASTP program (26), the *metE* gene, encoding cobalamin-independent methionine synthase in *Synechococcus* sp. strain PCC 73109, was identified on a plasmid (unnamed plasmid 5; GenBank accession number CP014003.1) by its high amino acid sequence identity (73%) and similarity (85%) to the *metE*-2 gene product of *Synechococcus* sp. strain JA-3-3Ab (CyanoBase ID CYA_0167) (see Fig. S1 in the supplemental material). The amino acid sequence

of MetE is available in GenBank under accession number AMA10842.1. The availability of this gene from a very closely related *Synechococcus* sp. strain led us to question whether this *metE* gene could complement the cobalamin auxotrophy of *Synechococcus* sp. strain PCC 7002.

The *metE* gene from *Synechococcus* sp. strain PCC 73109 complements the cobalamin auxotrophy of *Synechococcus* sp. strain PCC 7002. MetE synthesis driven by the strong P_{cpcBA} promoter (5, 40) in strain AAP001 occurs in both the presence and the absence of exogenous cobalamin, and His_{10}-tagged MetE was detected in cells under both conditions by immunoblotting (Fig. 1B). WT *Synechococcus* sp. strain PCC 7002 is initially able to grow in A^+ B_{12}^- medium because of cellular reserves, residual MetH, and cobalamin carried over from the stock cultures derived from A^+ medium plates or liquid cultures. For this reason, multiple serial transfers in medium A^+ B_{12}^- were required to dilute the cobalamin and MetH to the point at which cells could no longer grow. After three serial transfers, WT *Synechococcus* sp. strain PCC 7002 is unable to grow in medium A^+ B_{12}^-. Strain AAP001 was still able to grow after similar treatment (Fig. 1C). Complementation with *metE* is sufficient to eliminate the obligate requirement for exogenous cobalamin in *Synechococcus* sp. strain PCC 7002, although strain AAP001 had a slightly lower growth rate (see below). Consistent with the notion that strain AAP001 no longer requires cobalamin, the growth rate of this strain was the same in the presence or absence of cobalamin (data not shown).

Deletion of the native *metH* gene from *metE*-complemented *Synechococcus* sp. strain PCC 7002. *Synechococcus* sp. strain PCC 7002 contains a cobalamin-dependent methionine synthase gene (CyanoBase ID SYNPCC7002_A2466 [*metH*]), and bioinformatic analyses have suggested that this is probably the only enzyme that requires cobalamin as a coenzyme. Thus, the inability of this strain to synthesize cobalamin might explain its obligate requirement for exogenous cobalamin (41). Preliminary growth experiments supported this interpretation. Cells were grown in the absence of cobalamin with or without added methionine (1 mM). In the absence of added methionine, cells were unable to grow in medium A^+ B_{12}^-, as expected. Although *Synechococcus* sp. strain PCC 7002 apparently lacks a methionine transporter, cells were able to grow with supplied methionine even after multiple serial transfers to deplete cells of cobalamin. These cells grew in medium A^+ B_{12}^- at approximately half the rate of the WT in medium A^+ (data not shown). This result suggested that methionine synthase was likely to be the only cellular function absolutely dependent on cobalamin or, at minimum, was a cell growth rate-limiting factor under these conditions.

To study this behavior further, a *metH* deletion construct was made by cloning the upstream and downstream flanking regions of *metH* surrounding an erythromycin resistance cassette (*ermC*) from plasmid pRL409 (30, 31) (see Fig. S2A in the supplemental material). This construct was transformed into strain AAP001 to produce a *metH* null mutant, AAP002. Full segregation of the *metH* and Δ*metH*::*ermC* alleles in the deletion strain was confirmed by PCR (see Fig. S2B). Compared to WT *Synechococcus* sp. strain PCC 7002, this strain grew ~20% slower under photoautotrophic conditions (Fig. 2). The calculated doubling times in exponential phase were 4.22 ± 0.14 h for the WT strain and 5.08 ± 0.18 h for strain AAP002. In addition, strain AAP002 no longer required antibiotics to maintain plasmid pAQ1Ex-P_{cpcBA}[*metE*], as the deletion of *metH* makes *metE* essential for viability (data not shown).

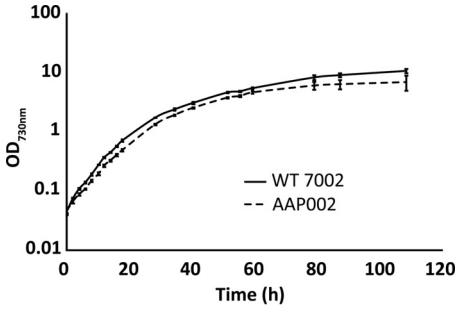

FIG 2 Comparison of the growth of strain AAP002 in medium A^+ B_{12}^- and the WT strain in medium A^+ containing cobalamin. The average values of three independent cultures of each strain are plotted, and the error bars show the standard deviations.

The 5′ untranslated region of the *metE* gene of *Synechococcus* sp. strain PCC 73109 contains a putative cobalamin riboswitch. The *Synechococcus* sp. strain PCC 73109 *metE* locus, comprising the *metE* gene and ~600 bp upstream of the *metE* start codon, was analyzed with the online riboswitch prediction tool RibEx (35). This tool identified a B_{12} box (21), a conserved motif commonly found in cobalamin riboswitches. Other elements were identified that correspond to a system that should control gene expression at the transcriptional level, including a putative terminator helix and a terminator-like poly-T (U) tract (22) (Fig. 3). These elements were identified 328 nucleotides upstream of the start codon of the *metE* gene. The function, if there is any, of the remainder of the leader sequence is unknown.

The putative riboswitch-containing *metE* promoter of *Synechococcus* sp. strain PCC 73109 represses *yfp* expression in the presence of exogenous cobalamin. The *yfp* gene, encoding YFP, has been used as a versatile reporter that can be assayed in whole cells by spectrofluorometry (5). To assess the functionality of the potential cobalamin riboswitch in the *metE* promoter, strain AAP003 was grown in the absence or presence of cobalamin. The P_{metE} promoter that controls the expression of *yfp* in this strain contains the putative cobalamin riboswitch and all of its associated elements (Fig. 4A). YFP fluorescence was detected in AAP003 cells grown in medium A^+ B_{12}^-; however, the YFP fluorescence signal was strongly repressed when cells were grown in A^+ growth medium containing exogenous cobalamin (Fig. 4B). Compared to the fluorescence emission of WT *Synechococcus* sp. strain PCC 7002, a small amount of YFP fluorescence was detected even in the presence of cobalamin in AAP003 cells (Fig. 4B). These data support the *in silico* prediction of a cobalamin riboswitch in the *metE* promoter region that controls *metE* expression via repression in the presence of exogenous cobalamin.

YFP expression is enhanced but still regulated by cobalamin when the *metE* promoter is fused with the *cpcBA* promoter from *Synechocystis* sp. strain PCC 6803. The *metE* promoter (P_{metE}) of *Synechococcus* sp. strain PCC 73109 enables repression of YFP synthesis in the presence of cobalamin in strain AAP003. In the absence of cobalamin, only very low levels of YFP expression occurred (Fig. 4B). To enhance the expression of *yfp*, a fused promoter was constructed by using the strong *cpcBA* promoter from the cyanobacterium *Synechocystis* sp. strain PCC 6803 (5, 40). In plasmid pAQ3Ex-P_{fused}[*yfp*], the *cpcBA* promoter (P_{cpcBA})

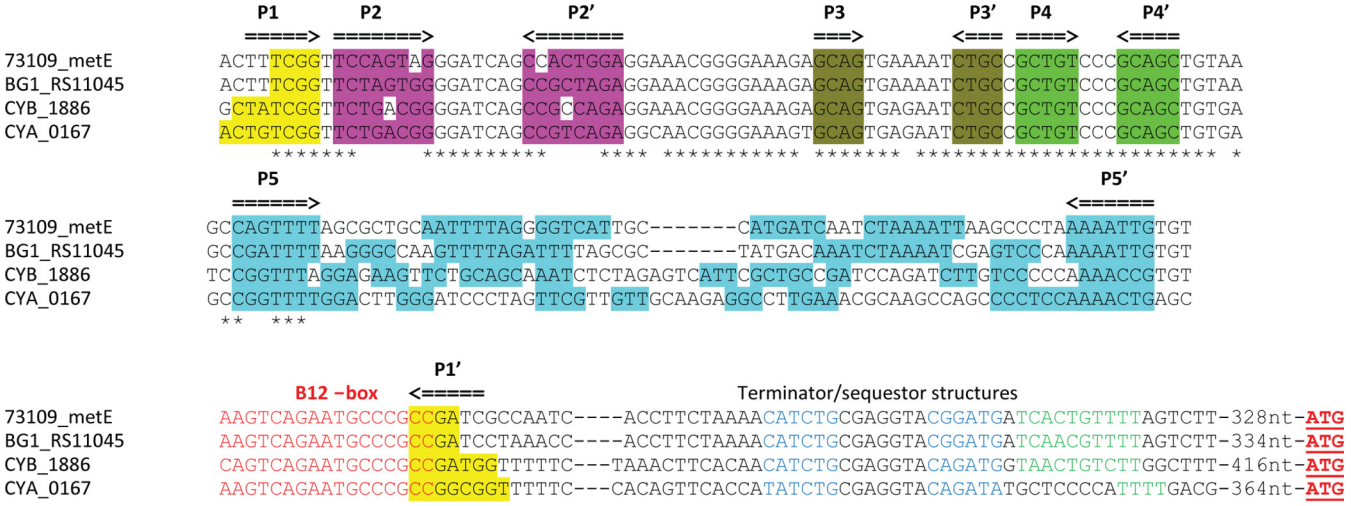

FIG 3 Multiple-sequence alignment of the regions upstream of the *metE* gene of *Synechococcus* spp. and identification of conserved RNA secondary structures of the B_{12} riboswitches. The alignment shows the regions upstream of the *metE* gene of *Synechococcus* sp. strain PCC 73109 (73109_metE), *Synechococcus* sp. strain JA-3-3A (CYA_0167), *Synechococcus* sp. strain JA-3-3Ab (CYB_1886), and *Synechococcus* sp. strain NKBG15041c (BG1_RS11045). The arrows above the sequences indicate the complementary stems of the RNA secondary structure. Base-paired positions are highlighted in matching colors. The sequences of conserved B_{12} box elements are in red font. Secondary structures of putative terminators/ribosomal sequesters are in blue font; poly-T tracts in terminators are in green font. Start codons are in bold red font and underlined.

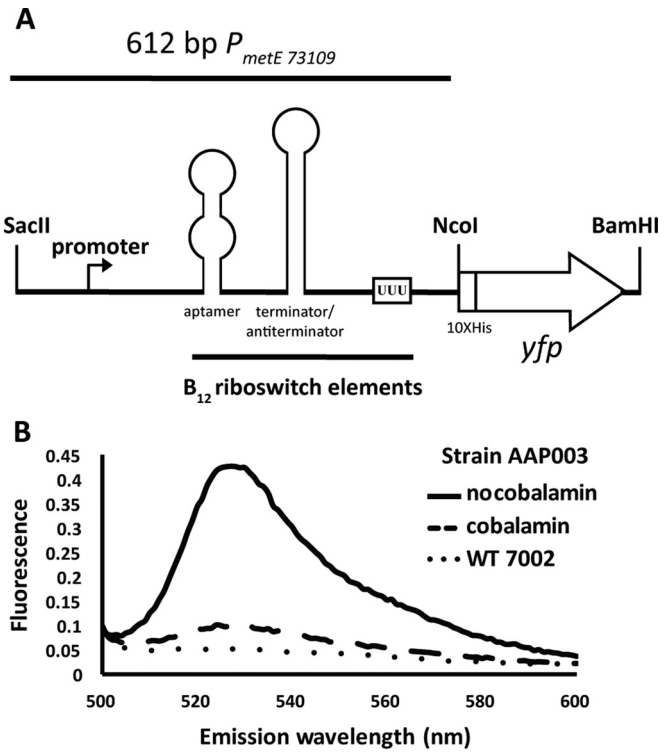

FIG 4 The *metE* promoter from *Synechococcus* sp. strain PCC 73109 contains a putative cobalamin riboswitch. (A) Representation of P_{metE} in plasmid pAQ3Ex-P_{metE}[*yfp*]. (B) Detection of YFP expression in strain AAP003. Fluorescence emission spectra of YFP *in vivo* with *Synechococcus* sp. strain PCC 7002 normalized at an OD_{730} of 1.0 in growth medium A^+ with or without exogenous cobalamin. The dotted line shows fluorescence emission from WT *Synechococcus* sp. strain PCC 7002 grown in medium A^+ as a negative control. The dashed line shows YFP fluorescence emission in strain AAP003 in the presence of cobalamin in A^+ medium, and the solid line shows YFP fluorescence emission for the same strain grown in medium A^+ B_{12}^-.

(38) was fused upstream of P_{metE} and cloned into pAQ3Ex to control *yfp* expression (Table 1; Fig. 5A). Plasmid pAQ3Ex-P_{fused}[*yfp*] was transformed into strain AAP004 to produce strain AAP005. In contrast to the results obtained with AAP003, strain AAP005 had much higher levels of YFP fluorescence in the absence of exogenous cobalamin (Fig. 5B). A low background level of YFP production still occurred in the presence of cobalamin, but the YFP fluorescence intensity increased >6-fold in the absence of cobalamin. The fused hybrid $P_{cpcBA/metE}$ promoter in pAQ3Ex-Pf_{used}[*yfp*] includes the transcription start sites for both the P_{metE} (based on sequence alignment; results not shown) and P_{cpcBA} promoters (40, 42, 43). Results in Fig. 5B demonstrate that this does not affect the strong expression directed by P_{cpcBA} or the cobalamin-dependent repression directed by the P_{metE} promoter.

A cytosine-to-thymine double transition mutation in the conserved 3′ region of the B_{12} box causes loss of cobalamin regulation. Mutations in conserved regions of cobalamin riboswitches are often employed to demonstrate that cobalamin can regulate gene expression through a riboswitch mechanism (16). A transition mutation of the conserved CC motif in the 3′ end of the B_{12} box-P1 helix interface to TT (UU in RNA) was introduced to obtain plasmid pAQ3Ex-$P_{metE\text{-}1}$[*yfp*] (Fig. 6A). Figure 6B shows that YFP expression no longer occurs in response to exogenous cobalamin when *yfp* expression is controlled by promoter $P_{metE\text{-}1}$ instead of WT promoter P_{metE}.

The cobalamin riboswitch in the *metE* promoter of *Synechococcus* sp. strain PCC 73109 probably acts as a transcription terminator in the presence of cobalamin. It was of interest to determine whether the cobalamin riboswitch operates at the transcriptional or translational level. RT-PCR of total RNA from *Synechococcus* sp. strain PCC 73109 with specific primers for the *metE* gene of *Synechococcus* sp. strain PCC 73109 showed that *metE* transcripts were not detectable in cells grown in the presence of cobalamin (Fig. 7A). Conversely, *metE* transcripts were readily

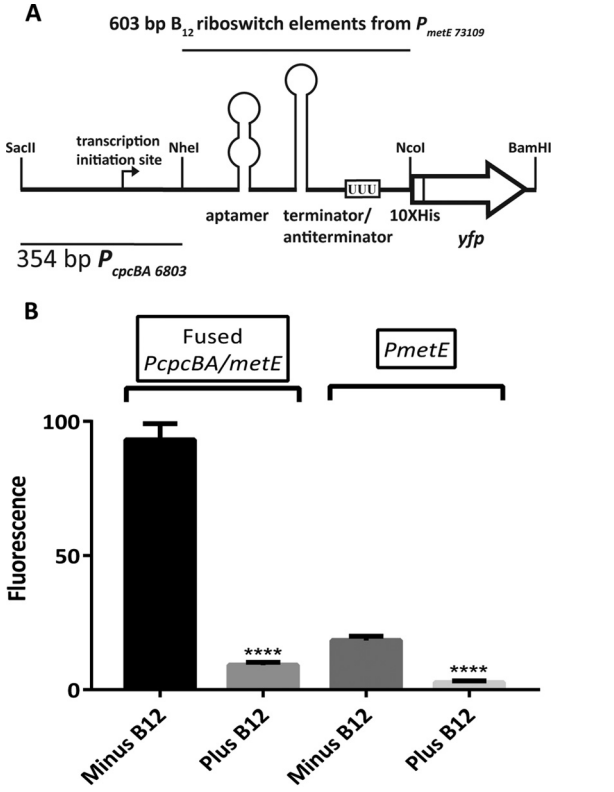

FIG 5 A fused promoter (P_{fused}) can control the expression of YFP in response to cobalamin. (A) Fused promoter P_{fused} construct (derived from P_{cpcBA} and P_{metE}) in plasmid pAQ3Ex-P_{fused}[*yfp*]. (B) *In vivo* fluorescence measurements of YFP in strains AAP005 and AAP003. *Synechococcus* sp. strain PCC 7002 was standardized at an OD_{730} of 1.0 in growth medium A^+ with or without exogenous cobalamin. Each bar represents the average emission amplitude of YFP at 527 nm of three biological replicates after normalization by using the highest fluorescence amplitude as 100%. The error bars represent the standard deviations of these measurements. The asterisks represent statistical significance with *P* values as follows: none, $P > 0.05$; ****, $P \leq 0.0001$.

detectable when *Synechococcus* sp. strain PCC 73109 cells were grown in the absence of exogenous cobalamin (Fig. 7B). This result strongly implies, but further experiments are required to confirm definitively, that the cobalamin riboswitch in the *metE* untranslated leader sequence operates as a transcription terminator when cells are grown in the presence of cobalamin.

DISCUSSION

Few studies have examined the role of cobalamin in *Synechococcus* sp. strain PCC 7002, even though this compound is an essential nutrient for the growth of this organism and other cyanobacteria (6). In fermenting bacteria, cobalamin is associated with essential functions in the metabolism of certain small molecules (e.g., 1,3-propanediol) and is used as a coenzyme in some reactions of the Wood-Ljungdahl pathway (44), but cobalamin is also important in processes including methionine and ribonucleotide biosynthesis (8). With the advent of bioinformatics and the ability to sequence and study complete genomes, the role of cobalamin in *Synechococcus* sp. strain PCC 7002 was revisited with an emphasis on the cobalamin-dependent methionine synthase (*metH*) gene, which is apparently the only gene whose product requires this vitamin as a coenzyme in this cyanobacterium. The absence of the

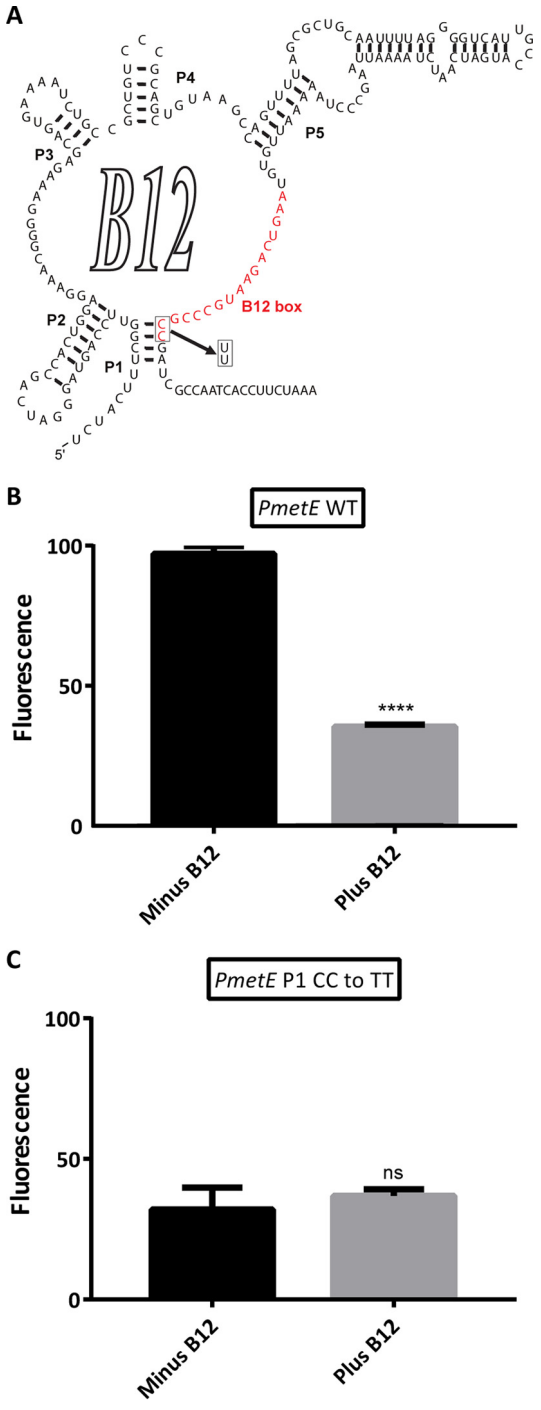

FIG 6 Mutational analysis of the putative cobalamin riboswitch in the P_{metE} promoter. (A) Putative secondary structure of the cobalamin riboswitch in the *metE* leader region of *Synechococcus* sp. strain PCC 73109 in plasmid pAQ3-$P_{metE\text{-}1}$[*yfp*]. The boxed bases represent the B_{12} box-P1 helix interface, where a CC-to-TT (UU in the RNA structure) transition mutation was introduced to generate $P_{metE\text{-}1}$. (B, C) Expression of YFP in the P_{metE} promoter variants in the presence or absence of cobalamin. (B) Fluorescence amplitudes of strain AP006 (P_{metE} promoter) in the presence or absence of exogenous cobalamin. (C) Fluorescence amplitudes of strain AP007 ($P_{metE\text{-}1}$, CC-to-UU mutation) in the presence or absence of exogenous cobalamin. Each bar represents the average emission amplitude of YFP at 527 nm of three biological replicates after normalization by using the highest fluorescence value as 100%. The error bars represent the standard deviations of these measurements. The asterisks represent statistical significance with *P* values as follows: none, $P > 0.05$; ****, $P \leq 0.0001$; ns, not significant.

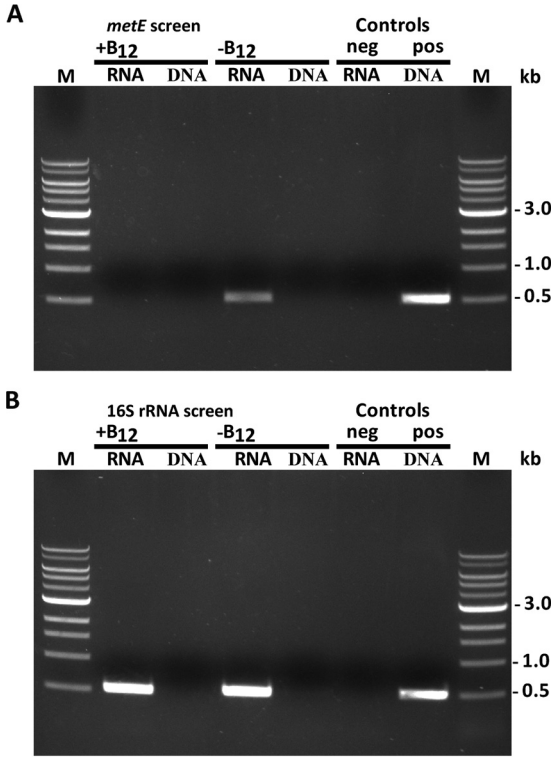

FIG 7 The cobalamin riboswitch represses the expression of *yfp* in the presence of cobalamin. (A) RT-PCR evaluation of the *Synechococcus* sp. strain PCC 73109 *metE* gene in which the total RNA template (RNA) was set to 1 ng/μl from cultures grown in the presence of 4 μg of cyanocobalamin liter^{-1} (+B_{12}) or in medium lacking cobalamin (–B_{12}). DNA lanes represent RT-PCRs lacking reverse transcriptase. The negative control (neg) refers to an RT-PCR mixture containing all of the reagents and amplified under standard RT-PCR conditions but lacking a template to survey for extraneous nucleic acid contamination (no-template control). The positive control (pos) refers to the use of *metE* RT-PCR primers in a PCR with *Synechococcus* sp. strain PCC 73109 genomic DNA as the template. (B) RT-PCR evaluation of 16S rRNA in the total RNA of *Synechococcus* sp. strain PCC 73109 grown in the presence or absence of cobalamin. Conditions are exactly the same as those for panel A, except that primers specific for the 16S rRNA were utilized. All PCR products are 504 bp in size. Lanes M contained molecular size markers.

cobalamin-independent methionine synthase (*metE*) gene and the occurrence of *metH* in *Synechococcus* sp. strain PCC 7002 indicate that this cyanobacterium requires cobalamin for the synthesis of L-methionine (12, 45). The only other alternative would be to take up this amino acid from the surrounding milieu with a specialized transporter (46). The inability to obtain sufficient L-methionine from external sources would render cobalamin an essential component for the otherwise photoautotrophic lifestyle of *Synechococcus* sp. strain PCC 7002.

In a previous study, Wilhelm and Trick (6) investigated the role of cobalamin in *Synechococcus* sp. strain PCC 7002 by comparing cells that had been grown under cobalamin-deficient and cobalamin-replete conditions. The cell densities achieved correlated with the levels of cobalamin in the medium, and cells grown in limiting cobalamin had lower levels of chlorophyll *a* and exhibited changes in the integrity of the thylakoid membranes. Lower cobalamin levels also led to the appearance of several outer membrane proteins, which was the first evidence that a TonB-like active-transport uptake system for cobalamin transport might be regulated by cobalamin itself (47). Nevertheless, a direct correlation between these phenotypes and cobalamin was never demonstrated.

Cobalamin has been suggested to be an important molecule in aquatic systems where extensive exchange of nutrients occurs among diverse organisms and has been shown to play a limiting role in marine systems with cyanobacteria and eukaryotic phytoplankton (48, 49). *Synechococcus* sp. strain PCC 7002 is similar to other types of marine phytoplankton that are unable to produce cobalamin *de novo* and require external sources of this key nutrient (49). However, unlike some of its marine photoautotrophic eukaryotic counterparts that still retain auxotrophy for cobalamin when grown with exogenous L-methionine (50), *Synechococcus* sp. strain PCC 7002 is capable of slow cobalamin-independent growth if a relatively high level of methionine (1 mg ml^{-1}) is added to the growth medium (data not shown). The genome of *Synechococcus* sp. strain PCC 7002 does not appear to encode a methionine transporter but does encode a predicted ABC transporter for branched-chain amino acids (KEGG Pathway entry syp02010), which might account for some methionine transport.

In this study, we confirmed that *metH* is responsible for the cobalamin auxotrophy of *Synechococcus* sp. strain PCC 7002, as it is the sole route for *de novo* methionine biosynthesis in this cyanobacterium. Providing an alternate route for methionine biosynthesis via the expression of *metE* allowed cells to grow in cobalamin-free medium; this rendered *metH* unnecessary, as evidenced by the facile deletion of the *metH* gene (see Fig. S2 in the supplemental material). Cobalamin-independent strain AAP002 grew a bit (~20%) more slowly than its WT counterpart when both were grown under photoautotrophic conditions. There are several possible reasons for the lower growth rate, including the lower turnover rate of MetE than of MetH, which utilizes cobalamin as a coenzyme to facilitate the conversion of L-homocysteine into L-methionine (51). Alternatively, the constitutive overexpression of *metE* from the high-copy-number pAQ1Ex expression system and the strong *cpcBA* promoter in plasmid pAQ1Ex-P_{cpcBA}[*metE*] could perturb cellular metabolites and siphon off important metabolic resources. The adoption of an alternate pathway for methionine biosynthesis could itself affect the global metabolic fluxes, thereby influencing the growth rate.

The identification and validation of a cobalamin riboswitch, with potential transcriptional regulation, in the 5′-upstream untranslated region of the *metE* gene from *Synechococcus* sp. strain PCC 73109 provide a novel genetic tool for controlling gene expression. They also represent an efficient system for characterizing putative cobalamin riboswitches from other organisms that lack available genetic systems by experimentally using YFP as a reporter system in cobalamin-independent *Synechococcus* sp. strain PCC 7002. Future experimentation could also utilize this system to identify genes responsible for uptake of the large cobalamin molecule (16) into *Synechococcus* sp. strain PCC 7002.

The development of a cobalamin-independent version of *Synechococcus* sp. strain PCC 7002 uncovered the unique role of cobalamin in this marine cyanobacterium that serves as a model organism. Development of this strain favors the use of *Synechococcus* sp. strain PCC 7002 for industrial large-scale culturing without the need to provide external cobalamin. The possible implementation of MetE as a selection marker that would simply require the removal of cobalamin is also more feasible than im-

plementing commonly used selection methods based on antibiotic resistance and other methods that require expensive reagents and/or the development of mutant host strains (52). With this development, another step has been taken to establish *Synechococcus* sp. strain PCC 7002 as a versatile and cost-effective model organism for industrial applications.

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