

Characterization of Cyanate Metabolism in Marine *Synechococcus* and *Prochlorococcus* spp.[∇]

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Cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* are the most abundant photosynthetic organisms on earth, occupying a key position at the base of marine food webs. The *cynS* gene that encodes cyanase was identified among bacterial, fungal, and plant sequences in public databases, and the gene was particularly prevalent among cyanobacteria, including numerous *Prochlorococcus* and *Synechococcus* strains. Phylogenetic analysis of *cynS* sequences retrieved from the Global Ocean Survey database identified >60% as belonging to unicellular marine cyanobacteria, suggesting an important role for cyanase in their nitrogen metabolism. We demonstrate here that marine cyanobacteria have a functionally active cyanase, the transcriptional regulation of which varies among strains and reflects the genomic context of *cynS*. In *Prochlorococcus* sp. strain MED4, *cynS* was presumably transcribed as part of the *cynABDS* operon, implying cyanase involvement in cyanate utilization. In *Synechococcus* sp. strain WH8102, expression was not related to nitrogen stress responses and here cyanase presumably serves in the detoxification of cyanate resulting from intracellular urea and/or carbamoyl phosphate decomposition. Lastly, we report on a cyanase activity encoded by *cynH*, a novel gene found in marine cyanobacteria only. The presence of dual cyanase genes in the genomes of seven marine *Synechococcus* strains and their respective roles in nitrogen metabolism remain to be clarified.

Cyanase (EC 4.2.1.104) converts cyanate to carbon dioxide and ammonia in a bicarbonate-dependent reaction: $\text{NCO}^- + \text{HCO}_3^- + 2\text{H}^+ \rightarrow 2\text{CO}_2 + \text{NH}_3$ (16). The enzyme is encoded by *cynS* and is found in a wide range of organisms: *Cyanobacteria*, *Proteobacteria* (including enterobacteria), some Gram-positive bacteria, fungi, and plants. Transcriptional regulation and enzymatic activity were initially studied in *Escherichia coli* strain B/1 (1). In response to cyanate addition, transcription was induced as a polycistronic message of *cynS*, together with a cyanate transporter gene (1). Twenty years later, the protein structure and subunit organization of *E. coli* CynS were determined at 1.65-Å resolution (38). The cyanase monomer was found to be composed of two domains: an N-terminal domain with similarity to the DNA-binding α -helix bundle motif and an “open-fold” C-terminal domain with no structural homology to other proteins. The dimer structure of the cyanase subunit revealed intertwined C-terminal domains with five dimers forming a decameric cyanase holoenzyme. The proposed active site contains three conserved residues—Arg-96, Glu-99, and Ser-122—so that five catalytic sites found in the active decamer form an inner ring around a hollow core (38).

Cyanase activity in cyanobacteria was first described for the freshwater *Synechococcus* sp. strain PCC6301 (UTEX 625) and cyanate decomposition did not require preexposure of cells to cyanate (24). Instead, the decomposition of exogenous cyanate by *Synechocystis* sp. strain PCC6803 and *Synechococcus elon-*

gatus PCC7942 was found to be light dependent (10). Based on the sequence similarity, *cynS* was identified in *Synechocystis* sp. strain PCC6803, *Synechococcus elongatus* PCC7942 (14), and freshwater *Synechococcus* sp. strain PCC6301 (10). In the *Synechococcus* strains, *cynS* was transcribed as a part of an operon, together with *cynABD*, encoding a ABC-type cyanate transporter, while in *Synechocystis* it was cotranscribed with four molybdenum-cofactor biosynthesis genes (14). Transcription of the operon was negatively regulated by ammonium and required the presence of NtcA, a global nitrogen (N) regulator of cyanobacteria (14). Comparative genomics of marine cyanobacteria revealed *cynS* in the majority of *Synechococcus* (26, 31) and in some *Prochlorococcus* (11, 33). The physiological and ecological roles of cyanase in marine cyanobacteria have not yet been elucidated. In the presence of a specific transporter, cyanase may play a role in cyanate assimilation. Marine cyanobacteria strains that possess the *cynABD* genes, encoding an ABC-type cyanate transporter, grew at near-maximal growth rates with cyanate as the sole N source (17). The CynABD complex was recently shown to also contribute to nitrite uptake in *Synechococcus elongatus* PCC7942 (22). Conversely, transport systems for CO_2 , HCO_3^- , NO_3^- , NO_2^- , Cl^- , PO_4^{2-} , and SO_4^{2-} do not contribute to cyanate acquisition (10). The great majority of cyanobacteria that contain *cynS* in fact lack the genes for cyanate acquisition, suggesting a role for CynS in the detoxification of internally generated cyanate, which accumulates as a by-product of the urea cycle or via the degradation of carbamoyl phosphate (33). Here, we characterize transcriptional regulation of *cynS* and the resulting cyanase activity in marine cyanobacteria. Furthermore, we report on a novel source of cyanase activity associated with a conserved hypothetical gene in seven marine *Synechococcus* strains. Based on

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TABLE 1. Sequences of primers used in RT-PCR amplification, their annealing temperatures, and expected PCR product sizes

Target gene	Primer ^a	Sequence (5'-3')	T _a (°C) ^b	Size (bp)	
<i>Prochlorococcus</i> sp. MED4	<i>cynA</i>	RTcA-Med4 F	GGAGGTAGCTAAGGCTATTT	52.5	198
		RTcA-Med4 R	CCTCCTAGATCCCATCTTAT		
	<i>cynS</i>	RTcS-Med4 F	CCTACGGATCCCTCTTATCTA	52	163
		RTcS-Med4 R	CTAGAACCCTATCTCCCTTT		
	<i>ntcA</i>	RTnA-Med4 F	AGAGGAGCAGTAAGGTTATC	51	116
RTnA-Med4 R		TCAGACCTATGTCCTGTTAG			
PMM0615	RTorf1206 F	CCCTGAACCTTTATAGACACC	52	202	
	RTorf1206 R	GACTTTGTCTTCCCATAG			
<i>Synechococcus</i> sp. WH8102	<i>cynA</i>	cA-8102 F	GCCTCTATTCACCTACTAGTTCCCC	53	828
		cA-8102 R	GCGAATTATGCAACAAATCCTACT		
	<i>cynS</i>	cS-8102 F	AGGTTTGGGTTGCATCTTTG	52.5	235
		cS-8102 R	TCTCCGAAATGCTCCCTGAAT		
	16S rRNA	16S-8102 F	CATCATGCCCTTACATCCT	56	103
	16S-8102 R	AACTGAGCCACGGTTTATGG			
<i>Synechococcus</i> sp. WH7803	<i>cynS</i>	RTcS-2 F	GGCCACAGCATCAGCGGAGG	63	283
		RTcS-2 R	GGTGATCTTACGCGATCGCC		
	<i>orf0252</i>	RTorf0252 F	GTGCCCGGTATGTCTTCCTT	59	150
	RTorf0252 R	ATTGGCGTCTGTGTTGAGGT			

^a F, forward; R, reverse.

^b T_a, annealing temperature.

this activity, we have named it *cynH* (for cyanate hydratase), and we refer to this gene as such throughout this report.

MATERIALS AND METHODS

Strains and media. *Prochlorococcus* sp. strain MED4 was grown in the seawater-based PRO99 medium (25), while *Synechococcus* sp. strains WH8102 and WH7803 were grown in artificial seawater medium (41), supplemented to a final concentration of 0.8 mmol of ammonium chloride (NH₄Cl; J. T. Baker, Deventer, MO) liter⁻¹, up to mid-log phase. They were maintained at 25 ± 1°C with gentle agitation at 80 to 90 rpm on a model G2 gyratory shaker (New Brunswick Scientific Co., New Brunswick, NJ) with continuous illumination provided by “warm-white” fluorescence tubes at 20 to 25 μmol of photons m⁻² s⁻¹. For N nutrition experiments, NH₄Cl was replaced with 0.8 mmol of nitrate liter⁻¹, 0.8 mmol of freshly prepared sodium cyanate (NaOCN; Aldrich) liter⁻¹, 0.4 mmol of freshly prepared urea (Amresco) liter⁻¹, or 0.8 mmol of sodium chloride liter⁻¹ to produce an N-free medium. Cultures supplemented with fresh ammonium were used as a control.

For protein expression, we used HMS174 (Novagen/EMD Biosciences, Inc.), Rosetta pLysS (Novagen/EMD Biosciences, Inc.), and BL21-CodonPlus-RIL (Stratagene/Agilent Technologies) *E. coli* strains. Cloning and propagation of recombinant plasmids was performed according to the protocols of the manufacturers. For a negative control of specificity of the cyanate activity, maltose-binding protein (MBP)-fused NtcA and MBP itself were overexpressed in *E. coli* strains Rosetta pLysS and BL21-CodonPlus-RIL, respectively.

Sequence analysis. Protein sequence data of the *cynS* and *cynH* genes and their genomic context were obtained from the genome of *Synechococcus* sp. strain WH7803 available from GenBank, using the DNA sequence viewer and annotation tool Artemis (32). Further genomic data for comparative genomic context study, alignments, and phylogenetic analyses were obtained from the nr public database at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/Blast.cgi>) and the All Metagenomic ORF Peptides subject database of the Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis (CAMERA; <http://camera.calit2.net>) by BLASTN and BLASTP searches. Multiple protein sequence alignments were performed by using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (8). Phylogenetic relationships were analyzed with MrBayes 3.1 (15), with nucleotide frequencies and parameters for the GTR+gamma invariant model estimated from the data. Two independent runs of four chains were run for 2 million generations and sampled every 100 generations; comparison of parameter estimates from the two runs indicated conver-

gence (13). The secondary structures of CynS and CynH were determined by using the Jpred Prediction Server (www.compbio.dundee.ac.uk/www-jpred).

Transcriptional regulation. Cyanobacterial cells (200 ml) grown with different N sources were harvested 3, 6, 9, 12, and 24 h after medium replacement and centrifuged at 10,500 × g for 6 min at 4°C. Cell pellets were resuspended in 0.5 ml of TRI Reagent (Ambion) and immediately frozen at -20°C. Further RNA isolation was performed according to standard procedure recommended by the manufacturer. Prior to the analysis, RNA samples were treated with the DNA-free kit (Ambion) to eliminate DNA contamination, and the RNA purity was confirmed by PCR using the primer pairs listed in Table 1. Final nucleic acid concentrations were determined photometrically (NanoDrop). An ImProm-II reverse transcription system (Promega) kit was used for two-step reverse transcription-PCR (RT-PCR) analysis. For the first step, a standard reaction was applied with the gene-specific reverse primers listed in Table 1. Subsequent PCRs with specific primer sets were performed in a final volume of 50 μl containing 1 to 2 μl of cDNA, 0.5 μmol of each primer liter⁻¹, 0.25 mmol of each deoxyribonucleotide triphosphate liter⁻¹, 1.25 U of *Taq* DNA polymerase (Peqlab), and 10× PCR buffer containing 15 mmol of MgCl₂ liter⁻¹. Using a PTC200 Thermo Cycler (MJ Research, Inc.), the reaction mix was preincubated at 94°C for 5 min, followed by cycles of denaturation at 94°C for 45 s, primer annealing for 30 s (see Table 1 for primer-specific temperatures), and elongation at 72°C for 45 s. Samples of 6 μl were promptly collected after 20, 25, and 30 cycles. Equal volumes of PCR products from the three sets (20, 25, and 30 cycles) were run on 1.5% agarose gels and visualized with ethidium bromide (Sigma). Quantification of gene expression level was performed with ImageJ analysis software (rsbweb.nih.gov/ij/). The set chosen for quantification was always the one before saturation of the PCR amplification was reached (Fig. 1). In the rare case of early saturation, cDNA was PCR amplified for 16 to 18 cycles instead. The reference genes in the RT-PCR analyses were PMM0615 of *Prochlorococcus* sp. MED4 (cell wall hydrolase/autolysin [COG0388]) and *orf0250* of *Synechococcus* sp. WH7803 (Ycf48-like photosystem II stability/assembly factor [COG4447]). These genes were chosen since they are part of the cyanobacterial core genomes and do not alter their transcript level, as assessed by microarray (H. Zer, A. Singer, and A. F. Post, unpublished data). For *Synechococcus* sp. WH8102, the reference was the 16S rRNA gene. The transcript levels of the genes of interest determined were normalized to those of the reference genes. The regression lines describing gene expression over time were compared while testing the hypotheses of coincidence, parallelism, and equality of intercepts (as described in *Master of Applied Statistics* by Pia Veldt Larsen [<http://statmaster.sdu.dk/courses/st111/module09/>]). The *P* value was calculated from the *F* value (www.graphpad.com/quickcalcs/) obtained with the following equation:

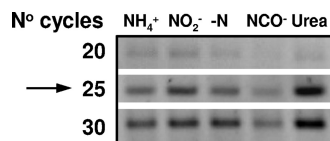


FIG. 1. Typical example of RT-PCR amplification of *cynS* cDNA for *Synechococcus* sp. WH7803. Samples (50 μ l) were run for 30 cycles, and 6- μ l subsamples were collected at five-cycle intervals. PCR products from each cycle set were run on a 1.5% agarose gel and visualized by staining with ethidium bromide. The band density was determined for the set in which subsamples had clearly not reached saturation phase (arrow), assuming that they most closely resembled the phase of exponential amplification.

$$F = \frac{[Ss(\text{time}) + Ss(\text{time} \cdot \text{type})]/2}{Ms(\text{residual})}$$

(where Ss is the sum of squares and Ms is the mean square) from the multivariate analysis of variance (MANOVA)-derived variables.

Cloning of cyanase genes. Genomic DNA of *Synechococcus* sp. WH7803 was extracted by using phenol-chloroform as described previously (28). The complete coding sequences of *Synechococcus* sp. WH7803 *cynS* (424 bp) and *cynH* (201-bp) were PCR amplified with the primer combinations (i) cSEcoRI2-F (5'-AGAAAGGGGAATTCATGAGTTTCGCCGATC-3') and cSPstI-R (5'-CACGATTCAAGCTGCAGTTACCATTTTTGTAAGGAAGG-3') and (ii) scSEcoRI-F (5'-AGTTCGTGGAATTCATGAGTGCTCTTTCCGTTCC-3') and scSPstI-R (5'-GCCCGAGGGCTGCAGTTACGGGGAGTTCGAGATAGG-3'), respectively. The forward/reverse primers contain EcoRI and PstI restriction sites, respectively, to facilitate synthesis of the MBP fusion construct. PCRs (50 μ l) were performed with Phusion high-fidelity DNA polymerase (Finnzymes) and 1.2 ng of DNA template μ l⁻¹. PCRs were run over 30 cycles of denaturation (98°C, 30 s), annealing (58°C for *cynS* and 66°C for *cynH*, 20 s), and elongation (72°C, 20 s), followed by a final 5 min of elongation. PCR products were purified on 1.2% Tris-acetate-EDTA-buffered agarose gels and eluted with the Wizard SV Gel and PCR Clean-Up System. Both the amplicons and pMBP1 vector (kindly provided by P. Sheffield, University of Virginia) were digested with EcoRI and PstI. Purified amplicons (150 ng) were ligated downstream of *malE* on the pMBP1 vector in a 1:3 molar ratio. In-frame assembly of fusion constructs was verified from DNA sequence analysis after transformation into a suitable *E. coli* host strain.

Overexpression of cyanase genes. After transformation into *E. coli*, the expression of the CynS-MBP and CynH-MBP fusion proteins was tested in several strains suitable for protein overexpression. In order to determine optimal conditions for protein expression, we tested different IPTG (isopropyl- β -D-thiogalactopyranoside) concentrations, temperatures, and incubation times. Accumulation of recombinant protein was then confirmed by SDS-PAGE analysis using whole-cell lysate of IPTG-induced cells. The cells were centrifuged at 20,000 \times g for 1 min at 4°C, and the cell pellet was kept overnight at -20°C to ease further lysis. The next day, the cell pellet was resuspended in column buffer (20 mmol Tris-HCl [pH 7.5], 200 mmol of NaCl, and 1 mmol of EDTA liter⁻¹) containing 100 μ g of lysozyme ml⁻¹, 1 mmol of phenylmethylsulfonyl fluoride liter⁻¹, 0.1 mg of DNase ml⁻¹, and 0.1 mmol of MgSO₄ liter⁻¹; incubated on ice for 30 min; and then sonicated. Membrane debris was removed by centrifugation at 20,000 \times g for 15 min at 4°C. Both pellet and soluble fractions were analyzed for recombinant protein accumulation by using SDS-PAGE. The experimental conditions for the expression and accumulation of recombinant proteins in the soluble fraction of specific *E. coli* strains were as follows: MBP-CynS was expressed in *E. coli* strain HMS174 incubated for 4 h at 37°C after the addition of 0.4 mmol IPTG liter⁻¹; MBP-CynH was expressed in *E. coli* strain Rosetta pLysS incubated for 4 h at 22°C after the addition of 0.1 mmol IPTG liter⁻¹; MBP-NtcA was expressed in *E. coli* strain Rosetta pLysS incubated for overnight at 17°C after the addition of 0.2 mmol IPTG liter⁻¹; and MBP was expressed in *E. coli* strain BL21-CodonPlus-RIL incubated for 3 h at 37°C after the addition of 0.3 mmol IPTG liter⁻¹. All *E. coli* strains were supplemented with 100 μ g of ampicillin ml⁻¹, and the Rosetta pLysS strain was supplemented with 34 μ g of chloramphenicol ml⁻¹.

SDS-PAGE and Western blotting. The identity of the induced protein was confirmed by Western blotting, where the cell lysate from a noninduced culture, alongside pellet and eluted fractions from an induced culture, were separated on 14.5% acrylamide gels by SDS-PAGE. Proteins were transferred to a polyvinyl-

idene difluoride membrane at 4°C using transfer buffer containing 54 mmol of Tris liter⁻¹, 384 mmol of glycine liter⁻¹, and 20% (vol/vol) methanol. After blocking the membrane with 5% (wt/vol) skimmed milk powder (Difco) dissolved in TBS (10 mmol of Tris-HCl [pH 7.5] liter⁻¹, 250 mmol of NaCl liter⁻¹) with 0.06% (vol/vol) Tween 20, it was washed three times with TBS buffer containing 0.01% Tween 20 (TTBS). Primary antibody (MBP antibody [R29.6]; ab65; Abcam) diluted 1:1,000 was added, followed by incubation overnight at 4°C, and then washed with TTBS buffer and exposed to a blocking buffer containing peroxidase-conjugated anti-mouse IgG secondary antibody (Jackson Immuno-research Laboratories, Inc.) diluted 1:5,000. Chemiluminescence detection was carried out with an EZ-ECL Enhanced Chemiluminescence Detection Kit for HRP (Biological Industries, Ltd.) using a LAS-3,000 Image Analyzer (Fujifilm).

Purification of recombinant protein. Purification of CynS-MBP and CynH-MBP fusion proteins and MBP was performed using amylose resins (E8021) according to New England Biolabs protocols. In brief, cell pellets were resuspended in 1/10 culture volume of phosphate buffered saline (PBS, 0.05 mmol liter⁻¹, pH 7.6) complemented with 0.2 mg of lysozyme ml⁻¹, 0.05 mg of DNase ml⁻¹, 10 mmol of MgCl₂ liter⁻¹, 1 mmol of dimethyl sulfoxide liter⁻¹, and 1:200 protease cocktail inhibitor, and the cells were disrupted by sonication. After centrifugation at 10,000 \times g for 15 min, the supernatant was mixed with the amylose resin, and after binding for 1 h at 4°C, the column was washed gently with PBS buffer. Recombinant protein was eluted after the addition of 10 mmol of free maltose liter⁻¹ in PBS buffer.

Cyanase activity assay. The *in vitro* cyanase activity of the *Synechococcus* sp. WH7803 associated with the CynS and CynH fusion proteins was measured as described previously (1) with small modifications. In brief, 20- μ g recombinant protein aliquots were used for activity measurements using two controls. The first control was supplemented with all reaction mixture ingredients except cyanate and tested for background ammonia levels occasionally introduced with the recombinant protein mixture. The second control consisted of the reaction mixture without a protein aliquot and tested the spontaneous decomposition of cyanate. The reactions were initiated by the addition of sodium cyanate (NaOCN; Aldrich) and terminated by the addition of 225 μ l of Nessler reagent (K₂HgI₄; Aldrich) diluted 1:3 with double-distilled water. The temperature of the reaction solution was adjusted to the desired value prior to cyanate addition. Reactions were performed in 48-well plates (Nunc) and analyzed on a Microplate Reader Synergy2 (BioTek Instruments, Inc.) within 5 min after termination of the reaction. The specificity of the CynS and CynH activity was demonstrated in parallel control experiments containing MBP by itself or MBP-NtcA, proteins that lack enzymatic activity. Cyanase inhibition reactions were performed after 200- μ mol liter⁻¹ Na-azide additions. All control reactions were performed at 26°C. One unit of cyanase activity was defined as the amount of enzyme required to catalyze the formation of 1 μ mol of ammonia per min. Temperature optima for the fusion proteins were estimated by performing enzymatic reactions at five different temperatures in the range of 4 to 50°C. Protein concentrations were determined with a Bradford assay (3).

RESULTS

Phylogenetic analysis. Cyanase, encoded by *cynS*, is a well-characterized enzyme in *E. coli*. Orthologs to *cynS* are commonly found in a wide range of microorganisms, including marine unicellular cyanobacteria. In an attempt to better define cyanase evolution in cyanobacteria, we performed alignments and phylogenetic analyses of both genomic and environmental CynS sequences. Among 12 marine *Synechococcus* genomes, 11 were found to possess one or more *cynS* orthologs. The only *Synechococcus* lacking *cynS* was WH5701, a strain representative of halotolerant (40), estuarine *Synechococcus*, ancestral to marine *Synechococcus*. Among 12 *Prochlorococcus* genomes only three carried a *cynS* ortholog (17). Figure 2 shows an alignment of translated *cynS* sequences of *Synechococcus* and *Prochlorococcus*, along with cyanase of *E. coli*. With an overall 37 to 44% identity, the alignment revealed a higher degree of sequence conservation for the C-terminal region compared to the N-terminal region. Moreover, amino

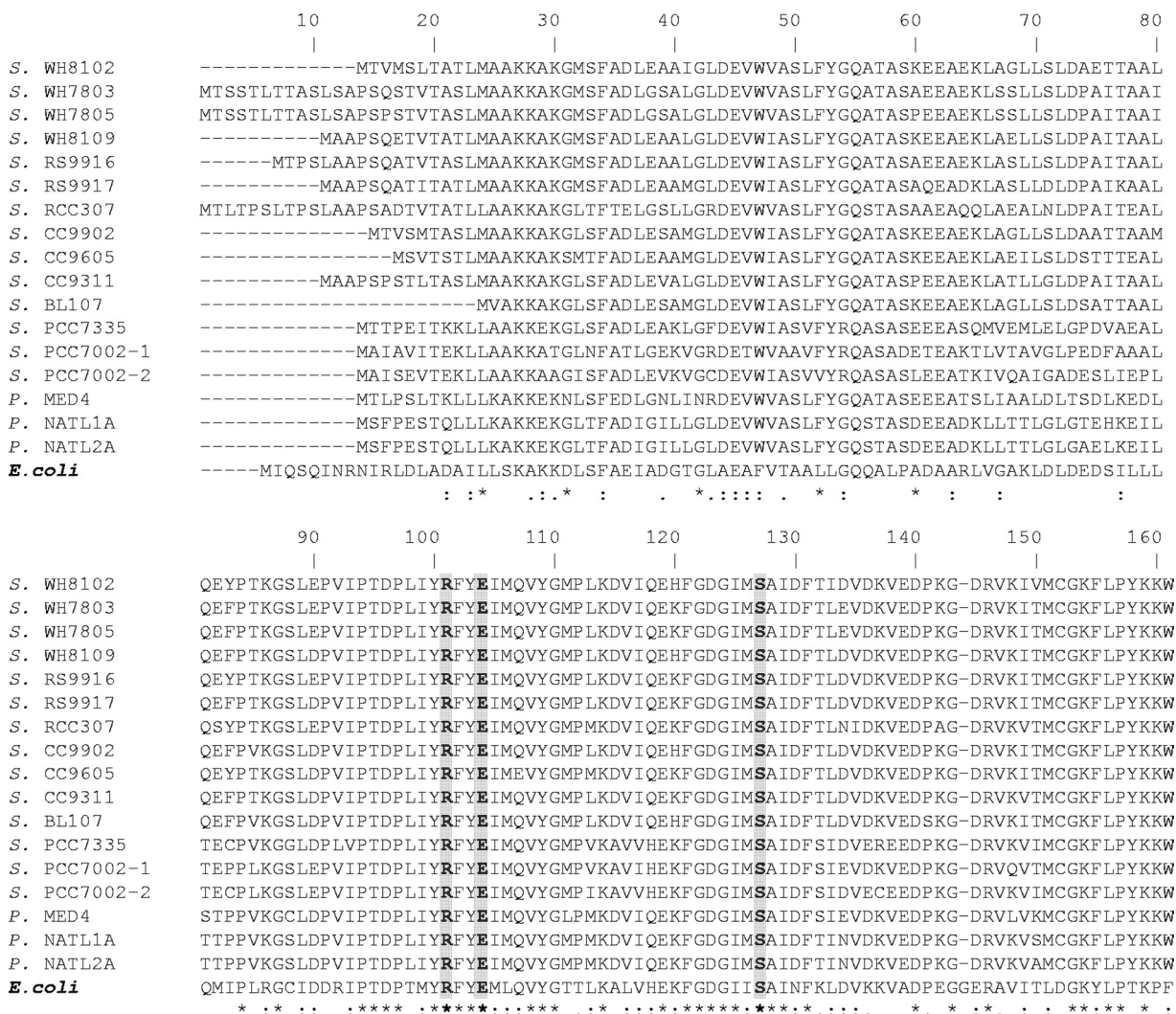


FIG. 2. Alignment of the amino acid sequences for 17 putative cyanase genes in marine *Prochlorococcus* (*P.*) sp. and *Synechococcus* (*S.*) sp. strains, along with that of the characterized *E. coli* cyanase. Fully conserved residues are labeled with “*”, conserved replacements are labeled with “:”, and functional similarity are labeled with “.”. The proposed active-site residues Arg-96, Glu-99, and Ser-122 are shown in boldface against a gray background. The GenBank accession numbers for cyanase sequences in alignment are as follows: *Synechococcus* sp. strains WH8102 (NP_898579), WH7803 (YP_001226218), WH7805 (ZP_01124911), WH8109 (ZP_05789360), RS9916 (ZP_01471501), RS9917 (ZP_01079240), RCC307 (YP_001228741), CC9902 (YP_378288), CC9605 (YP_382939), CC9311 (YP_732088), BL107 (ZP_01469110), PCC7335 (ZP_05037924), PCC7001 (ZP_05043889, and YP_001733904); *Prochlorococcus* sp. strains MED4 (NP_892492), NATL1A (YP_001013899), and NATL2A (YP_292581); and *E. coli* strain K-12 (NP_414874) (4).

acid residues that are proposed to contribute to the catalytic activity of the *E. coli* protein (38) were fully conserved in all *Synechococcus* and *Prochlorococcus* CynS.

Using CynS from *Synechococcus* sp. WH7803 and *Roseovarius* sp. strain 217 as queries in the BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), we retrieved 107 full-length CynS sequences from genomes of bacterial isolates and strains, 9 fungal CynS sequences, and a single archaeal sequence. In addition, 147 translated open reading frames (ORFs) derived from the Global Ocean Survey (GOS) (27) were retrieved by using the CAMERA BLAST Wizard Tool. After a preliminary alignment, we discarded truncated and incoherent sequences. Subsequently, 90 translated environmental sequences >120 amino acids in length, from surface waters of the Atlantic, Pacific, Indian, and Southern Oceans, were aligned with 29

CynS proteins of known origin. The Archaeal CynS was chosen as an outgroup for the construction of a phylogenetic tree. Tree topology suggests that cyanobacterial *cynS* evolved from a common ancestor near the base of the bacterial radiation. Of a total of 90 GOS-derived cyanase sequences, 56 clustered with cyanobacterial CynS, and they were affiliated with known *Synechococcus* and *Prochlorococcus* CynS (Fig. 3). Of these, 77% clustered with *Synechococcus*, and the remaining 23% clustered with *Prochlorococcus*. Branching patterns for *cynS* closely mirror those of 16S and *ntcA* topologies (28, 33), suggesting that cyanobacterial *cynS* distributions resulted from vertical evolution with limited (if at all) contribution of early lateral gene transfer events. Interestingly, *cynS* has thus far not been identified among any of the marine diazotrophic cyanobacteria.

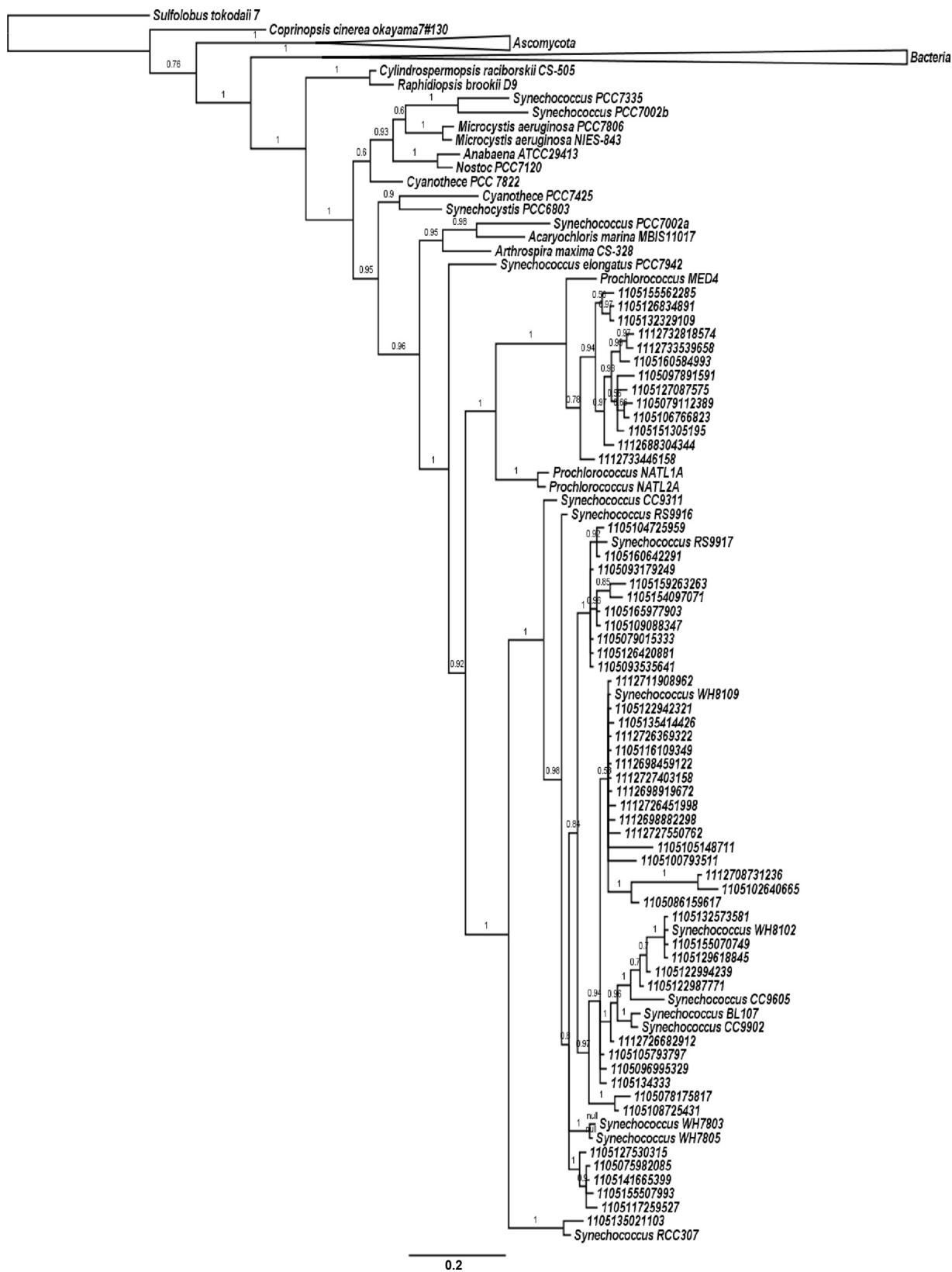


FIG. 3. Tree topology resulting from Bayesian phylogenetic analysis of translated environmental *cynS* sequences (135 amino acids) derived from the Global Ocean Survey database, along with *CynS* sequences retrieved from GenBank. Detailed relationships among cyanobacterial clades are shown, while bacterial and fungal branches are collapsed for the purpose of presentation. The scale bar provides a distance measure for two substitutions per 100 nucleotides between sequences. Posterior probabilities are given at the nodes using a scale from 0 to 1. The denomination “JCVI PEP” has been omitted from all environmental sequences in order to improve the presentation.

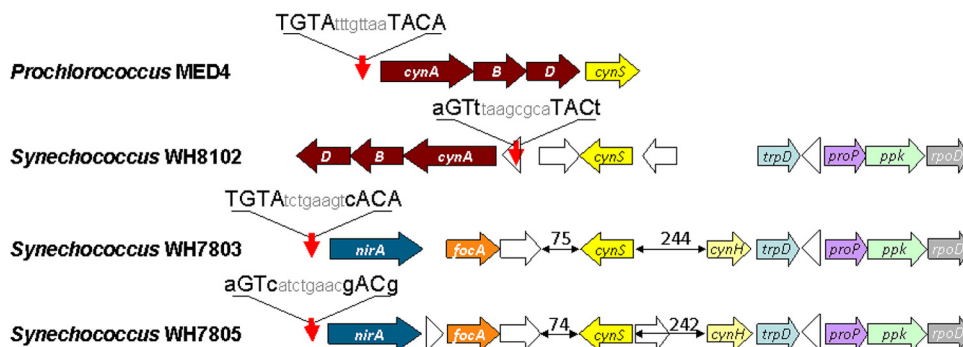


FIG. 4. Schematic representation of the genome regions that contain the cyanate utilization genes in *Prochlorococcus* sp. strain MED4 and *Synechococcus* spp. strains WH8102, WH7803, and WH7805. Vertical red arrows indicate location of putative NtcA binding sites shown above the arrows, and numbers indicate the nucleotide distance between two adjacent genes. Horizontal arrows indicate *cynABD* genes encoding for the cyanate transporter (brown), *nirA* (COG0155, dark blue); *focA* (COG2116, orange), *cynS* (bright yellow), *cynH* (pale yellow), *trpD* (COG0547E, blue), *proP* (COG2814G, purple), *ppk* (COG0855P, green), and *rpoD* (COG0568K, gray) and ORFs encoding unidentified proteins (white).

Transcriptional regulation of *cynS*. Consistent with the fact that cyanate may serve as an N source, cyanate acquisition genes form an integral part of the regulon controlled by NtcA, a global N-stress regulator in marine cyanobacteria (33). However, NtcA control over *cynS* transcription has not been clearly established. Whole-genome microarray analyses for *Prochlorococcus* sp. MED4 (37) showed elevated transcript levels for genes encoding urea (*urtA*) and cyanate (*cynA*) transporters in N-deprived cells. However, despite the *cynABD* and *cynS* organization as an NtcA-controlled operon, the authors of that study reported that *cynS* was not differentially expressed (37). In *Synechococcus* sp. WH8102, *cynABD* and *cynS* are separated by 1,932 bp with two putative ORFs (SYNW2488 and SYNW2489) between them. A putative NtcA binding site was found upstream of *cynA* but not of *cynS* (Fig. 4), suggesting that *cynS* transcription may be uncoupled from N stress responses in strain WH8102. Likewise, the promoter region of *cynS* in *Synechococcus* sp. WH7803 lacked an NtcA binding motif. Here, we aimed at confirmation of the microarray result for *ntcA*, *cynA*, and *cynS* in N-starved *Prochlorococcus* sp. MED4 by quantitative RT-PCR. We further expanded transcription studies of these genes to cells supplemented with different N sources (Fig. 5). We further monitored *cynA* and *cynS* responses in two *Synechococcus* strains in an effort to tease apart their contribution to N-scavenging (uptake and conversion of cyanate to ammonium and CO₂) and detoxification (cyanate conversion only) pathways.

In order to monitor the N status of *Prochlorococcus* sp. MED4 during the experiment, *ntcA* transcript levels were determined alongside those of *cynA* and *cynS*. *ntcA* transcript levels, of cells maintained under different N regimes, increased with time before reaching a maximum after 6 h (cyanate, urea) or 9 h (nitrate, no N) of incubation, compared to basal levels in the presence of ammonium. We found as a general trend that during the first 9 h the transcription patterns of *cynA* and *cynS* followed that of *ntcA* in *Prochlorococcus* sp. MED4, indicating that their transcription occurred in response to changes in N-source and/or its availability (Fig. 5A to C). After 9 h, the transcript had reached steady-state levels or decreased in all treatments. In *Synechococcus* sp. WH8102, the transcription pattern of *cynA* (Fig. 5D) was similar to that in *Prochlorococcus* sp. MED4 (Fig. 5A).

However, the increase of *cynS* transcript was minor and no clear pattern could be discerned in any of the treatments (Fig. 5E). In *Synechococcus* sp. WH7803 lacking cyanate and urea acquisition capacity, basal transcription of *cynS* was determined in cells grown with ammonium. After 3 h, *cynS* transcript accumulated in cells grown with nitrate or urea or in N-free medium. However, in the cyanate-grown culture, transcript levels decreased after 6 h and returned to the levels seen in ammonium-grown cells. After 12 h, *cynS* remained strongly transcribed in N-starved cells only (Fig. 5F). Figure 6 illustrates the parallel pattern of *cynA* and *cynS* transcript accumulation observed for *Prochlorococcus* sp. MED4 (panel A). In contrast, a significantly different pattern ($P < 0.05$) was observed in *Synechococcus* sp. WH8102, suggesting that the *cynA* and *cynS* respond to different N signals and controls in this strain.

Genomic context of *cynS*. The genomic context of *cynS* was different in different marine cyanobacterial strains. In *Prochlorococcus* sp. strains NATL1A and NATL2A, *cynS* was positioned among conserved hypothetical genes. In *Prochlorococcus* sp. MED4, *cynS* was positioned immediately downstream of *cynABD* (Fig. 4), and it was probably transcribed as part of a polycistronic message (Fig. 6), as in *E. coli*. In marine *Synechococcus* genomes *cynS* is confined to a 60-kb region that contains the major N-acquisition genes (33). It is typically found downstream of *nirA* (assimilatory nitrite reductase) and *focA* (formate/nitrite transporter) genes and flanked by four genes with a fully conserved localization on these genomes: *trpD*, *proP*, *ppk*, and *rpoD* (respectively encoding for glycosyl transferase family protein, an unidentified permease, polyphosphate kinase, and an alternative RNA polymerase sigma factor; Fig. 4). Interestingly, *Synechococcus* sp. WH7805 carries an ORF near *cynS* that was identified as a cyanate hydratase (i.e., cyanase) in the automated annotation pipeline (Fig. 4). The predicted amino acid sequence appeared unique and orthologs were found in seven marine *Synechococcus* genomes, as well as on a clone GRIST19 from a metagenomic library obtained from the Atlantic Ocean (GenBank accession no. EU795157). *Synechococcus* sp. strain CC9311 was found to possess two copies of *cynH*. Sequence comparison revealed no significant similarity between known CynS sequences and the

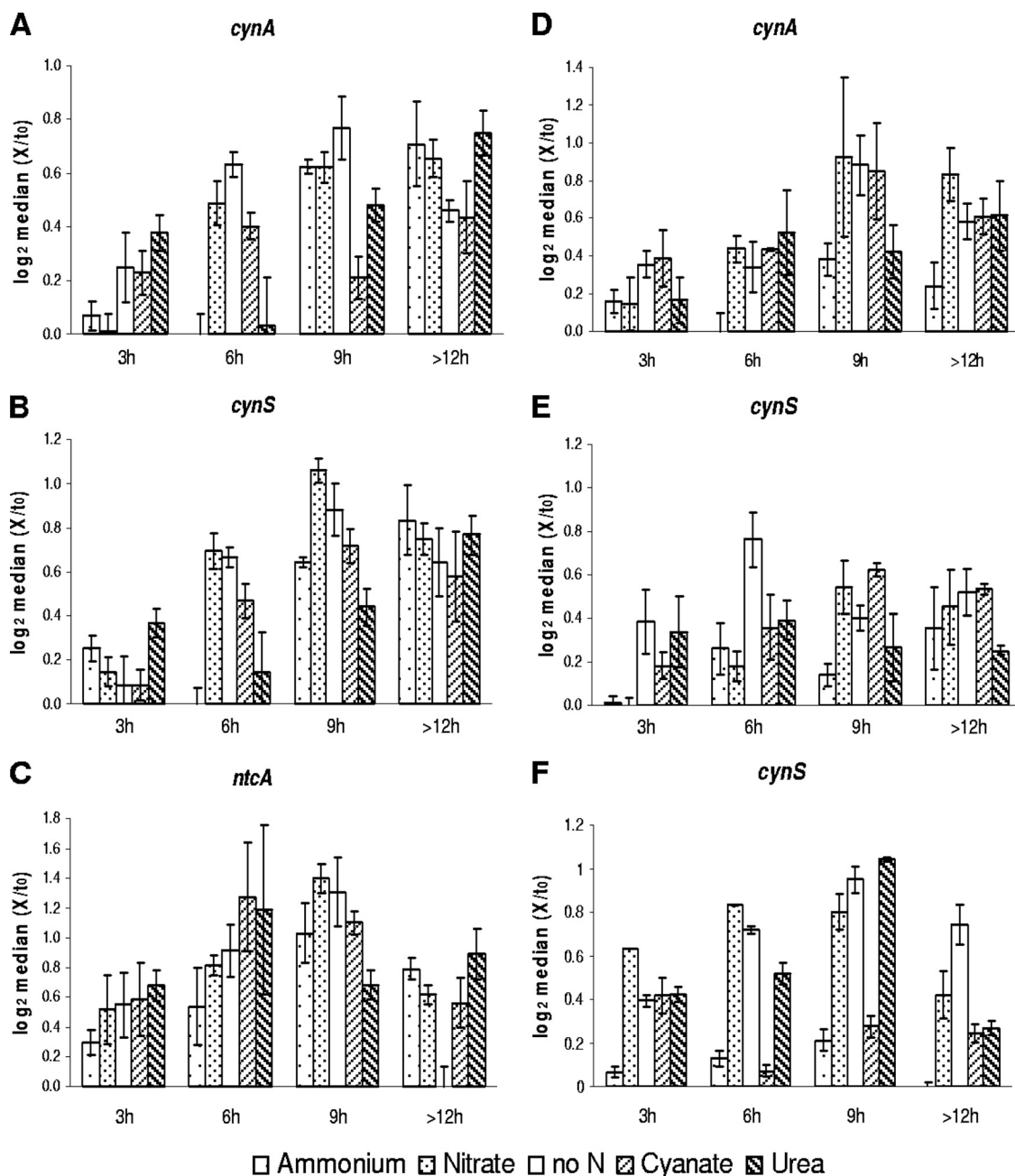


FIG. 5. Transcript accumulation of *cynA* (A and D), *ntcA* (C) and *cynS* (B, E, and F), in *Prochlorococcus* sp. MED4 (A to C), *Synechococcus* sp. WH8102 (D and E), and *Synechococcus* sp. WH7803 (F) cells grown on ammonium or on alternative N sources or deprived of combined N for 3, 6, 9, and >12 h after resuspension in fresh medium. The data are \log_2 of median values of three replicates, normalized to their initial transcription level, with 25th to 75th percentiles.

short protein encoded by *cynH*. Moreover, CynH could not be assigned to a functional protein family, since no known structural domains were identified in its amino acid sequence by Superfamily 1.73 (42) and Phyre Server 0.2 (20). In the sections below we describe experiments pertaining to cyanase activity associated with overexpressed fusion constructs of WH7803 *cynS* and *cynH*.

CynS and CynH overexpression. Overexpression of the CynS-MBP and CynH-MBP fusion proteins in different *E. coli* hosts after IPTG induction was found to be optimal in the *E.*

coli strains HMS174 and Rosetta pLysS, respectively. The recombinant proteins were purified on amylose resin, and the protein presence in the elution fraction was confirmed by SDS-PAGE (Fig. 7). After elution, purified fusion proteins appeared as an ~50-kDa peptide for CynS-MBP, while the apparent molecular mass of CynH-MBP was slightly smaller (~48 kDa). Both were in close approximation to the estimated molecular masses for *Synechococcus* sp. WH7803 CynS (14.3 kDa) and CynH (6.2 kDa) fused to MBP (42.1 kDa). Purified fusion proteins appeared as a single band following the second

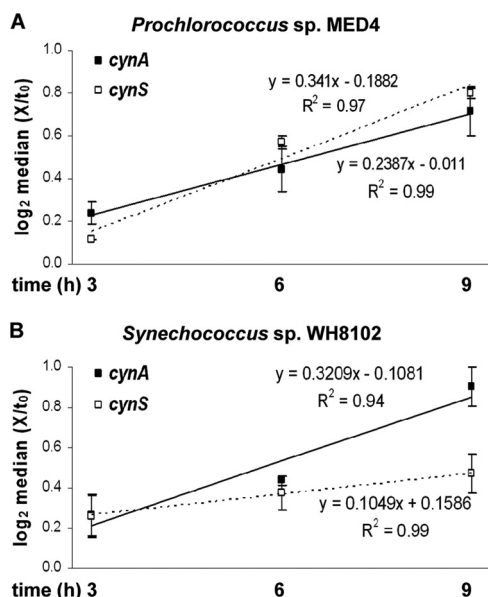


FIG. 6. Median log-normalized values of *cynA* and *cynS* transcription for *Prochlorococcus* sp. MED4 (A) and *Synechococcus* sp. WH8102 (B) cultures grown on alternative N sources or with no N for 3, 6, and 9 h after medium replacement. The error bars represent 25th to 75th percentiles. Linear regression values and R^2 values for *cynA* (continuous) and *cynS* (dashed) accumulation are presented above and below the respective trend line.

elution off the maltose resin (Fig. 7B and C). We confirmed the identity of the purified protein by immunoblotting with monoclonal antibodies against MBP (not shown). The levels of fusion protein were below detection for both constructs in crude lysate of noninduced cells but were readily identified in lysate of IPTG-induced cells. A distinct cross-reactivity with the α -MBP antibody was obtained in both supernatant and pellet fractions, implying that the recombinant protein was in part directed to inclusion bodies. In the elution fraction for CynH-MBP, a single band indicated the presence of the fusion protein in a stable configuration. For CynS, however, two bands were detected, presumably representing CynS-MBP and a product resulting from spontaneous cleavage of the fusion protein.

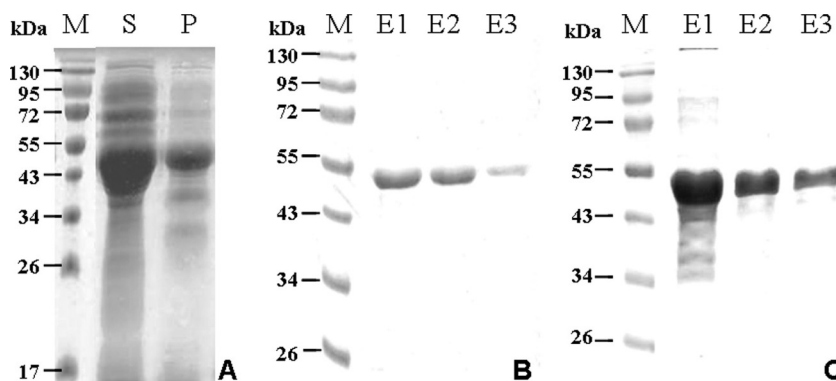


FIG. 7. (A) IPTG-induced expression of CynS and its accumulation in soluble (lane S) and particulate (lane P) fractions of *E. coli* cell lysates. (B and C) Purification of CynS (B) and CynH (C) recombinant proteins using amylose resin-based affinity chromatography. M, molecular weight markers; E1 to E3, elution fractions.

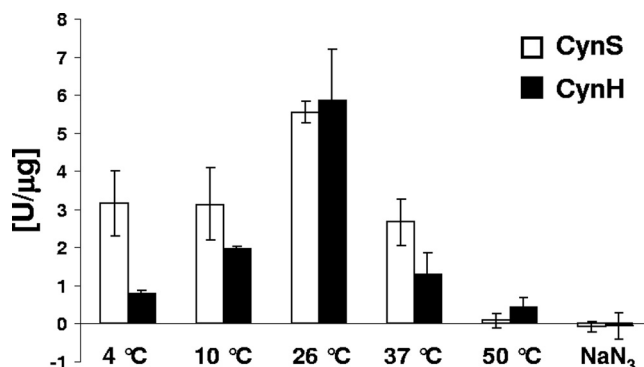


FIG. 8. Characterization of cyanase activity of CynS and CynH fusion constructs at different temperatures and their sensitivity to NaN_3 (200 $\mu\text{mol liter}^{-1}$) addition. Freshly prepared sodium cyanate (2 mmol liter^{-1}) was added to the reaction mix (50- mmol liter^{-1} PBS buffer [pH 7.6], 3 mmol of sodium bicarbonate liter^{-1}) complemented with aliquots of recombinant protein (20 μg) and incubated at the desired temperature for 10 min. Ammonium accumulation was determined with Nessler reagent. The activities shown are averages from at least nine replicates from three independent experiments.

Aliquots of the second eluted fraction for both fusion constructs were subsequently tested for cyanase activity. Measured as ammonium liberated from cyanate, cyanase activity of CynS was maximal (5.56 U mg^{-1}) at 26°C, and rapidly dropped to 50% of this maximum activity at both higher and lower temperatures (Fig. 8). The protein became rapidly inactive at higher temperatures and only residual activity (0.09 U mg^{-1}) was detected at 50°C (Fig. 8). Furthermore, we report here on a distinct cyanase activity associated with the gene product of *cynH*, identified as a cyanate hydratase in genome annotations, a characterization that thus far lacked experimental evidence. In general, cyanate dependent ammonium accumulation rates were similar to those obtained with CynS: highest cyanase activities (5.87 U mg^{-1}) were measured at 26°C and activities declined at both higher and lower temperatures to 0.80 U mg^{-1} at 4°C and 0.43 U mg^{-1} at 55°C. Cyanase activities were derived specifically from either CynS or CynH, as ammonium failed to accumulate when MBP or overexpressed NtcA-MBP (an N regulatory protein) were added to the reaction mix (data not shown). No ammonium accumulation was observed for

CynS nor CynH in the presence of the cyanase inhibitor Na-azide (Fig. 8).

DISCUSSION

Cyanase serves several functions, the most pronounced being the detoxification of cyanate generated in various metabolic pathways (7, 35). Besides detoxification, microorganisms use cyanase in the assimilation of cyanate from the environment. *E. coli* transports cyanate into the cell via a cyanate permease encoded by *cynX* (2, 34). Cyanobacteria utilize cyanate following its acquisition via an ABC-type transport system (10, 17, 24). It has been proposed that cyanate and urea play an important role in the N cycle of marine oligotrophic environments (17). In the present study we characterized the evolution, marine distribution, and transcriptional regulation of *cynS* (cyanase) and the activity associated with its gene product. We further report on a novel cyanase encoded by *cynH* in marine *Synechococcus*.

CynS tree topologies show cyanobacteria as a well-defined branch emerging at the base of the bacterial lineage. Cyanase was found in *Synechocystis* sp. PCC6803 (19), *Synechococcus elongatus* PCC7942 (39), the filamentous diazotrophs *Anabaena* sp. strain ATCC 29413 and *Nostoc* sp. strain PCC7120 (9), *Synechococcus* sp. strains PCC7002 and PCC7335 from brackish, estuarine waters (29), toxic bloom-forming *Microcystis* (18), and members of the unicellular marine *Synechococcus* and *Prochlorococcus* (the present study). A total of >60% of the GOS-derived cyanase sequences were identified as cyanobacterial. This includes 10 clones from a hypersaline lagoon that clustered with *Synechococcus* sp. strain RS9917, a euryhaline ecotype (6, 12).

Cyanobacteria likely acquired cyanase during the very early stages of their evolution. Tree topologies of cyanobacterial CynS matched phylogenies based on 16S rRNA, ITS and *ntcA* (12, 21, 28, 30, 33), and branching was supported by strong posterior probabilities. Based on these observations, we suggest that *cynS* was common in ancestral cyanobacteria and *cynS* was lost from many modern cyanobacteria. Our tree topology suggests that the importance of lateral gene transfer of *cynS* was minor; however, it might occur among related species. Thus, the estuarine *Synechococcus* sp. PCC7002 carries two *cynS* orthologs that share 74% identity at the amino acid level (*Synechococcus* PCC7002a and PCC7002b in Fig. 3). *Synechococcus* sp. PCC7002a, encoded by a stand-alone *cynS* gene, clustered with CynS of the endosymbiont *Acaryochloris marina* MBIC11017, which is ancestral to *Synechococcus* sp. PCC7002 (36). The *Synechococcus* sp. PCC7002b homolog is most closely related to CynS of *Synechococcus* sp. PCC7335 and partakes in an NtcA-regulated *cynABDS* operon, which is very similar to our observations for *Prochlorococcus* sp. strain MED4. Thus, the cyanase gene is involved in lateral gene transfer, suggesting that different CynS may carry out distinct functions in the cyanobacterial cell. We propose that CynS by itself may provide the cell with means to detoxify internally generated cyanate, whereas the *cynABDS* operon encodes the utilization of external cyanate. The presence of two cyanase homologs on a single genome suggests that both functions play distinct roles in the N metabolism and N assimilation of cyanobacteria.

In an attempt to set apart the cyanate detoxification and utilization functions of cyanase, we studied transcriptional regulation of *cynS* and *cynA* alongside that of the N-regulatory gene *ntcA*. In *Prochlorococcus* sp. MED4, in contrast to *Synechococcus* sp. WH8102, *cynABDS* genes showed coordinated expression in response to N deprivation (Fig. 6). This is in agreement with the gene arrangement in the strains examined (Fig. 4). In *Synechococcus* sp. WH7803, which lacks the transporter genes, N-independent regulation of *cynS* was observed. This implies that *cynS* in *Prochlorococcus* sp. MED4 takes part in utilization of external cyanate, whereas the presence of *cynS* in *Synechococcus* genomes indicates a requirement to detoxify an intracellular cyanate. There are several possible sources for the intracellular cyanate in cyanobacteria. A substrate of the urea cycle, carbamoyl phosphate is known to decompose spontaneously to cyanate and phosphate (2). Urea undergoes spontaneous transformation to cyanate by an isomeric change (5, 23). The origin of urea in *Synechococcus* and *Prochlorococcus* cells is unclear, since they lack the *arg* gene product that facilitates urea hydrolysis (33). However, marine cyanobacteria may convert excess arginine to spermidine by sequential action of arginine decarboxylase (EC 4.1.1.19), agmatine ureaohydrolase (EC 3.5.3.11), and spermidine synthase (EC 2.5.1.16). Hence, despite a lack of arginase (EC 3.5.3.1), the toxic cyanate can transform from urea produced by agmatine ureaohydrolase.

In an attempt to confirm cyanase activity by CynS from unicellular marine cyanobacteria, the *cynS* was cloned as a fusion construct with MBP (CynS-MBP), overexpressed in an *E. coli* background, and subsequently purified on amylose resin. Using enzyme assays, we clearly identified CynS as a functionally active cyanase. Similarly to cyanases described in other studies (2, 9, 38), the CynS-MBP construct showed bicarbonate-dependent cyanate degrading activity that was inhibited by Na-azide, the latter preventing binding of substrate to the holoenzyme (38). Cyanase activity was also confirmed for a short peptide, a product of an ORF that was tentatively annotated as cyanate hydratase, and we propose to rename this ORF *cynH*. The identification of an additional cyanase raises questions about its origin and physiological importance. The *cynH* gene was found in seven marine *Synechococcus* genomes, as well as on the metagenomic clone GRIST19. Although located on the same genomic region, *cynS* is separated from *cynH* by 210 to 244 bp and is transcribed in the opposite orientation (Fig. 4). It is unlikely that *cynH* resulted from (partial) gene duplication since its amino acid sequence does not align with any part of *cynS*. The two genes are thus paralogs. Nine *cynH* sequences shared a high degree of identity in the C-terminal half (Fig. 9B) similar to CynS, suggesting that the catalytic domain of CynH is confined to this region. The catalytic site of CynS contains Arg (R), Glu (E), and Ser (S) residues (38) in a configuration conserved across bacteria, fungi, and plants (9). Interestingly, we identified three fully conserved amino acid residues in CynH, identical to those of CynS, and their configuration is reminiscent of the active site of CynS. Moreover, secondary structure predictions indicated the presence of an α -helix followed by a short β -sheet in the C-terminal domain of CynH in agreement with the secondary structure of the C-terminal domain of CynS (38) (Fig. 9). No reliable prediction for tertiary structure of CynH is available

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