Characterization of Cyanate Metabolism in Marine Synechococcus and Prochlorococcus spp.

Nina A. Kamennaya and Anton F. Post

Department of Plant and Environmental Sciences, Hebrew University of Jerusalem, Edmond J. Safra Campus, Jerusalem 91904, Israel, and Bay Paul Center for Comparative Molecular Biology and Evolution, Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Received 30 May 2010/Accepted 27 October 2010

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 \( \alpha \)-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 dimer 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 elongatus 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 Synechocystis strains, cynS was transcribed as 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 \( \text{CO}_2 \), \( \text{HCO}_3^- \), \( \text{NO}_3^- \), \( \text{NO}_2^- \), \( \text{Cl}^- \), \( \text{PO}_4^{2-} \), and \( \text{SO}_4^{2-} \) 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...
the 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 WH7802 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 gyration 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 (Stratagen/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 strain WH7803 available from GenBank, using the DNA sequence viewer and annotation tool Artemis (32). Further genomic data for comparative genomic

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer*</th>
<th>Sequence (5’-3’)</th>
<th>Tₐ (°C)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prochlorococcus</em> sp. MED4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cynA</td>
<td>RTcA-Med4 F</td>
<td>GGAGGTAAGCTAAGGCTATT</td>
<td>52.5</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>RTcA-Med4 R</td>
<td>CTTCCCTGATCCCTATATT</td>
<td>52</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>RTcS-Med4 F</td>
<td>CTTCAAGCTTCGTATACCTTA</td>
<td>51</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>RTcS-Med4 R</td>
<td>CTAGAACCTTATCCCTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RTnA-Med4 F</td>
<td>AGAAGACCTAAGGTTATCC</td>
<td>52</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>RTnA-Med4 R</td>
<td>TCGACATGTCCGTITAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RTtorf1206 F</td>
<td>CCGTTATCTTATAGACACC</td>
<td>52</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>RTtorf1206 R</td>
<td>GACCTTTGCTTTCCTCCATAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. WH8102</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cynA</td>
<td>eA-8102 F</td>
<td>GCCCTCTATCATCTAGTTCCCC</td>
<td>53</td>
<td>828</td>
</tr>
<tr>
<td></td>
<td>eA-8102 R</td>
<td>GCGAATATCGAACAATCTTACT</td>
<td>52.5</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>eS-8102 F</td>
<td>AGGTTTTGGTGTCATCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>eS-8102 R</td>
<td>TCTCCGAATGCTCTGTAAT</td>
<td>56</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>16S rRNA</td>
<td>CATCATGCCCCCTACATCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S-8102 F</td>
<td>AACTGAGCCACGGTTATAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S-8102 R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. WH7803</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cynS</td>
<td>RTcS-2 F</td>
<td>GGGCCACAGCTACGCGGAGG</td>
<td>63</td>
<td>283</td>
</tr>
<tr>
<td></td>
<td>RTcS-2 R</td>
<td>GGTGATCTTACGCGATGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RTorfp252 F</td>
<td>GTGCCGGTGATCTTGTCTTT</td>
<td>59</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>RTorfp252 R</td>
<td>ATGGCGCTCTGTGGTGAAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* F, forward; R, reverse.

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 RT reactions with specific primer sets were performed in a final volume of 50 μl containing 1 μl of cDNA, 0.5 μmol of each primer 1 μl, 0.25 mmol of each deoxyribonucleotide liter⁻¹, 2.5 U of Taq DNA polymerase (Peglab), and 10 μl 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 45 s at 4°C (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 (rsweb.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 orf250 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:
proteins in E. coli strains were supplemented with 0.3 mmol IPTG liter−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-aceate-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 restriction sites, respectively, to facilitate synthesis of the MBP fusion construct. PCRs (50 µl) were performed with Phuson high-fidelity DNA polymerase (Finnzymes) and 1.2 ng of DNA template µl−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-aceate-EDTA-buffered agarose gels and eluted with the Wizard SV Gel and PCR Clean-Up System. Both the amplicons and 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 restriction sites, respectively, to facilitate synthesis of the MBP fusion construct.

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′-AGAAGGGGAGATCATAGTGTCCGACGTC-3′) and cSsPlsR-R (5′-CAACGATTGACGCGCTTACCTTTTTGTTAAGGAGG-3′), and (ii) cSsEcoRI-R (5′-AGTGGGATCCATGAGTGCTCTTTTCCGTTCC-3′) and cSsPlsF-F (5′-GCCGGAGGGCTGCAGTTACGGGGATGCAGATA GG-3′), respectively. The forward/reverse primers contain EcoRI and PstI restriction sites, respectively, to facilitate synthesis of the MBP fusion construct. PCRs (50 µl) were performed with Phuson high-fidelity DNA polymerase (Finnzymes) and 1.2 ng of DNA template µl−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-aceate-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 restriction sites, respectively, to facilitate synthesis of the MBP fusion construct.

Cyanase activity assay. The in vitro cyanase activity of the Synechococcus sp. WH7803 associated with the CynS and CynH fusion proteins was tested in parallel control experiments containing MBP by itself or MBP-NtcA proteins that lack enzymatic activity. Cyanase inhibition reactions were performed after 200-µmol 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 µmol 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 by 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

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 [R296; 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 Immunoresearch 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).

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 polyvinylpiridinylene-
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. WH7083 and Roseovarius 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, Ghu-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_0124911), WH8109 (ZP_05789360), RS9916 (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_01013899), and NATL2A (YP_292581); and E. coli strain K-12 (NP_414874) (4).

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 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.
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 ppoD (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
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 a single band following the second

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 log2 of median values of three replicates, normalized to their initial transcription level, with 25th to 75th percentiles.
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 αH9251-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.

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. 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.

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 Na-azide (200 µmol 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 *A. 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* along with 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 urea-hydrolase (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 arginine urea-hydrolase. 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 CynS (38) (Fig. 9). No reliable prediction for tertiary structure of CynH is available.
due to the low similarity to any known protein and a lack of defined motifs. The dual role the cyanase presents in Synechococcus cyanS and cyanH genes in marine Synechococcus remains to be clarified.

ACKNOWLEDGMENTS

Plasmids with recombinant MBP-NtcA constructs were kindly provided by H. Zer and B. Rihtman. The Niedersachsen State Fund at the Hebrew University, the Israel Science Foundation (grant 135/05), and the NATO Science for Peace program (grant SfP 98216) all provided financial support.

REFERENCES


