

## Involvement of the *cynABDS* Operon and the CO<sub>2</sub>-Concentrating Mechanism in the Light-Dependent Transport and Metabolism of Cyanate by Cyanobacteria<sup>∇</sup>

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The cyanobacteria *Synechococcus elongatus* strain PCC7942 and *Synechococcus* sp. strain UTEX625 decomposed exogenously supplied cyanate (NCO<sup>-</sup>) to CO<sub>2</sub> and NH<sub>3</sub> through the action of a cytosolic cyanase which required HCO<sub>3</sub><sup>-</sup> as a second substrate. The ability to metabolize NCO<sup>-</sup> relied on three essential elements: proteins encoded by the *cynABDS* operon, the biophysical activity of the CO<sub>2</sub>-concentrating mechanism (CCM), and light. Inactivation of *cynS*, encoding cyanase, and *cynA* yielded mutants unable to decompose cyanate. Furthermore, loss of CynA, the periplasmic binding protein of a multicomponent ABC-type transporter, resulted in loss of active cyanate transport. Competition experiments revealed that native transport systems for CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, NO<sub>2</sub><sup>-</sup>, Cl<sup>-</sup>, PO<sub>4</sub><sup>2-</sup>, and SO<sub>4</sub><sup>2-</sup> did not contribute to the cellular flux of NCO<sup>-</sup> and that CynABD did not contribute to the flux of these nutrients, implicating CynABD as a novel primary active NCO<sup>-</sup> transporter. In the *S. elongatus* strain PCC7942  $\Delta$ *chpX*  $\Delta$ *chpY* mutant that is defective in the full expression of the CCM, mass spectrometry revealed that the cellular rate of cyanate decomposition depended upon the size of the internal inorganic carbon (C<sub>i</sub>) (HCO<sub>3</sub><sup>-</sup> + CO<sub>2</sub>) pool. Unlike wild-type cells, the rate of NCO<sup>-</sup> decomposition by the  $\Delta$ *chpX*  $\Delta$ *chpY* mutant was severely depressed at low external C<sub>i</sub> concentrations, indicating that the CCM was essential in providing HCO<sub>3</sub><sup>-</sup> for cyanase under typical growth conditions. Light was required to activate and/or energize the active transport of both NCO<sup>-</sup> and C<sub>i</sub>. Putative *cynABDS* operons were identified in the genomes of diverse *Proteobacteria*, suggesting that CynABDS-mediated cyanate metabolism is not restricted to cyanobacteria.

Cyanate (NCO<sup>-</sup>) metabolism has been studied extensively in *Escherichia coli* and to various degrees in a range of heterotrophic and autotrophic bacteria (references 4, 23, 41, and 50 and references therein). Key to this process is the enzyme cyanase (EC 4.2.1.104) which catalyzes the bicarbonate-dependent decomposition of cyanate to CO<sub>2</sub> and NH<sub>3</sub> (2, 15, 21, 47) according to the following reaction: NCO<sup>-</sup> + HCO<sub>3</sub><sup>-</sup> + 2H<sup>+</sup> → 2CO<sub>2</sub> + NH<sub>3</sub>. The net formation of CO<sub>2</sub> also means that cyanase cocatalyzes the irreversible dehydration of HCO<sub>3</sub><sup>-</sup> (16).

Kinetic, isotopic, and X-ray crystallographic studies show that cyanase binds both NCO<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> in the active site forming a dianion intermediate that enzymatically decarboxylates to CO<sub>2</sub> and carbamate (3, 28, 49). Spontaneous decarboxylation of the carbamate subsequently yields a second CO<sub>2</sub> and NH<sub>3</sub>. Assimilation of cyanate-derived NH<sub>3</sub> and CO<sub>2</sub> then proceeds through conventional metabolic pathways providing a unique source of nitrogen (N) for growth in a variety of bacteria and a source of carbon (C) for autotrophic metabolism (7, 15, 30, 41, 50, 53).

In *E. coli*, the coexpression of carbonic anhydrase (CA) is vital to maintain ongoing cyanate metabolism (17, 23). Mutants lacking CA activity do not readily catalyze cyanate decomposition, are unable to grow with NCO<sup>-</sup> as the sole N source, and are far more susceptible than wild-type cells to the

toxic effects of cyanate itself on growth (18, 22–24). CA involvement is related to the absolute requirement by cyanase for HCO<sub>3</sub><sup>-</sup> (rather than CO<sub>2</sub>) as a substrate in the reaction. Physiological studies (17, 18, 22, 23) indicate that in the absence of CA, the CO<sub>2</sub> generated from cyanate decomposition diffuses out of cells faster than it can be hydrated nonenzymatically to HCO<sub>3</sub><sup>-</sup>. This leads to a cellular depletion of HCO<sub>3</sub><sup>-</sup> and cessation of cyanate metabolism through substrate deprivation. CA prevents this cellular depletion by trapping CO<sub>2</sub> and catalytically regenerating HCO<sub>3</sub><sup>-</sup> within cells at a rate that is not limiting for cyanase.

Carbonic anhydrase, cyanase, and a hydrophobic protein designated as CynX are encoded by *cynT*, *cynS*, and *cynX* (4, 44), respectively, which are arranged in an operon in *E. coli*, ensuring the coordinated expression of the two enzymes required for cyanate decomposition. Expression of the *cynTSX* operon is induced by exogenous cyanate and positively regulated by CynR (45), a member of the LysR family of regulatory proteins. The *cynR* gene is located immediately upstream of the *cynTSX* operon but is transcribed in the opposite direction.

The photoautotrophic cyanobacterium *Synechococcus* sp. strain UTEX 625 also converts exogenous cyanate to CO<sub>2</sub> and NH<sub>3</sub> as described in the reaction above (30). Inhibitor studies (30) have shown that cyanate-derived NH<sub>3</sub> is rapidly incorporated by this cyanobacterium via the central nitrogen assimilation pathway, and it has recently been suggested that NCO<sup>-</sup> can serve as the sole source of N for growth of the globally important marine cyanobacterium *Synechococcus* sp. strain WH8102 (35, 43). CO<sub>2</sub> arising from cyanate decomposition is also rapidly assimilated by *Synechococcus* sp. strain UTEX 625

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through the photosynthetic carbon reduction (Calvin) cycle. Consequently,  $\text{NCO}^-$  supports photosynthetic oxygen evolution (30). Biochemical and molecular studies have demonstrated cyanase activity in whole-cell extracts of *Synechococcus* sp. strain UTEX 625, *Synechococcus elongatus* strain PCC7942 and *Synechocystis* sp. strain PCC6803, which is absent from derived strains carrying engineered mutations within *cynS* homologs (19, 20, 30).

Unlike *E. coli*, the decomposition of exogenous cyanate by *Synechococcus* sp. strain UTEX625 is light dependent (30). The expression of *cynS* is not induced by exogenous cyanate, but it is negatively regulated by  $\text{NH}_3$  and controlled by the global nitrogen regulator NtcA (19, 43). Sequence analysis also indicates that an operon similar to *E. coli cynTSX* is absent from the genomes of cyanobacteria examined to date. Although monocistronic *cynT* homologs have been identified and characterized as part of the  $\text{CO}_2$ -concentrating mechanism (CCM) of cyanobacteria (5, 39, 40), a core requirement for CA activity in cyanobacterial  $\text{NCO}^-$  decomposition has not been demonstrated. Instead, it has been proposed that the active  $\text{HCO}_3^-$  transport systems that normally provide inorganic carbon ( $\text{C}_i$ ) ( $[\text{CO}_2] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}]$ ) for photosynthesis may fulfill the role that is played by CA in *E. coli* (30).

The ability of heterotrophs and autotrophs to utilize exogenous  $\text{NCO}^-$  as a source of N and C presumably relies upon the transport of this anion into cells. Transport studies in *E. coli* indicate that  $\text{N}^{14}\text{CO}^-$  uptake involves an energy-dependent, saturable transporter with a  $K_m$  of 400  $\mu\text{M}$  and a  $V_{\text{max}}$  of 4.4  $\text{nmol min}^{-1} 10^9 \text{ cells}^{-1}$ . CynT was initially implicated as the cyanate permease (46). But as it is now known that CynT is a soluble, cytoplasmic CA, its role in the observed intracellular accumulation of  $\text{N}^{14}\text{CO}^-$  likely reflects the trapping of cyanate-derived  $^{14}\text{CO}_2$ , as  $\text{H}^{14}\text{CO}_3^-$ , within the cells (17). Since these studies were carried out, CynX has been recognized through amino acid sequence similarity to be a member of the major facilitator superfamily of transport proteins (36). However, an *E. coli* mutant defective in *cynX* was found to metabolize cyanate in a manner similar to the wild-type strain (18). Consequently, if CynX is a cyanate permease, there may be multiple pathways for  $\text{NCO}^-$  uptake in *E. coli*. Similarly, the absence of *cynX* homologs in cyanobacteria suggests the occurrence of an alternate path for cyanate transport in these organisms. Thus, there appear to be fundamental differences in the molecular components required to support cyanate metabolism in *Synechococcus* sp. strain UTEX625 and *E. coli*.

In the present study, we have used radiochemical and mass spectrometric techniques to investigate the transport of cyanate by cyanobacteria and to follow the fate of cyanate-derived  $\text{CO}_2$  during metabolism. To determine the specificity of cyanate transport, we examined the role played by the major nutrient anion transporters in the acquisition of cyanate. We also investigated the role of an ABC-type (ATP-binding cassette) transporter that is encoded upstream of *cynS* and forms a putative operon (*cynABDS*) in *S. elongatus* strain PCC7942 and *Synechococcus* sp. strain PCC6301 but is absent in *Synechocystis* sp. strain PCC6803 (19, 20). Classification of this ABC transporter as a cyanate permease is based primarily on the proximity of *cynABD* to *cynS* (19, 20), but it is annotated as an  $\text{NO}_3^-$  or  $\text{HCO}_3^-$  transporter due to the high degree of amino acid sequence similarity between CynA and the respec-

tive periplasmic binding proteins NrtA and CmpA (29). The hypothesis that the  $\text{C}_i$  transport systems of cyanobacteria are essential to support ongoing cyanate metabolism was tested in a mutant lacking  $\text{CO}_2\text{-HCO}_3^-$  transport capability.

## MATERIALS AND METHODS

**Strains and growth conditions.** The unicellular cyanobacteria *Synechococcus* sp. strain UTEX 625 (University of Texas Culture Collection, Austin TX; also known as *Synechococcus* sp. strain PCC6301), *S. elongatus* strain PCC7942, and *Synechocystis* sp. strain PCC6803 (Pasteur Culture Collection, Paris, France) were grown photoautotrophically in BG11 medium containing 17.5 mM  $\text{NaNO}_3$ . The presence of  $\text{NO}_3^-$  ensured that cyanase was fully induced (19). Cells were grown either under  $\text{C}_i$  limitation in standing culture, where diffusion of atmospheric  $\text{CO}_2$  was the sole means of  $\text{CO}_2$  delivery, or under high- $\text{C}_i$  growth conditions where  $\text{CO}_2$  was supplied by gassing buffered {25 mM 1,3-bis[Tris(hydroxymethyl)-methylamino]propane [BTP], pH 8 } cell suspensions with sterile air containing 5% (vol/vol)  $\text{CO}_2$  at 200  $\text{ml min}^{-1}$  (8). The *cynA* and *cynS* mutants of *S. elongatus* strain PCC7942 were grown in the presence of kanamycin (10  $\mu\text{g ml}^{-1}$ ), spectinomycin (40  $\mu\text{g ml}^{-1}$ ), or ampicillin (100  $\mu\text{g ml}^{-1}$ ). The  $\Delta\text{chpX}$   $\Delta\text{chpY}$  mutant of *S. elongatus* strain PCC7942 (52) was kindly provided by G. D. Price (Australian National University) and was grown in the presence of kanamycin (12  $\mu\text{g ml}^{-1}$ ) and chloramphenicol (5  $\mu\text{g ml}^{-1}$ ). *E. coli* DH5 $\alpha$  (Gibco-BRL) was grown on Luria-Bertani medium (38) and supplemented with ampicillin (100  $\mu\text{g ml}^{-1}$ ) or spectinomycin (50  $\mu\text{g ml}^{-1}$ ), when appropriate.

**Insertional inactivation of *cynA* and *cynS*.** Targeted mutants of *S. elongatus* strain PCC7942 were constructed by homologous recombination using antibiotic resistance cassettes to disrupt the coding sequences of the *cynA* and *cynS* genes.

Nonreplicating, pUC19-based plasmids containing either *cynA* or *cynS* (20) were cut with a restriction endonuclease at a unique ClaI or unique BglIII site present within the midportion of the respective genes. The *npIII* gene, conferring kanamycin resistance, was ligated into the restricted site within *cynA* while the *aadA* gene, conferring spectinomycin resistance, was ligated into the restricted site within *cynS*. The resulting plasmids pP12::ORF440 ( $\text{Amp}^r \text{Kan}^r::\text{cynA}$ ) and pXH::ORF146 ( $\text{Amp}^r \text{Spt}^r::\text{cynS}$ ) were propagated in *E. coli* DH5 $\alpha$ , purified, and transformed into *S. elongatus* strain PCC7942 using the method described by Laudenbach and Grossman (26). Transformants recovered from BG11 plates were maintained initially in the dark and then at low light (10  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ; 400 to 700 nm) on 5% (vol/vol)  $\text{CO}_2$  in air at 30°C and supplemented with 5 mM sodium thiosulfate along with the appropriate selective antibiotic (10  $\mu\text{g ml}^{-1}$  kanamycin or 40  $\mu\text{g ml}^{-1}$  spectinomycin). Transformants were serially streaked on fresh plates three times to encourage complete segregation. Mutants arising from homologous recombination between plasmid and *cynA* or *cynS* were recovered by replica plating and identified as colonies that were ampicillin sensitive. Genomic DNA was subsequently isolated (25) from putative, homozygous mutants and analyzed by Southern hybridization (38) to confirm the presence and location of the insert.

**Database queries.** Homologs of the *S. elongatus* strain PCC7942 CynA, CynS, CmpA, and NrtA proteins were identified by searching the NCBI database (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>) with the resident BLASTp program (1) using proteins AAB58736, AAB02940, YP\_400505, and YP\_400256, respectively, as individual queries. Phylogenetic trees were constructed using the TreeView program from multiple protein sequence alignments generated by Clustal X (48).

**Preparation of cells.** Cells (4 to 6  $\mu\text{g}$  of chlorophyll [Chl]  $\text{ml}^{-1}$ ) used for transport and physiological studies were harvested in mid-log phase, washed three times by centrifugation, and suspended in  $\text{C}_i$ - and  $\text{Na}^+$ -free 25 mM BTP–23.5 mM HCl buffer to a final density of 7 to 18  $\mu\text{g}$  of Chl  $\text{ml}^{-1}$ . All experiments were conducted at 30°C and pH 8 to 8.3. The [Chl] of cell suspensions was measured spectrophotometrically at 665 nm after extraction in methanol (8).

**$\text{O}_2$  evolution and chlorophyll *a* fluorescence yield.** Photosynthetic  $\text{O}_2$  evolution was measured in a Clarke-type electrode (Hansatech, Norfolk, United Kingdom) (8). Chl *a* fluorescence quenching was measured with a pulse amplitude modulation fluorometer (PAM 101; H. Walz, Efeltrich, Germany) as described previously (6). In both of the *Synechococcus* strains used, the extent of Chl *a* fluorescence quenching is directly proportional to the magnitude of the intracellular  $\text{C}_i$  pool (6). Fluorescence was, therefore, used as a surrogate to continuously monitor the formation and dissipation of the  $\text{C}_i$  pool in real time. Fluorescence quenching was expressed as a percentage of the variable fluorescence,  $F_V^*$ , such that  $F_V^* = F_M^* - F_O$ , where  $F_M^*$  is the maximum fluorescence yield and  $F_O$  is the instantaneous low-fluorescence yield (6).

**Mass spectrometry (MS) and measurement of dissolved gas fluxes.** The concentrations of  $^{16}\text{O}_2$ ,  $^{12}\text{CO}_2$ ,  $^{13}\text{CO}_2$ , and  $^{14}\text{CO}_2$  ( $m/z$  values of 32, 44, 45, and 46, respectively) dissolved in reaction media or cell suspensions or arising from the metabolism of cyanate were measured using a magnetic sector MS (model MM 14-80 SC; VG Gas Analysis, Middlewich, United Kingdom.) (30). The membrane-covered inlet to the MS was inserted into a port in the side of a transparent, temperature-controlled reaction vessel to allow direct measurements of dissolved gas concentrations over time. The response of the instrument was calibrated by the addition of known concentrations of  $^{16}\text{O}_2$ ,  $\text{K}_2^{12}\text{CO}_3$ ,  $\text{K}_2^{13}\text{CO}_3$ , or  $\text{NaH}^{14}\text{CO}_3$ . The content of the reaction vessel was isolated from the atmosphere using a Plexiglass stopper fitted with a dual O-ring. A capillary bore in the stopper facilitated the addition of reagents to the cell suspension.

Cell suspensions in the reaction vessel were illuminated with a tungsten-halogen projector lamp ( $300 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ; 400 to 700 nm). Simultaneous measurements of  $\text{CO}_2$  and  $\text{O}_2$  fluxes and Chl  $a$  fluorescence yield were obtained by placing the convergent end of the four-armed fiber optic bundle of the fluorometer at the surface of the MS reaction vessel at a  $90^\circ$  angle to the white light source.

**Radiochemical analysis of  $\text{NCO}^-$ ,  $\text{HCO}_3^-$ , and  $\text{CO}_2$  transport.** Transport and intracellular accumulation were assayed using the silicone fluid filtering centrifugation technique with  $\text{N}^{14}\text{CO}^-$ ,  $\text{H}^{14}\text{CO}_3^-$ , or  $^{14}\text{CO}_2$  as substrate, essentially as described by Miller et al. (32). To determine the level of acid-stable products of photosynthesis and the acid-labile intracellular pool of  $\text{NCO}^-$  or  $\text{C}_i$ , the cell samples were subsequently processed as described by Espie et al. (10). Intracellular concentrations of  $\text{NCO}^-$  or  $\text{C}_i$  were calculated using an internal cellular water volume of  $49 \mu\text{mol} \text{mg}^{-1}$  of Chl (9).

Four variations on the transport assay protocol were used. With method A, cells were incubated in the light ( $300 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ; 400 to 700 nm) in the  $\text{O}_2$ -electrode chamber and allowed to deplete the medium of  $\text{C}_i$ , as indicated by the cessation of  $\text{O}_2$  evolution. A cell sample ( $100 \mu\text{l}$ ) was then removed and placed into a  $400\text{-}\mu\text{l}$  microcentrifuge tube containing  $100 \mu\text{l}$  of silicone fluid (middle layer, silicone oils AR20:AR200 at 1.75:1 (vol/vol); Wacker Chemie, Munich, Germany) and  $100 \mu\text{l}$  of terminating solution (bottom layer, 2 M KOH in 10% [vol/vol] methanol). The tube was placed in a Beckman microcentrifuge E, illuminated from above ( $300 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) for a further 1 min, and the experiment was started by the addition of  $\text{KO}^{14}\text{CN}$  to the cells using a  $10\text{-}\mu\text{l}$  Hamilton syringe. Uptake was stopped at timed intervals by centrifuging (1 min at  $12,500 \times g$ ) the cells through the silicone fluid and into the terminating solution. In method B, when  $\text{N}^{14}\text{CO}^-$  accumulation was measured in mutant cells or when long-term uptake assays were conducted,  $\text{KO}^{14}\text{CN}$  was introduced directly into the  $\text{O}_2$ -electrode chamber. Then, under constant illumination,  $100\text{-}\mu\text{l}$  samples were removed and layered on top of the silicone fluid/termination solution contained in microcentrifuge tubes. The experiment was terminated as above. For method C, another approach was used in experiments to evaluate the effect of  $\text{HCO}_3^-$  on  $\text{NCO}^-$  transport and the effect of  $\text{NCO}^-$  on  $\text{HCO}_3^-$  transport. In these experiments, the silicone fluid was overlaid with  $50 \mu\text{l}$  of buffer containing  $\text{KO}^{14}\text{CN} + \text{NaHCO}_3$  or  $\text{KOCN} + \text{NaH}^{14}\text{CO}_3$ . The headspace was flushed with  $\text{N}_2$ . Cells ( $50 \mu\text{l}$ ) depleted of  $\text{C}_i$  were then introduced into the microcentrifuge tubes in such a way as to create an  $\text{N}_2$ -filled gap between the cell suspension and the reaction buffer. The sealed tubes (six at once) were then placed in the microcentrifuge and illuminated for 1 min. To start the experiments, the cell suspensions and reaction buffers were rapidly mixed by briefly (for less than 1 s) engaging the drive of the microcentrifuge. The uptake experiments were terminated as above. For method D, when the effect of  $\text{NCO}^-$  on  $^{14}\text{CO}_2$  transport was determined,  $\text{NCO}^-$  and  $^{14}\text{CO}_2$  were added in rapid sequence to cells already in a microcentrifuge tube. Uptake was terminated after 10 s as described above. The  $^{14}\text{CO}_2$  was created by adding  $\text{NaH}^{14}\text{CO}_3$  to 10 mM phthalic acid buffer, pH 4.

**KOCN and  $\text{C}_i$  contamination.** Reagent grade KOCN was obtained from BDH Chemicals (Toronto, Canada) and was crystallized three times from a water-ethanol mixture prior to use (21).  $\text{KO}^{14}\text{CN}$  was obtained from Dupont Canada (Mississauga), and  $\text{KO}^{13}\text{CN}$  was from MSD Isotopes (Montreal). Stock solutions were made by dissolving crystallized KOCN in  $\text{C}_i$ -free water and by dissolving  $\text{KO}^{14}\text{CN}$  or  $\text{KO}^{13}\text{CN}$  in  $\text{C}_i$ -free 25 mM BTP buffer, pH 8. At pH 8, virtually all the KOCN existed in the form of the  $\text{NCO}^-$  anion ( $\text{pK}_a$  of  $\text{HOCN}$  is 3.7 at  $27^\circ\text{C}$ ) (27) and slowly decomposed ( $0.01\% \text{h}^{-1}$ ) forming  $\text{CO}_2$  and  $\text{NH}_3$ . The level of contaminant  $^{12}\text{CO}_2$ ,  $^{13}\text{CO}_2$ , or  $^{14}\text{CO}_2$  present in KOCN solutions, " $\text{C}_i$ -free" buffers, and other solutions was routinely determined by MS. Equilibrium  $\text{C}_i$  concentrations were subsequently calculated for the experimental temperature and pH as described by Stumm and Morgan (42). Typically, freshly crystallized and freshly prepared 1 mM KOCN stock solutions contained  $7.1 \pm 2 \mu\text{M}$   $^{12}\text{C}_i$  ( $n = 7$ ) while 1 mM  $\text{KO}^{14}\text{CN}$  contained  $6.5 \pm 1.8 \mu\text{M}$   $^{14}\text{C}_i$  ( $n = 7$ ), and 1 mM  $\text{KO}^{13}\text{CN}$  contained  $86.5 \pm 9.7 \mu\text{M}$   $^{13}\text{C}_i$  ( $n = 7$ ). These values increased over

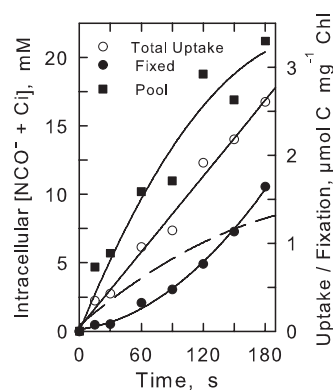


FIG. 1. Time course (180 s) of  $\text{NCO}^-$  uptake in the light for *Synechococcus* sp. strain UTEX 625 standing culture cells provided with 1 mM  $\text{KO}^{14}\text{CN}$ , as determined by method A. Shown are total uptake ( $\circ$ ), the acid-stable products of C-fixation ( $\bullet$ ), and the acid-labile intracellular pool of  $\text{NCO}^- + \text{C}_i$  ( $\blacksquare$ ). Data are the average of three replicates  $\pm 10.4\%$  (maximum). Also shown (dashed line) is the calculated intracellular pool of  $\text{NCO}^-$  corrected for intracellular  $\text{C}_i$ .

time. The  $\text{KO}^{14}\text{CN}$  stock solutions contained less than 2% acid-stable material, and its presence was corrected for in calculations. In some experiments, the level of  $\text{C}_i$  contamination in solutions could be reduced to near-zero by first allowing cells to photosynthetically deplete the reaction medium. But in all cases, appropriate corrections were made to the specific activities of radiochemicals and to concentrations of reactants to account for the presence of contaminant  $\text{C}_i$ .

Cyanate is relatively stable in alkaline solution (hydrolysis rate of  $0.01\% \text{h}^{-1}$ ) but rapidly decomposes to  $\text{CO}_2$  and  $\text{NH}_3$  below about pH 4.5 (27). Consequently, unreacted  $\text{N}^{14}\text{CO}^-$  was removed from samples by acidification and driving off the resulting  $^{14}\text{CO}_2$  by evaporation.

**Nucleotide sequence accession numbers.** The nucleotide sequence of the *cynABDS* region determined in this study has been deposited in the GenBank database at NCBI under accession numbers AF001333 and U59481.

## RESULTS

**Cyanate uptake and the fate of cyanate-derived  $^{14}\text{C}$ .** Figure 1 shows a typical time course of  $^{14}\text{C}$  uptake by *Synechococcus* sp. strain UTEX 625 (PCC6301) cells provided with 1 mM  $\text{KO}^{14}\text{CN}$ . The intracellular  $^{14}\text{C}$  was found in two major fractions: an acid-stable fraction composed of metabolic intermediates and an acid-labile fraction consisting of unreacted  $\text{N}^{14}\text{CO}^-$  and  $^{14}\text{C}_i$ . Over the course of the experiment (180 s), the intracellular acid-labile pool of  $^{14}\text{C}$  rose to about 21 mM.

Interpretation of cyanate uptake experiments (Fig. 1 and 2) is complicated by the presence of  $^{14}\text{C}_i$  which invariably contaminated the  $\text{KO}^{14}\text{CN}$  (7 to  $25 \mu\text{M}/1 \text{ mM}$ ). This  $^{14}\text{C}_i$  was undoubtedly transported rapidly into the cells by the high-affinity  $\text{C}_i$  transport systems and fixed by the Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase, thereby contributing to both the acid-labile and acid-stable fractions (9, 12, 31, 32). In fact, nearly all the  $^{14}\text{C}$  fixed during the uptake experiments (Fig. 1) entered the acid-stable fraction via the photosynthetic carbon reduction cycle as evidenced by the fact that darkness, DCMU (3[3,4-dichlorophenyl]-1,1-dimethylurea), and the Calvin cycle inhibitor glycolaldehyde reduced  $^{14}\text{C}$  incorporation by 98%, 98%, and 94%, respectively (Table 1). Over the course of 25-min experiments (Fig. 2), 18 to 26% of the total added C (as KOCN) was found in the acid-stable fraction. This level of incorporation exceeded the  $^{14}\text{C}_i$  available in  $\text{KO}^{14}\text{CN}$  solutions by at least 36-fold. During the short-

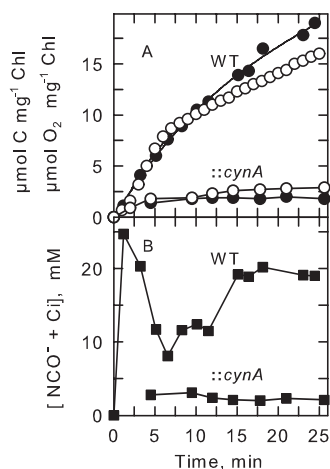


FIG. 2. Typical time course (25 min) of  $\text{NCO}^-$  uptake and utilization by *S. elongatus* PCC7942 wild type (WT) and the *cynA* mutant ( $::cynA$ ). Cells grown in standing culture with low  $\text{C}_i$  concentrations were provided with 1 mM  $\text{KO}^{14}\text{CN}$  in the light. Uptake and accumulation of  $^{14}\text{C}$  was determined as described in method B. (A) Simultaneous measurements of cyanate-dependent  $\text{CO}_2$  fixation (●) and  $\text{O}_2$  evolution (○) for the wild type and the *cynA* mutant. (B) Intracellular accumulation of acid-labile  $\text{NCO}^- + \text{C}_i$  (■) for the wild type and the *cynA* mutant ( $::cynA$ ).

term experiments (Fig. 1),  $^{14}\text{C}$  incorporation exceeded the available  $^{14}\text{C}_i$  by fourfold, indicating that most of the  $^{14}\text{C}$  found in the acid-stable fraction was derived from the  $^{14}\text{C}$  originally present in  $\text{KO}^{14}\text{CN}$ .

The incorporation of  $^{14}\text{C}$  into acid-stable products initially lagged behind the accumulation of the acid-labile internal pool of  $^{14}\text{C}$  (Fig. 1). But as this pool increased in size so, too, did the rate of C incorporation, approaching a steady-state rate as the internal pool approached a maximum. The dynamics of these two fractions were indicative of a precursor-product relationship in which the acid-labile C ( $\text{NCO}^-$  and  $\text{C}_i$ ) was first transported and accumulated within the cells and, subsequently, incorporated into acid-stable products of photosynthesis.

Cyanate supported light-dependent  $\text{O}_2$  evolution at a linear rate for about 10 min, after which the rate declined to a lower steady-state rate (Fig. 2). The decline in the rate of  $\text{O}_2$  evolution was paralleled by a decline in C-fixation and coincided in time with a decrease in the internal  $\text{NCO}^- + \text{C}_i$  pool (Fig. 2). With time, the internal pool stabilized at a new steady-state level leading to a lower steady-state rate of cyanate-dependent  $\text{O}_2$  evolution and C-fixation. Biphasic time courses of  $\text{O}_2$  evolution were commonly observed when cells were provided with  $\text{KOCN}$  exceeding about 100  $\mu\text{M}$ . A near 1:1 stoichiometry was found between added  $\text{KOCN}$ ,  $\text{O}_2$  evolution, and  $\text{CO}_2$  fixation (Fig. 2) (30), consistent with the complete decomposition of cyanate to  $\text{NH}_3$  and  $\text{CO}_2$  and subsequent fixation of  $\text{CO}_2$  into acid-stable products of photosynthesis.

The ability of *Synechococcus* sp. UTEX 625 to utilize cyanate-derived  $\text{CO}_2$  to support photosynthesis was affected by the level of  $\text{C}_i$  experienced during growth. For cells grown in standing culture (low  $\text{C}_i$  concentration, approximately 20  $\mu\text{M}$ ), with air bubbling (intermediate  $\text{C}_i$  concentration, approximately 180  $\mu\text{M}$ ), or bubbling with 5%  $\text{CO}_2$  (high  $\text{C}_i$  concentration), analysis of cyanate response curves indicated  $K_{0.5}$  (the

substrate concentration that yields one-half the maximum rate) ( $\text{NCO}^-$ ) values for  $\text{O}_2$  evolution of  $0.34 \pm 0.04$ ,  $0.43 \pm 0.13$ , and  $0.70 \pm 0.05$  mM and maximum rates of  $138 \pm 10$ ,  $187 \pm 34$ , and  $233 \pm 43$   $\mu\text{mol O}_2 \text{ mg}^{-1}$  of  $\text{Chl h}^{-1}$  ( $\pm$  standard deviation;  $n = 3$ ), respectively. Since growth on  $\text{C}_i$  also affects the level of induction of the CCM (5), the data suggest a possible role for the CCM in modulating cyanate utilization.

**Active transport and accumulation of  $\text{NCO}^-$ .** Cyanate uptake was stimulated by light and inhibited by DCMU, suggesting that photosynthetic electron transport may be involved in energizing or activating  $\text{NCO}^-$  transport (Table 1). An acid-labile pool of C was also formed in the presence of glycolaldehyde, indicating that uptake was not strictly tied to the photosynthetic metabolism of cyanate. Oxalate, a membrane-impermeant inhibitor of both *E. coli* and cyanobacterial cyanase (3, 30), had little effect on either the acid-labile or acid-stable fractions (Table 1), indicating that cyanase was absent from the periplasm. This finding is consistent with previous biochemical studies which show cyanase activity in the soluble, cytoplasmic fraction of cell lysates (19, 30). Consequently,  $\text{NCO}^-$  and/or  $\text{HOCN}$  must permeate the inner membrane prior to decomposition.

In order to distinguish between  $\text{N}^{14}\text{CO}^-$  uptake and accumulation and  $^{14}\text{C}_i$  uptake and accumulation (Fig. 1), we conducted a series of experiments in the presence of unlabeled  $^{12}\text{C}_i$  (Table 2). Through dilution with  $^{12}\text{C}_i$ , the specific activity of the  $^{14}\text{C}_i$  present as a contaminant in  $\text{KO}^{14}\text{CN}$  solutions was reduced by up to 1,000-fold, effectively eliminating the contribution of  $^{14}\text{C}_i$  uptake to the intracellular acid-labile pool. Table 2 shows that the initial rate (20 s) of  $^{14}\text{C}$  uptake by cells presented with 1 mM  $\text{KO}^{14}\text{CN}$  was 48% or 49% of the control value in the presence of 2 or 5 mM  $^{12}\text{C}_i$ , respectively. Similarly, the internal pool was reduced to 45% or 46% of the control value, while  $^{14}\text{C}$ -fixation declined to 47% to 42% of the control. Slightly larger decreases in the rate of uptake and intracellular accumulation were observed over 120-s time intervals while  $^{14}\text{C}$ -fixation declined by as much as 75% (Table 2). Consequently, we estimated that 40% to 45% of the total acid-labile intracellular pool of  $^{14}\text{C}$  was comprised of  $\text{N}^{14}\text{CO}^-$ . Based on this estimate, the intracellular concentration of cyanate would be expected to be about 2.7 mM at 20 s, 6.3 mM at 120 s (Table 2), and 8.4 mM at 180 s (Fig. 1). This latter value is 8.4-fold higher than the initial concentration of  $\text{KOCN}$  provided to the cells. Assuming a membrane potential of

TABLE 1. Characterization of  $\text{NCO}^-$  uptake and metabolism in *Synechococcus* sp. strain UTEX 625

Experimental conditions <sup>a</sup>	$\text{N}^{14}\text{CO}^-$ uptake (% of control)	$^{14}\text{C}$ fixation (% of control)
Light (control)	100	100
Dark	6.3	2.4
Light + 25 $\mu\text{M}$ DCMU	7.9	2.2
Light + 15 mM glycolaldehyde	59.1	5.6
Light + 15 mM oxalate	88	99.7
Light + 1 mM ethoxzolamide	39	32.3

<sup>a</sup>  $\text{NCO}^-$  uptake and C fixation were measured by the silicone fluid filtering centrifugation technique using method A. Cells grown in standing culture were incubated with 1 mM  $\text{KO}^{14}\text{CN}$  for 2 min in BTP/HCl buffer, pH 8.0, containing 25 mM NaCl at 30°C and illuminated at 300  $\mu\text{mol m}^{-2} \text{ s}^{-1}$ . Data are the average of 5 experiments in triplicate  $\pm 6.5\%$ .

TABLE 2. Effect of [ $^{12}\text{C}_i$ ] on  $\text{N}^{14}\text{CO}^-$  transport by *Synechococcus* sp. strain UTEX 625 grown in standing culture<sup>a</sup>

Experimental condition (with 1 mM $\text{KO}^{14}\text{CN}$ )	% of control value at 20 s			% of control value at 2 min		
	$\text{N}^{14}\text{CO}^-$ uptake	Pool	$^{14}\text{C}$ fixation	$\text{N}^{14}\text{CO}^-$ uptake	Pool	$^{14}\text{C}$ fixation
0 mM $\text{KHCO}_3$	100	100 <sup>b</sup>	100 <sup>c</sup>	100	100 <sup>d</sup>	100 <sup>e</sup>
2 mM $\text{KHCO}_3$	48.2 ± 9.3	45.4 ± 16.2	47.5 ± 21.6	38.4 ± 15.2	41.0 ± 12.9	24.9 ± 10.6
5 mM $\text{KHCO}_3$	49.4 ± 7.3	46.4 ± 19.3	42.9 ± 12.2	39.6 ± 14.6	41.5 ± 18.7	30.4 ± 19.7

<sup>a</sup> Values were determined using method C in BTP-HCl buffer, pH 8, containing 25 mM NaCl. Light was supplied at 300  $\mu\text{mol}$  of photons  $\text{m}^{-2}\text{s}^{-1}$ . Data are the average of seven experiments ± standard deviations.

<sup>b</sup> Control value, 6.0 ± 2.0 mM.

<sup>c</sup> Control value, 0.06  $\mu\text{mol}$  of C  $\text{mg}^{-1}$  Chl.

<sup>d</sup> Control value, 11.0 ± 7.0 mM.

<sup>e</sup> Control value, 0.23  $\mu\text{mol}$  of C  $\text{mg}^{-1}$  Chl.

−0.12 V (37), the electrochemical potential for  $\text{NCO}^-$  was calculated to be 16.6  $\text{kJ mol}^{-1}$  (−0.63 V), indicating that a component of  $\text{NCO}^-$  uptake involved carrier-mediated, active transport.

**Transport specificity.** The reduction in  $^{14}\text{C}$  accumulation in the presence of  $^{12}\text{C}_i$  (Table 2) may also indicate a competition between  $\text{C}_i$  and  $\text{NCO}^-$  during transport. Consequently, the possibility that  $\text{NCO}^-$  transport was gratuitously mediated via one of the  $\text{C}_i$  transport systems or one of the major nutrient anion transport systems was tested in a series of competition experiments in the presence of  $\text{CO}_2$ ,  $\text{HCO}_3^-$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Cl}^-$ ,  $\text{PO}_4^{2-}$ , or  $\text{SO}_4^{2-}$  (Fig. 3 and 4).

Physiologically,  $\text{C}_i$  transport in *Synechococcus* sp. strain UTEX 625 (PCC6301) and *S. elongatus* strain PCC7942 is comprised of an active,  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  uptake activity, an active  $\text{Na}^+$ -independent  $\text{HCO}_3^-$  uptake activity, and high- and low-affinity active  $\text{CO}_2$  uptake activities (6, 9, 12, 31). These transport activities are thought to involve the membrane proteins SbtA, CmpABCD, NdhF3/NdhD3/ChpY, and NdhF4/NdhD4/ChpX, respectively (reviewed in reference 5). If one or more of these transporters is involved in  $\text{NCO}^-$  transport, then  $\text{NCO}^-$  must

also be an inhibitor of  $\text{C}_i$  transport activity. Figure 3A and B show that neither  $\text{Na}^+$ -independent  $\text{HCO}_3^-$  transport nor  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport was inhibited by up to a 24-fold excess of KOCN. However, in cells grown with low  $\text{C}_i$  concentrations in standing culture and with high  $\text{C}_i$  concentrations,  $\text{CO}_2$  transport was reduced by up to 50% with 1 mM KOCN (Fig. 3C). Thus,  $\text{NCO}^-$  transport may occur through the  $\text{CO}_2$  transport system. The fact that  $\text{NCO}^-$  is an isoelectronic structural analog of  $\text{CO}_2$  supports this view. However, although  $^{14}\text{CO}_2$  transport assays were conducted rapidly, the  $^{12}\text{CO}_2$  generated from the decomposition of cyanate (Fig. 4) may also have contributed to the apparent inhibition of  $^{14}\text{CO}_2$  transport. Thus, the involvement of the  $\text{CO}_2$  transport systems in  $\text{NCO}^-$  transport requires further analysis (see below).

The involvement of nitrate/nitrite transport systems in  $\text{NCO}^-$  transport was tested in a different way. The *Synechococcus* strains grown under high- $\text{C}_i$  conditions have a greatly reduced capacity for  $\text{HCO}_3^-$  transport and rely primarily on the low affinity  $\text{CO}_2$  uptake system for  $\text{C}_i$  acquisition (5). Consequently, the contribution (if any) of the  $\text{CO}_2$  transport systems to  $\text{NCO}^-$  transport can be minimized. When cells grown with high  $\text{C}_i$  concentrations were presented with  $\text{N}^{13}\text{CO}^-$ , a large and sustained rise in the extracellular  $^{13}\text{CO}_2$  concentration was observed that was light dependent (Fig. 4). The ad-

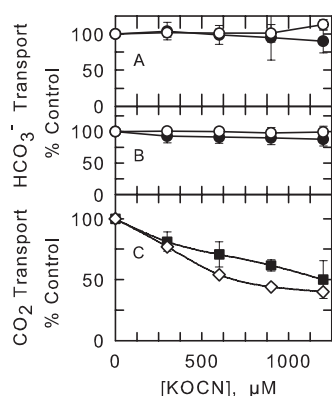


FIG. 3. Effect of KOCN on  $\text{HCO}_3^-$  and  $\text{CO}_2$  transport in *Synechococcus* sp. strain UTEX 625 cells grown in standing culture. The initial rate (20 s) of  $\text{HCO}_3^-$  transport was measured (method C) at substrate concentrations of 50  $\mu\text{M}$  (●) and 250  $\mu\text{M}$  (○) in pH 8 buffer to determine the effect of increasing [KOCN] on  $\text{Na}^+$ -independent  $\text{HCO}_3^-$  transport in the presence of 100  $\mu\text{M}$  NaCl (A) or  $\text{Na}^+$ -dependent  $\text{HCO}_3^-$  transport in the presence 30 mM NaCl (B). (C) The initial rate (10 s) of  $\text{CO}_2$  transport (■) was measured at 10  $\mu\text{M}$  in the presence of 100  $\mu\text{M}$  NaCl (method D). Also shown is the effect of KOCN on  $\text{CO}_2$  transport in cells grown under high- $\text{C}_i$  conditions (◇). The data are the average of six determinations ± standard deviations.

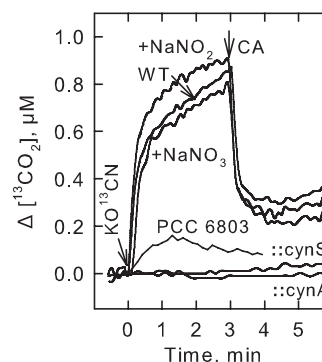


FIG. 4. KOCN-dependent  $\text{CO}_2$  efflux by cells grown under high- $\text{CO}_2$  conditions. Illuminated wild-type (WT) *S. elongatus* PCC7942 cells were provided with 1 mM  $\text{KO}^{13}\text{CN}$ , and the efflux of  $^{13}\text{CO}_2$  was followed by MS over time in the absence or presence of 25 mM  $\text{NaNO}_2$  or  $\text{NaNO}_3$ . Bovine CA was added to the suspension to illustrate that  $\text{CO}_2$  was the  $\text{C}_i$  species arising in the cell suspensions. Also shown are the  $\text{CO}_2$  fluxes arising from the *cynA* and *cynS* mutants and from *Synechocystis* sp. strain PCC6803 grown in high- $\text{CO}_2$  concentrations.

dition of bovine CA to cell suspensions reduced the  $[^{13}\text{CO}_2]$ , indicating that it was  $\text{CO}_2$  and not  $\text{HCO}_3^-$  that exited the cells and that the  $\text{CO}_2$  was present at a concentration well above its chemical equilibrium with  $\text{HCO}_3^-$ . The use of  $\text{KO}^{13}\text{CN}$  as substrate confirmed that the  $^{13}\text{CO}_2$  arising in the medium was derived directly from cyanate uptake and decomposition rather than from respiration or any other process (30). Consequently, in vivo cyanate uptake and decomposition can be followed in real time by measuring KOCN-dependent  $\text{CO}_2$  efflux via MS (Fig. 4).

When *S. elongatus* strain PCC7942 was presented with  $\text{KO}^{13}\text{CN}$  in the presence of either 25 mM  $\text{NaNO}_3$  or 25 mM  $\text{NaNO}_2$ , both the qualitative and quantitative patterns of  $^{13}\text{CO}_2$  formation were very similar to the control pattern observed with cells in buffer alone (Fig. 4) or in the presence of 25 mM  $\text{NaCl}$  (not shown). Thus, neither  $\text{NO}_3^-$  nor  $\text{NO}_2^-$  interfered with the process of cyanate uptake or its decomposition to  $\text{CO}_2$ . Since the nitrate/nitrite transport systems were saturated at these levels, we conclude that these transporters were not involved in the transport of  $\text{NCO}^-$ . Similarly, neither phosphate nor sulfate affected the patterns of KOCN-dependent  $\text{CO}_2$  efflux (not shown).

In cells grown under low- $\text{C}_i$  conditions, the steady-state rate of KOCN-dependent  $\text{O}_2$  evolution in the light was also not substantially reduced by the presence of nutrient anions. Measured rates (100  $\mu\text{M}$  KOCN;  $n = 3$ ) in the presence of 25 mM  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{Cl}^-$ ,  $\text{PO}_4^{2-}$ , and  $\text{SO}_4^{2-}$  were 92.0%, 97.1%, 104%, 94.1%, and 89.2% of the control value (25 mM BTP, pH 8). Collectively, the data indicated that none of the major inorganic anion transport systems played a significant role in the active transport of  $\text{NCO}^-$ .

***cynS* is required but not sufficient to promote the metabolism of exogenous  $\text{NCO}^-$ .** Southern hybridization analysis of  $\text{ClaI}$ -digested DNA from *S. elongatus* strain PCC7942 wild-type and the *cynS* mutant was carried out using a 187-bp  $\text{EcoRI}$ - $\text{BglII}$  restriction fragment from *cynS* as the probe. A single hybridization signal of approximately 7.0 kb was obtained from DNA from the *cynS* mutant compared to a 5.0-kb band from wild-type DNA (not shown). The 2-kb difference corresponded well to the expected insertion of the  $\text{Spt}^t$  cassette into *cynS* and indicates that only a single disrupted copy of *cynS* was present in the genome of the *S. elongatus* strain PCC7942 *cynS* mutant.

Previous studies have shown that targeted interruption of *cynS* in *S. elongatus* strain PCC7942 results in the loss of intracellular cyanase activity (19). Consistent with this is the observation that our *cynS* mutant was unable to utilize exogenously supplied KOCN to support photosynthetic  $\text{O}_2$  evolution (Fig. 5A). The inability of the *cynS* mutant to utilize  $\text{NCO}^-$  was not related to a defect in the photosynthetic utilization of cyanate-derived  $\text{CO}_2$  since it grew photoautotrophically in a normal fashion under low- $\text{C}_i$  conditions and responded in a comparable manner to wild-type cells when supplied with 25  $\mu\text{M}$   $\text{C}_i$ . The *cynS* mutant was also able to form and retain a large internal pool of  $\text{C}_i$  when provided with exogenous 25  $\mu\text{M}$   $\text{C}_i$ , as judged by an increase in Chl *a* fluorescence quenching (Fig. 5B). The addition of KOCN also elicited substantial fluorescence quenching (43% of  $F_v^*$  after 5 min) in wild-type cells which corresponded to an internal  $\text{C}_i$  pool of about 28 to 30 mM (6). KOCN-dependent fluorescence

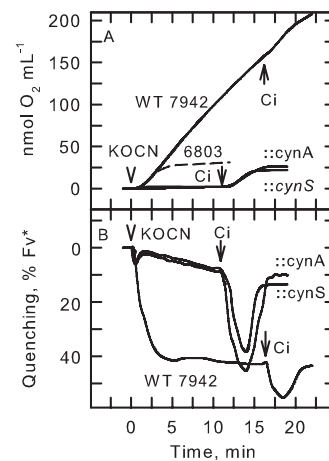


FIG. 5. Measurements of KOCN-dependent  $\text{O}_2$  evolution (A) and Chl *a* fluorescence quenching (B) in *S. elongatus* PCC7942 wild-type (WT) cells and the *cynA* and *cynS* mutants. Cells grown under low- $\text{C}_i$  conditions were allowed to deplete the medium of  $\text{C}_i$  in the light. Cells were then provided with 1 mM KOCN, and  $\text{O}_2$  evolution and Chl *a* fluorescence quenching were measured simultaneously over time. Once a steady state was achieved,  $\text{C}_i$ -dependent  $\text{O}_2$  evolution and Chl *a* fluorescence quenching were also measured following the addition of 25  $\mu\text{M}$   $\text{C}_i$  to the cell suspensions. Also shown in panel A is the response of *Synechocystis* sp. strain PCC6803 to the addition of 1 mM KOCN.

quenching preceded the onset of steady-state photosynthesis, consistent with an initial uptake and subsequent decomposition of cyanate to form an internal pool of  $\text{CO}_2$ . These responses to KOCN were completely absent in the *cynS* mutant (Fig. 5B), as was the rise in  $\text{CO}_2$  concentration following KOCN addition to cells grown under high- $\text{C}_i$  conditions (Fig. 4). The disruption of *cynS* thus led to the loss of intracellular cyanase activity (19) and to an inability to metabolize exogenous cyanate in vivo.

Although the cyanobacterium *Synechocystis* sp. strain PCC6803 also expresses a high level of cyanase activity when grown with nitrate as its N source (19), the wild-type cells were unable to utilize exogenous cyanate to support  $\text{O}_2$  evolution in the light (Fig. 5A). In addition, *Synechocystis* sp. strain PCC6803 cells grown under high- $\text{C}_i$  conditions failed to create the sustained rise in extracellular  $\text{CO}_2$  concentration in the light that was characteristic of cyanate decomposition (Fig. 4), in spite of the presence of abundant and functional cyanase (19). These data suggest that molecular components beyond *cynS* are required to facilitate the metabolism of exogenous cyanate in the two *Synechococcus* strains and that some or all of these components are lacking naturally or are not expressed in *Synechocystis* sp. strain PCC6803.

***cynA* is required for cyanate transport.** Immediately upstream of *cynS* in *S. elongatus* strain PCC7942 and *Synechococcus* sp. strain PCC6301, but not *Synechocystis* sp. strain PCC6803, is a three-gene cluster (GenBank accession numbers AF001333 and U59481) initially designated as *orf440*, *orf263*, and *orf289* and later renamed *cynA*, *cynB*, and *cynD*, respectively (19, 20). The *cyn* genes are all transcribed in the same direction (*cynA* to *cynS*) and are separated by 9, 3, and 10 nucleotides, respectively. Putative ribosome binding sites are located 7 to 10 bp upstream of start codons in all but *cynD* (16).

A potential transcription termination sequence is located downstream of *cynS* (20) while an NtcA-dependent promoter is located upstream of *cynA* between -334 and -293 (19) (GenBank accession number AB005890). Sequence analysis, therefore, predicts that *cynA*, *cynB*, *cynD*, and *cynS* form an operon. This view is supported by Northern hybridization analysis which indicates that both *cynA* (20) and *cynS* (19, 20) are present on a transcript approximately 4.3 kb in size.

The deduced amino acid sequences of CynA, CynB, and CynD show significant sequence identity to proteins that are members of the ABC-type superfamily of membrane transport proteins (19, 20). The protein encoded by *cynB* has characteristics typical of the integral membrane permease component of the transport system while CynD contains ATP-binding motifs characteristic of the peripheral membrane component involved in the energization of transport (19, 20). CynA, a putative periplasmic binding protein, has a high degree of amino acid similarity to its paralogs NtrA (46%) and CmpA (45%). The latter two periplasmic proteins are known to bind  $\text{NO}_3^-/\text{NO}_2^-$  and  $\text{HCO}_3^-$ , respectively (29, 34). Thus, CynA may be a monovalent anion binding protein.

To determine if the *cynABDS* operon is involved in cyanate transport, we characterized a *S. elongatus* strain PCC7942 *cynA* mutant, which was created by interrupting the *cynA* gene with a Kan<sup>r</sup> cassette. Southern hybridization analysis of PstI-digested DNA from *S. elongatus* strain PCC7942 wild type and the *cynA* mutant strain, using a 687-bp PstI-ClaI restriction fragment from *cynA* as the probe, revealed the presence of a single disrupted copy of *cynA* in the genome of the mutant (not shown). The *cynA* mutant was unable to carry out KOCN-dependent  $\text{O}_2$  evolution or KOCN-dependent Chl *a* fluorescence quenching, although the mutant was capable of typical wild-type  $\text{C}_i$ -dependent  $\text{O}_2$  evolution and  $\text{C}_i$ -dependent Chl *a* fluorescence quenching (Fig. 5). Similarly, *cynA* mutant cells grown with high  $\text{C}_i$  concentrations did not evolve  $\text{CO}_2$  when presented with KOCN (Fig. 4), indicating that the lesion in *cynA* had disrupted their ability to utilize exogenous cyanate. Transport assays (Fig. 2) revealed a marked decline in intracellular  $\text{N}^{14}\text{CO}^-$  accumulation and the absence of sustained  $^{14}\text{C}$  fixation in the light compared to wild-type cells. In these experiments, sampling of the *cynA* mutant was begun 5 min after the addition of 2 mM  $\text{KO}^{14}\text{CN}$ . This allowed the mutant sufficient time to fix contaminating  $^{14}\text{C}_i$  into acid-stable products, as judged by  $\text{O}_2$  evolution and fluorescence measurements. Consequently, direct measurements of the acid-labile intracellular  $\text{N}^{14}\text{CO}^-$  pool were possible (Fig. 2B). The average intracellular concentration of  $\text{NCO}^-$  in the *cynA* mutant was  $2.1 \pm 1.1$  mM ( $n = 6$ ) after a 5-min exposure and  $3.1 \pm 1.9$  mM ( $n = 6$ ) after a 10-min exposure. In comparison, wild-type *S. elongatus* strain PCC7942 accumulated a total pool of  $27.1 \pm 8.1$  mM ( $n = 8$ ) after 5 min and  $37.1 \pm 11.1$  mM ( $n = 8$ ) after 10 min. From these data, internal concentrations of  $\text{NCO}^-$  of 12.2 and 16.7 mM, respectively, were calculated assuming that 45% of the measured pool in wild-type cells was free  $\text{NCO}^-$ . Consequently, the level of  $\text{NCO}^-$  accumulation in the *cynA* mutant is at least fivefold lower than in wild-type cells. Calculations using the Henderson-Hasselbach equation indicate that about one-half of the observed accumulation in the *cynA* mutant was accounted for by the diffusion of  $\text{HO}^-$  into the cells and its passive equilibration with  $\text{NCO}^-$ . Thus, the ability of

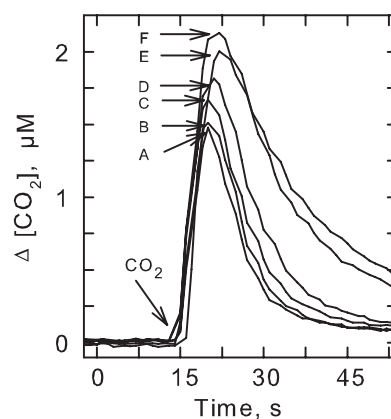


FIG. 6. Effect of KOCN on  $\text{CO}_2$  transport. *S. elongatus* PCC7942 *cynA* mutant cells grown under low- $\text{C}_i$  conditions were placed in the reaction chamber attached to the MS and allowed to equilibrate (25 mM BTP-HCl buffer, pH 8, at  $30^\circ\text{C}$ ) in the light ( $300 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) in the presence of KOCN.  $\text{CO}_2$  transport was initiated by adding  $3.5 \mu\text{M}$   $\text{CO}_2$  to the reaction vessel, and its concentration was followed over time. The difference between the curves obtained in the light (A, 0 mM KOCN, maximum uptake) and the dark (F, no uptake) was taken as a measure of  $\text{CO}_2$  transport. The effect of 1, 3, 5, or 10 mM KOCN (B, C, D, and E) on  $\text{CO}_2$  transport are also shown.

the *cynA* mutant to transport and concentrate  $\text{NCO}^-$  is severely impaired.

**Inhibition of  $\text{CO}_2$  transport by  $\text{NCO}^-$ .** Since the *cynA* mutant does not generate  $\text{CO}_2$  from  $\text{NCO}^-$ , this mutant was used to further examine KOCN inhibition of  $\text{CO}_2$  transport (Fig. 3) using an MS assay (11). Pure  $\text{CO}_2$  was introduced into the reaction cuvette, and its disappearance was followed over time (Fig. 6). The differences between the curves obtained in the light and the dark were a consequence of cellular uptake and a measure of  $\text{CO}_2$  transport (11). In contrast to wild-type cells (Fig. 3C), 1 mM KOCN had little effect on  $\text{CO}_2$  transport in the mutant (Fig. 6). However, at 10 mM KOCN,  $\text{CO}_2$  transport was reduced by nearly 90%. Although high concentrations of KOCN inhibited  $\text{CO}_2$  transport in the mutant, the lack of an effect at 1 mM indicated that the inhibition by 1 mM KOCN on  $^{14}\text{CO}_2$  transport in wild-type cells (Fig. 3C) was due mainly to competition with  $^{12}\text{CO}_2$  generated from the decomposition of cyanate.  $\text{NCO}^-$ , therefore, is an inhibitor of the  $\text{CO}_2$  uptake system but is not a transported substrate.

**The role of the CCM and the  $\text{C}_i$  transporters in cyanate metabolism.**  $\text{HCO}_3^-$  is also required by cyanase as a substrate, and it seems reasonable to assume that the CCM of *S. elongatus* strain PCC7942 plays a direct role in its provision in the light, particularly given that the net rate of cyanate decomposition may reach  $230 \mu\text{mol mg}^{-1}$  of Chl  $\text{h}^{-1}$ . The role of the internal  $\text{C}_i$  pool in promoting cyanate metabolism was tested using the  $\Delta\text{chpX} \Delta\text{chpY}$  mutant of *S. elongatus* strain PCC7942 grown on 5%  $\text{CO}_2$ . As this mutant lacks  $\text{CO}_2$  transport capabilities and  $\text{HCO}_3^-$  transport is repressed by growth under high- $\text{C}_i$  conditions, it has little or no ability to concentrate  $\text{C}_i$  internally (52). Consequently, diffusion of  $\text{C}_i$  largely controls the size of the internal  $\text{C}_i$  pool in the  $\Delta\text{chpX} \Delta\text{chpY}$  mutant both in the light and dark. In these experiments (Fig. 7), we controlled the size of the  $\text{C}_i$  pool by imposing diffusion gradients between  $\text{C}_i$ -free cells and the medium through the provi-

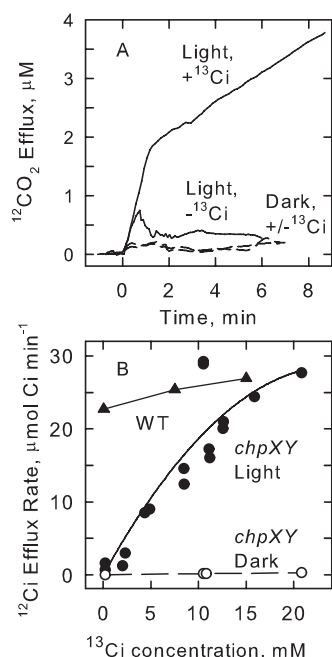


FIG. 7. Involvement of the cyanobacterial CCM in cyanate metabolism. (A) Time course of KOCN-dependent  $^{12}\text{CO}_2$  efflux in the light (solid lines) and dark (dashed lines). *S. elongatus* strain PCC7942  $\Delta\text{chpX} \Delta\text{chpY}$  cells grown on 5%  $\text{CO}_2$  were suspended in 100 mM EPPS (*N*-[2-hydroxyethyl]piperazine-*N'*-[3-propanesulfonic acid]) buffer, pH 8, and incubated in the light or dark for 5 min in the reaction cuvette, in the absence and presence of 10 mM  $\text{NaH}^{13}\text{CO}_3$  ( $^{13}\text{C}_i$ ). The experiment was started by the addition of 1 mM KOCN, and  $^{12}\text{CO}_2$  efflux was measured over time by MS. (B) Dependence of  $^{12}\text{C}_i$  efflux rate on  $^{13}\text{C}_i$  concentration. The average rate of  $^{12}\text{C}_i$  efflux, at a constant cell density ( $7.5 \mu\text{g}$  of Chl *a*  $\text{ml}^{-1}$ ), was calculated from  $^{12}\text{CO}_2$  efflux measurements over an 8-min interval as a function of  $^{13}\text{C}_i$  concentration for  $\Delta\text{chpX} \Delta\text{chpY}$  (*chpXY*) cells in the light (●) and dark (○) provided with 1 mM KOCN. Also shown (dashed line and ▲) is the dependence on  $^{13}\text{C}_i$  concentration of  $^{12}\text{CO}_2$  efflux in *S. elongatus* PCC7942 wild-type (WT) cells grown under low- $\text{C}_i$  conditions.

sion of various concentrations of exogenous  $^{13}\text{C}_i$ . Following equilibration between the medium and the cells, we measured the average rate of decomposition of 1 mM KOCN as  $^{12}\text{C}_i$  efflux over a 6- to 8-min period in the light and dark. A typical time course is shown for the  $\Delta\text{chpX} \Delta\text{chpY}$  mutant in the presence and absence of 10 mM  $\text{NaH}^{13}\text{CO}_3$  (Fig. 7A). The data confirm that cyanate transport was light dependent and demonstrated that the rate of cyanate decomposition was dependent upon and limited by the size of the internal  $\text{C}_i$  pool, approaching a maximum rate beyond 20 mM  $\text{C}_i$  (Fig. 7B). In wild-type cells grown under low- $\text{C}_i$  conditions, a 20 mM  $\text{C}_i$  pool is readily formed in the light when the external  $\text{C}_i$  concentration is about 20  $\mu\text{M}$  (6). Consequently, the  $\text{C}_i$  that contaminated buffers and KOCN in our experiments was a sufficient source of substrate for the CCM to provide an internal  $\text{HCO}_3^-$  concentration that was able to support a near-maximum rate of KOCN decomposition without supplementing the buffer with  $\text{C}_i$  (Fig. 7B). Supplementing the buffer with 7.5 or 15 mM  $\text{NaH}^{13}\text{CO}_3$  resulted in only a modest increase in the rate of cyanate decomposition by wild-type cells grown under low- $\text{C}_i$  conditions. This result is consistent with the internal  $\text{HCO}_3^-$

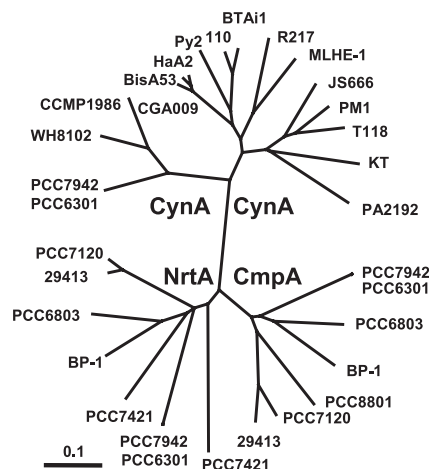


FIG. 8. Unrooted phylogenetic tree illustrating the relationship between CynA proteins identified from bacterial sources and NrtA and CmpA from cyanobacteria. Cyanobacterial species are as follows: *S. elongatus* PCC7942, *Synechococcus* sp. strain PCC6301, *Synechocystis* sp. strain PCC6803, *Thermosynechococcus elongatus* BP-1, *Cyanothece* sp. strain PCC7120, *Nostoc* sp. strain PCC7120, *Anabaena variabilis* ATCC 29413, *Gloeobacter violaceus* PCC7421, *Synechococcus* sp. strain WH8102, and *P. marinus* CCMP1986. Other organisms are *Rhodospseudomonas palustris* strains CGA009, BisA53, and HaA2; *Xanthobacter autotrophicus* Py2; *Bradyrhizobium japonicum* USDA 110; *Bradyrhizobium* sp. strain BTAi1; *Roseovarius* sp. strain R217; *Alkalilimnicola ehrlichei* MLHE-1; *Polaromonas* sp. strain JS666; *Rubrivivax gelatinosus* PM1; *Rhodospirillum rubrum* T118; *Methylobacillus flagellatus* KT; and *Pseudomonas aeruginosa* PA2192. Organisms are represented on the figure generally by the strain designation or culture collection number.

concentration being at a near-saturating level for cyanase when the external  $\text{C}_i$  concentration is extremely low.

There was no specific requirement for either the  $\text{Na}^+$ -dependent or  $\text{Na}^+$ -independent  $\text{HCO}_3^-$  transport systems to form the  $\text{C}_i$  pool as growth under high- $\text{C}_i$  conditions, which repressed these activities (5, 12), did not prevent cyanate uptake, decomposition (Fig. 4), or KOCN-dependent  $\text{O}_2$  evolution. Similarly, ethoxazolamide, a  $\text{CO}_2$  transport inhibitor, did not completely prevent  $\text{KO}^{14}\text{CN}$  accumulation and  $^{14}\text{C}$ -fixation in cells grown under low- $\text{C}_i$  conditions (Table 1) or KOCN-dependent  $\text{CO}_2$  efflux (not shown). We conclude that an internal  $\text{C}_i$  pool in the millimolar range (Fig. 7), independent of the actual means by which it is formed, is a prerequisite for KOCN metabolism in *S. elongatus* strain PCC7942. However, under normal physiological conditions (0.001 to 5 mM  $\text{C}_i$ ), one or more of the  $\text{C}_i$  transport systems is required to actively create an internal  $\text{C}_i$  pool if cyanate metabolism is to proceed at a significant rate (Fig. 7B).

**Occurrence of *cynABDS* among Bacteria.** A search of the NCBI database using the BLASTp program identified 98 bacterial strains (out of 598 as of 22 July 2006) bearing a protein with amino acid sequence similarity to CynS of *S. elongatus* strain PCC7942. These included 12 of 24 cyanobacterial strains. Gene clusters encoding putative ABC-type transporters were also found adjacent to *cynS* in 17 strains, including four cyanobacteria, *S. elongatus* strain PCC7942, *Synechococcus* sp. strain PCC6301, *Prochlorococcus marinus* strain CCMP1986, and *Synechococcus* sp. strain WH8102 (Fig. 8) (14,



43). Additional BLASTp searches using *S. elongatus* strain PCC7942 CynA as the query revealed that all 17 of the putative periplasmic substrate-binding proteins found encoded near *cynS* were highly related (E values less than  $10^{-85}$ ) and more closely related to one another than to NrtA or CmpA (Fig. 8). Similarly, when CynB from *S. elongatus* strain PCC7942 was the query, the permease components most similar to it were those that were encoded by permease components adjacent to *cynS* (E values less than  $10^{-59}$ ) (data not shown). However, this type of association broke down in the case of CynD, the ATP-binding protein that energizes transport. Since the substrate specificity of individual ABC-type transporters is determined by the binding protein and the permease subunits, while energization is a more generic function, it is likely that the newly identified *cynABDS*-like operons encode components for cyanate transport and degradation. Most of these ABC-transporters have previously been annotated as nitrate transporters and/or as simply possessing a twin-arginine signal sequence. In addition to the four cyanobacteria, *cynABDS*-like operons were found in seven strains of the *Alphaproteobacteria*, four strains of *Betaproteobacteria*, and two strains of *Gamma-proteobacteria* (Fig. 8). Most of these strains (11/17) were obligate or facultative chemo- or photoautotrophs. The remaining strains consisted of heterotrophs that oxidized a range of organic compounds and a single obligate methylotroph.

## DISCUSSION

**A model for cyanate metabolism.** The results of our investigation indicate that the transport and decomposition of exogenously supplied cyanate by strains of the freshwater cyanobacterium *Synechococcus* depend upon the products of the *cynABDS* operon and the biophysical activity of the CCM in a light-dependent manner. With the exception of *cynS*, the molecular components required to support the transport and cellular decomposition of cyanate are distinct from the components encoded by the *cynTSX* operon of *E. coli* and thus constitute a novel mechanism that facilitates the assimilation of C and N from cyanate. Indeed, it is likely that an *E. coli*-like system for cyanate metabolism would be deleterious in cyanobacteria because the cytosolic CA component would circumvent the operation of parts of the CCM.

Inactivation of *cynA*, which encodes the periplasmic binding protein of a putative multicomponent ABC-type transporter, results in the loss of active cyanate transport and the ability to decompose  $\text{NCO}^-$  to  $\text{CO}_2$  and  $\text{NH}_3$ . Similarly, inactivation of *cynS*, which codes for a cytosolic cyanase, yields a mutant that is also unable to decompose cyanate. As a result, both mutants lack cyanate-dependent  $\text{O}_2$  evolution and  $\text{CO}_2$  efflux. An additional novel aspect of cyanate metabolism in cyanobacteria is its dependence on light and photosynthetic electron transport. This dependence was multifaceted and traced to the light dependence of both cyanate transport and  $\text{C}_i$  transport. Presumably, ATP derived from photophosphorylation is used to energize cyanate transport through the membrane-bound ATP-binding protein CynD. Although oxidative phosphorylation also yields ATP, there is little active cyanate transport in the dark. These results suggest that cyanate transport activity is regulated by an additional step beyond energy supply or simple kinetic control mechanisms associated with substrate concen-

tration. The codependence of cyanate and  $\text{C}_i$  transport on light further suggests that the transporters may be controlled by a common activation/deactivation mechanism. Such a mechanism ensures that the transport of a potential toxin into the cells is coupled with the means to rapidly degrade it. However, ongoing  $\text{C}_i$  transport is not a prerequisite for the activation of  $\text{NCO}^-$  transport as evidenced by the fact that the  $\Delta\text{chpX}$   $\Delta\text{chpY}$  mutant of *S. elongatus* strain PCC7942 was able to decompose exogenous  $\text{NCO}^-$  when provided with an adequate supply of internal  $\text{HCO}_3^-$ , which was acquired through  $\text{CO}_2$  diffusion (Fig. 7).

**The role of the CCM in cyanate metabolism.** The rate of cyanate decomposition depends on the size of the internal  $\text{HCO}_3^-$  pool, which in turn is controlled by the activity of the CCM in the light (Fig. 7). In the absence of a functional CCM, cyanate decomposition was severely retarded at external  $\text{C}_i$  concentrations usually experienced during growth and points to the fact that  $\text{CO}_2$  diffusion is normally inadequate to supply intracellular  $\text{HCO}_3^-$  for cyanase. Coupling of cyanate decomposition to the CCM is a unique strategy for  $\text{HCO}_3^-$  acquisition that distinguishes *S. elongatus* strain PCC7942 from *E. coli* and, potentially, autotrophic cyanate metabolism from heterotrophic cyanate metabolism, which relies on the combined action of cyanase and CA. CA, which is crucial for the provision of  $\text{HCO}_3^-$  in the cytosol of *E. coli*, is confined to carboxysomes in the *Synechococcus* strains and functions in the opposite direction to supply  $\text{CO}_2$  for photosynthetic fixation (5, 39). A direct role for cyanobacterial CA in the trapping of  $\text{HCO}_3^-$  in the cytosol for cyanase is not indicated by virtue of the massive efflux of cyanate-derived  $\text{CO}_2$  that occurs in cells grown under high- $\text{C}_i$  conditions. The high capacity of the cyanobacterial CCM to concentrate  $\text{HCO}_3^-$  internally over a wide range of external  $\text{C}_i$  concentrations is an important factor which circumvents the limitation imposed by external  $\text{C}_i$  concentration and  $\text{CO}_2$  diffusion on the cellular capacity for cyanate decomposition and is a key reason why *Synechococcus* strains are able to have a 20-fold higher capacity for cyanate decomposition compared to *E. coli* (30).

The dependence of cyanate decomposition on the activity of the CCM also facilitates the assimilation of cyanate-derived  $\text{CO}_2$  through the photosynthetic carbon reduction cycle. This is most evident in cells grown in low  $\text{C}_i$  concentrations where a high-affinity CCM is fully induced. In these cells,  $\text{CO}_2$  efflux is substantially diminished (30), and this is reflected in a  $K_{0.5}$  ( $\text{NCO}^-$ ) value for photosynthetic cyanate utilization that is twofold lower compared to cells grown in high  $\text{C}_i$  concentrations. The superior retention of cyanate-derived  $\text{CO}_2$  by cells grown in low  $\text{C}_i$  concentrations coincides with the up-regulation of the high-affinity  $\text{CO}_2$  uptake system (51) that is predominantly localized to the thylakoid membranes. Presumably,  $\text{CO}_2$  loss from the cytoplasm is curtailed through its conversion to  $\text{HCO}_3^-$  by the light-driven action of the NdhF3/NdhD3/ChpY complex rather than carboxysome-localized CA. The trapped  $\text{HCO}_3^-$  then contributes to the overall internal pool available for photosynthetic fixation. Cyanate-derived  $\text{CO}_2$  that is lost from cells will undoubtedly be recovered by the  $\text{C}_i$  transport systems and eventually fixed, as evidenced by the 1:1 stoichiometry between added  $\text{NCO}^-$  and  $\text{CO}_2$  fixation ( $\text{O}_2$  evolution). In the short term, the proportion of  $\text{CO}_2$  that is lost from the cell to that which is retained will depend on the level

of induction of the CCM and the capacity of the cell for CO<sub>2</sub> fixation.

**Cyanate transport.** The high degree of amino acid sequence similarity between CynA and its paralogs NrtA and CmpA suggests that CynA may also perform similar transport functions in cellular metabolism. Consequently, the notion that CynABD acts as an NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> transporter or HCO<sub>3</sub><sup>-</sup> transporter in *S. elongatus* strain PCC7942 was investigated along with the hypothesis that native NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> transporters or HCO<sub>3</sub><sup>-</sup> transporters accept NCO<sup>-</sup> as an alternative substrate. In competition assays (Fig. 4), however, neither excess NO<sub>3</sub><sup>-</sup> nor NO<sub>2</sub><sup>-</sup> (as well as Cl<sup>-</sup>, PO<sub>4</sub><sup>2-</sup>, or SO<sub>4</sub><sup>2-</sup>) impaired the release of cyanate-derived CO<sub>2</sub> from whole cells, indicating that these anions did not interfere with the initial uptake of NCO<sup>-</sup>, as would be expected for alternative substrates of a common transporter. It seems unlikely, therefore, that CynABD is an NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> transporter that gratuitously transports NCO<sup>-</sup>. For the same reason, it is also unlikely that NCO<sup>-</sup> transport in *S. elongatus* strain PCC7942 is mediated by the bona fide NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> transporter, NrtABCD, as suggested for *Azotobacter chroococcum* (33).

The potential role of the HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub> transport systems in NCO<sup>-</sup> transport proved to be more difficult to unravel due to the nonenzymatic interconversion between HCO<sub>3</sub><sup>-</sup> and CO<sub>2</sub>, the presence of multiple C<sub>i</sub> transporters, the contamination of labeled KOCN solutions with labeled C<sub>i</sub>, the slow but spontaneous hydrolysis of cyanate-forming C<sub>i</sub>, and the rapid enzymatic decomposition of NCO<sup>-</sup> that yielded a large quantity of CO<sub>2</sub> within seconds of addition. <sup>14</sup>C-bicarbonate transport assays (Fig. 3) revealed that NCO<sup>-</sup> did not compete with H<sup>14</sup>CO<sub>3</sub><sup>-</sup> for uptake, consistent with a lack of involvement of CmpABCD or SbtA in the transport of NCO<sup>-</sup> by *S. elongatus* strain PCC7942. The roughly 50 to 60% reduction in <sup>14</sup>C accumulation observed in N<sup>14</sup>CO<sup>-</sup> transport assays (Table 2) when cells were supplied with excess <sup>12</sup>C<sub>i</sub> can be ascribed to the competitive elimination by H<sup>12</sup>CO<sub>3</sub><sup>-</sup> of the transport of H<sup>14</sup>CO<sub>3</sub><sup>-</sup> that contaminates KO<sup>14</sup>CN solutions rather than a direct inhibition of NCO<sup>-</sup> transport by HCO<sub>3</sub><sup>-</sup>, since a reciprocal inhibition of HCO<sub>3</sub><sup>-</sup> transport by NCO<sup>-</sup> did not occur. The fact that the effect of “cold” HCO<sub>3</sub><sup>-</sup> on N<sup>14</sup>CO<sup>-</sup> transport saturated at 2 mM suggests that CynABD is not an HCO<sub>3</sub><sup>-</sup> transporter since high HCO<sub>3</sub><sup>-</sup> concentrations should eliminate NCO<sup>-</sup> transport.

Inhibitor studies have provided evidence both for and against a role for the CO<sub>2</sub> transport system in NCO<sup>-</sup> transport (Fig. 3 and 6). While 1 mM NCO<sup>-</sup> appeared to inhibit <sup>14</sup>CO<sub>2</sub> transport in wild-type *S. elongatus* strain PCC7942 cells, 1 mM NCO<sup>-</sup> had no effect on CO<sub>2</sub> transport in the *cynA* mutant, as judged by MS. The most likely explanation for this apparent discrepancy is that cyanate-derived <sup>12</sup>CO<sub>2</sub> released from wild-type cells during transport experiments competed with <sup>14</sup>CO<sub>2</sub> for uptake, thereby reducing the internal concentration of <sup>14</sup>C. Since the *cynA* mutant does not decompose cyanate, cyanate-derived <sup>12</sup>CO<sub>2</sub> competition is not a complicating factor in determining whether or not cyanate inhibits CO<sub>2</sub> transport. Thus, these results lead us to conclude that cyanate is not a substrate of the CO<sub>2</sub> transport systems. This conclusion is also consistent with the observed inability of *Synechocystis* sp. strain PCC6803 to decompose externally supplied cyanate to CO<sub>2</sub> and NH<sub>3</sub>

(Fig. 4), in spite of possessing a functional CO<sub>2</sub> transport system to potentially deliver NCO<sup>-</sup> to the cytosol for cyanase.

The inability of *Synechocystis* sp. strain PCC6803 to decompose externally supplied cyanate in the light as well as the inability of the *S. elongatus* strain PCC7942 *ΔchpX ΔchpY* mutant to decompose cyanate in the dark at high internal HCO<sub>3</sub><sup>-</sup> concentrations (Fig. 7) indicates that the diffusive entry of HOCN contributes minimally to the gross uptake of cyanate. Thus, the collective data demonstrate that the *Synechococcus* strains are able to actively transport and concentrate NCO<sup>-</sup> through a specific pathway that involves CynA and, therefore, CynB and CynD. We are now in the process of determining if CynA is an NCO<sup>-</sup> binding protein. The detailed kinetics of cyanate transport have yet to be resolved, due to the technical difficulties of separating the initial flux of NCO<sup>-</sup> from the flux and reflux of CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>. It is clear, however, that a transient net flux of up to 230 μmol mg<sup>-1</sup> of Chl h<sup>-1</sup> is possible as this is the maximum steady-state rate of cyanate-dependent O<sub>2</sub> evolution. In our experiments, the ratio of [NCO<sup>-</sup>]<sub>in</sub>/[NCO<sup>-</sup>]<sub>out</sub> was in the range of 8 to 10 at 1 mM NCO<sup>-</sup>. These estimates were complicated by the need to apply a substantial correction for <sup>14</sup>C<sub>i</sub> accumulation and are subject to future refinement. Nevertheless, the data indicate that NCO<sup>-</sup> was concentrated above the level expected from diffusion alone.

*cynABDS*-like operons are present in a diverse range of cyanobacteria and proteobacteria, including the globally important oceanic cyanobacteria *Synechococcus* sp. strain WH8102 and *P. marinus* strain CCMP1986 (Fig. 8). It seems likely that the HCO<sub>3</sub><sup>-</sup> required for NCO<sup>-</sup> decomposition is provided by the CCM of these two organisms. A CCM has yet to be identified in autotrophic proteobacteria, such as *Rhodospseudomonas palustris* and *Bradyrhizobium* sp. strain BTAi1, which possess *cynABDS*-like operons. Interestingly, these organisms lack carboxysomes, a hallmark of the cyanobacterial CCM. Thus, it is possible that they may employ their own unique strategy for HCO<sub>3</sub><sup>-</sup> acquisition or utilize a cytosolic CA. Alternatively, NCO<sup>-</sup> degradation may be restricted to naturally occurring high C<sub>i</sub> environments where CO<sub>2</sub> diffusion could play a role. This latter route for HCO<sub>3</sub><sup>-</sup> acquisition may also be of particular importance for the heterotrophs which possess *cynABDS*-like operons.

**Physiological role.** One experimentally verified role for CynABDS in cellular metabolism is to provide a vehicle for the transport and decomposition of exogenous cyanate. Since the *Synechococcus* strains readily assimilate cyanate-derived CO<sub>2</sub> (Fig. 1 and 2) and NH<sub>3</sub> (30), CynABDS permits the utilization of cyanate as a niche source of C and N for growth. CynS alone cannot fulfill this metabolic role. Thus, CynABDS may be legitimately included as an input branch to the major C and N assimilatory pathways in cyanobacteria. The relatively high capacity for NCO<sup>-</sup> transport and decomposition may reflect a metabolic strategy for rapid utilization from environments where NCO<sup>-</sup> is only sporadically available and has a short half-life. Utilization of NCO<sup>-</sup> broadens the range of available C and N sources and may provide a small advantage over organisms that can only assimilate NH<sub>3</sub>, particularly in oligotrophic environments where C<sub>i</sub> and N sources limit growth (13, 14, 43). In other environments such as industrial waste waters, relatively high concentrations of NCO<sup>-</sup> are available, pro-

duced by bacterial consortia (heterotrophs and autotrophs) that are actively degrading thiocyanate (41, 50). Here, the opportunistic utilization of cyanate by cyanobacteria may reflect their participation in thiocyanate metabolism. We are currently investigating the possibility that cyanobacteria may also directly metabolize thiocyanate.

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