Involvement of the cynABDS Operon and the CO₂-Concentrating Mechanism in the Light-Dependent Transport and Metabolism of Cyanate by Cyanobacteria

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The cyanobacteria Synechococcus elongatus strain PCC7942 and Synechococcus sp. strain UTEX625 decomposed exogenously supplied cyanate (NCO⁻) to CO₂ and NH₃ through the action of a cytosolic cyanase which required HCO₃⁻ as a second substrate. The ability to metabolize NCO⁻ relied on three essential elements: proteins encoded by the cynABDS operon, the biophysical activity of the CO₂-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 CO₂, HCO₃⁻, NO₃⁻, NO₂⁻, Cl⁻, PO₄²⁻, and SO₄²⁻ did not contribute to the cellular flux of NCO⁻ and that CynABD did not contribute to the flux of these nutrients, implicating CynABD as a novel primary active NCO⁻ transporter. In the S. elongatus strain PCC7942 ΔchpX ΔchpY 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 (Ci) (HCO₃⁻ + CO₂) pool. Unlike wild-type cells, the rate of NCO⁻ decomposition by the ΔchpX ΔchpY mutant was severely depressed at low external Ci concentrations, indicating that the CCM was essential in providing HCO₃⁻ for cyanase under typical growth conditions. Light was required to activate and/or energize the active transport of both NCO⁻ and Ci. Putative cynABDS operons were identified in the genomes of diverse Proteobacteria, suggesting that CynABD-mediated cyanate metabolism is not restricted to cyanobacteria.

Cyanate (NCO⁻) 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 CO₂ and NH₃ (2, 15, 21, 47) according to the following reaction: NCO⁻ + HCO₃⁻ + 2H⁺ → 2CO₂ + NH₃. The net formation of CO₂ also means that cyanase coactivates the irreversible dehydration of HCO₃⁻ (16).

Kinetic, isotopic, and X-ray crystallographic studies show that cyanase binds both NCO⁻ and HCO₃⁻ in the active site forming a diamin diion intermediate that enzymatically decarboxylates to CO₂ and carbamate (3, 28, 49). Spontaneous decarbamylolation of the carbamate subsequently yields a second CO₂ and NH₃. Assimilation of cyanate-derived NH₃ and CO₂ 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⁻ 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 HCO₃⁻ (rather than CO₂) as a substrate in the reaction. Physiological studies (17, 18, 22, 23) indicate that in the absence of CA, the CO₂ generated from cyanate decomposition diffuses out of cells faster than it can be hydrated nonenzymatically to HCO₃⁻. This leads to a cellular depletion of HCO₃⁻ and cessation of cyanate metabolism through substrate deprivation. CA prevents this cellular depletion by trapping CO₂ and catalytically regenerating HCO₃⁻ 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 CO₂ and NH₃ as described in the reaction above (30). Inhibitor studies (30) have shown that cyanate-derived NH₃ is rapidly incorporated by this cyanobacterium via the central nitrogen assimilation pathway, and it has recently been suggested that NCO⁻ can serve as the sole source of N for growth of the globally important marine cyanobacterium Synechococcus sp. strain WH8102 (35, 43). CO₂ 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, 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 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 CO\(_2\)-concentrating mechanism (CCM) of cyanobacteria (5, 39, 40), a corequirement for CA activity in cyanobacterial NCO\(^{-}\) decomposition has not been demonstrated. Instead, it has been proposed that the active HCO\(_3^-\) transport systems that normally provide inorganic carbon (C\(_{1}\)) ([CO\(_3^{2-}\) + [HCO\(_3^-\)] + [CO\(_2\)\textsubscript{aq}]] for photosynthesis may fulfill the role that is played by CA in *E. coli* (30).

The ability of heterotrophs and autotrophs to utilize exogenic NCO\(^{-}\) as a source of N and C presumably relies upon the transport of this ion into cells. Transport studies in *E. coli* indicate that N\(^3\)CO\(^{-}\) uptake involves an energy-dependent, saturable transporter with a *K_m* of 400 \(\mu\)M and a *V_max* of 4.4 nmol min\(^{-1}\) \(10^9\) 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 N\(^3\)CO\(^{-}\) likely reflects the trapping of cyanate-derived H\(^1\)CO\(_3^-\), as H\(^1\)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 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 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 NO\(_3^-\) or HCO\(_3^-\) transporter due to the high degree of amino acid sequence similarity between CynA and the respective periplasmic binding proteins NrtA and CmpA (29). The hypothesis that the C\(_3\) transport systems of cyanobacteria are essential to support ongoing cyanate metabolism was tested in a mutant lacking CO\(_3^2-\)-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 PCC7930), *S. elongatus* strain PCC7942, and *Synechocystis* sp. strain PCC6803 (Pasteur Culture Collection, Paris, France) were grown photoautotrophically in BG11 medium containing 17.5 mM NaNO\(_3\). The presence of NO\(_3^-\) ensured that cyanate was fully utilized (19). Cells were grown either under C\(_3\) limitation in standing culture, where diffusion of atmospheric CO\(_2\) was the sole means of CO\(_2\) delivery, or under high-C\(_3\) growth conditions where CO\(_2\) was supplied by gassing buffered (25 mM 1,3-bis(Tris(hy- droxymethyl)-methylamino)propane [TRP], pH 8) cell suspensions with sterile air containing 5% (vol/vol) CO\(_2\), at 200 ml min\(^{-1}\) (8). The *cynT* and *cynS* mutants of *S. elongatus* strain PCC7942 were grown in the presence of kanamycin (10 \(\mu\)g ml\(^{-1}\)), spectinomycin (40 \(\mu\)g ml\(^{-1}\)), or ampicillin (100 \(\mu\)g ml\(^{-1}\)). The *ΔcynX* 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 \(\mu\)g ml\(^{-1}\)) and chloramphenicol (5 \(\mu\)g ml\(^{-1}\)). *E. coli* DH5\(_\alpha\) (Gibco-BRL) was grown on Luria-Bertani (LB) medium (38) supplemented with ampicillin (100 \(\mu\)g ml\(^{-1}\)) or spectinomycin (50 \(\mu\)g ml\(^{-1}\)), when appropriate.

**Insertional inactivation of *cynX* 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 *cynX* and *cynS* genes. Nonreplicating, pUC19-based plasmids containing either *cynX* or *cynS* (20) were cut with a restriction endonuclease at a unique ClaI or unique BglII site to produce an internal deletion that eliminates the respective genes. The resulting plasmids pP12::ORF440 (Amp\(^{\text{r}}\) Kan\(^{\text{r}}\)::*cynA*) and pXH::ORF146 (Amp\(^{\text{r}}\) Sptr::*cynA*) 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 LB1 plates were maintained initially in the dark and then at low light (10 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\); 400 to 700 nm) on 5% (vol/vol) CO\(_2\) in air at 30°C and supplemented with 5 mM sodium thiosulfate along with the appropriate selective antibiotic (10 \(\mu\)g ml\(^{-1}\) kanamycin or 40 \(\mu\)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 *cynX* or *cynS* were recovered by replica plating and identified as colonies that were ampicillin sensitive and resistant to the antibiotic mQ (25) or pLTD (22) that harbored the episomal vectors 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, CynM, 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 AABS8736, 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 \(\mu\)g of chlorophyll [Chl] ml\(^{-1}\)) used for transport and physiological studies were harvested in mid-log phase, washed three times by centrifugation, and suspended in C\(_3\)-free 25 mM BTP–23.5 mM HCl buffer to a final density of 7 to 18 \(\mu\)g of Chl 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).

**O\(_2\) evolution and chlorophyll \(a\) fluorescence yield.** Photosynthetic 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, Effeltrich, 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 pH gradient (6). Fluorescence was, therefore, used as a surrogate to determine C\(_3\) status (6). Fluorescence measurements were used to monitor the formation and dissipation of the C\(_3\) pool in real time. Fluorescence quenching was expressed as a percentage of the variable fluorescence, \(F_{v}/F_{m}\), such that \(F_{v}/F_{m} = F_{v}/F_{m0}\) for \(F_{m0}\) the maximum fluorescence yield and \(F_{v}\) the instantaneous low-fluorescence yield (6).
Mass spectrometry (MS) and measurement of dissolved gas fluxes. The concentrations of $^{14}\text{C}_{\text{O}}$, $^{12}\text{C}_{\text{O}}$, $^{13}\text{C}_{\text{O}}$, and $^{14}\text{C}_{\text{O}}$ (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, Middletown, 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 $^{16}\text{O}_{2}$, $^{12}\text{CO}_{2}$, $^{13}\text{CO}_{2}$, or 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 $\mu$mol of photons m$^{-2}$ s$^{-1}$; 400 to 700 nm). Simultaneous measurements of CO$_2$ and O$_2$ fluxes and Chl $\alpha$ 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° angle to the white light source.

Radiochemical analysis of NCO$^{-}$, HCO$^{3-}$, and CO$_2$ transport. Transport and intracellular accumulation were assayed using the silicone fluid filtering centrifugation technique with N$^{14}\text{CO}$, $^{12}\text{C}_{\text{O}}$, $^{13}\text{C}_{\text{O}}$, or $^{14}\text{C}_{\text{O}}$ 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 NCO$^{-}$ or C$\text{i}$, the cell samples were subsequently processed as described by Espie et al. (10). Intracellular concentrations of NCO$^{-}$ or C$\text{i}$ were calculated using an internal cellular water volume of 49 $\mu$l mg$^{-1}$ of Chl (9).

Four variations on the transport assay protocol were used. With method A, cells were incubated in the light (300 $\mu$mol of photons m$^{-2}$ s$^{-1}$; 400 to 700 nm) in the O$_2$-electrode chamber and allowed to deplete the medium of C$\text{i}$ as indicated by the cessation of O$_2$ evolution. A cell sample (100 $\mu$l) was then removed and placed into a 400-$\mu$l microcentrifuge tube containing 100 $\mu$l of silicone fluid (middle layer: silicone oils AR20:AR200 at 1.75:1 (vol/vol); Wacker Chemie, Munich, Germany) and 100 $\mu$l of terminating solution (bottom layer, 2 M KOH in 10% [vol/vol] methanol). The tube was placed in a Beckman microcentrifuge E (Beckman Instruments, Fullerton, CA). The sealed tubes (50 $\mu$l) were then introduced into the microcentrifuge tubes in such a way as to create an N$_2$-filled gap between the cell suspension and the reaction buffer. 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 N$^{14}\text{CO}$$^-$ accumulation was measured in mutant cells or when long-term uptake assays were conducted, KO$^{14}$CN was introduced directly into the O$_2$-electrode chamber. Then, under constant illumination, 100-$\mu$l samples were removed and layered on top of the silicone fluid/transport and the effect of NCO$^{-}$ on C$\text{i}$ transport.

RESULTS

Cyanate uptake and the fate of cyanate-derived $^{14}$C. Figure 1 shows a typical time course of $^{14}$C uptake by Synechococcus sp. strain UTEX 625 (PCC6301) cells provided with 1 mM KO$^{14}$CN. The intracellular $^{14}$C was found in two major fractions: an acid-stable fraction composed of metabolic intermediates and an acid-labile fraction consisting of unreacted N$^{14}$CO$^-$ and $^{14}$C$\text{i}$. Over the course of the experiment (180 s), the intracellular acid-labile pool of $^{14}$CO$_2$ rose to about 21 mM. Interpretation of cyanate uptake experiments (Fig. 1 and 2) is complicated by the presence of $^{14}$C$\text{i}$ which invariably contaminated the KO$^{14}$CN (7 to 25 $\mu$M) and its presence was corrected for in calculations. In some experiments, the level of C$\text{i}$ 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 C$\text{i}$.

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

Nucleotide sequence accession numbers. The nucleotide sequence of the cyanABDS region determined in this study has been deposited in the GenBank database at NCBI under accession numbers AF001333 and US94881.

The KO$^{14}$CN stock solutions contained less than 2% acid-stable material, and its presence was corrected for in calculations. In some experiments, the level of C$\text{i}$ 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 C$\text{i}$.

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The decline in the rate of O2 evolution in which the acid-labile C (NCO\textsuperscript{-}) two fractions were indicative of a precursor-product relationship of 14C (Fig. 1). But as this pool increased in size so, too, did the lagged behind the accumulation of the acid-labile internal pool.

The dynamics of these rate of C incorporation, approaching a steady-state rate as the internal pool approached a maximum. The accumulation of acid-labile NCO\textsuperscript{-} + C\textsubscript{i} (■) for the wild type and the cyn\textsuperscript{A} mutant (○).

In order to distinguish between N\textsuperscript{14}CO\textsuperscript{-} uptake and accumulation and 14C\textsuperscript{-} uptake and accumulation (Fig. 1), we conducted a series of experiments in the presence of unlabeled 14C\textsuperscript{-} (Table 2). Through dilution with 12C\textsuperscript{-}, the specific activity of the 14C\textsuperscript{-} present as a contaminant in KO\textsuperscript{14}CN solutions was reduced by up to 1,000-fold, effectively eliminating the contribution of 14C\textsuperscript{-} uptake to the intracellular acid-labile pool. Table 2 shows that the initial rate (20 s) of 14C\textsuperscript{-} uptake by cells presented with 1 mM KO\textsuperscript{14}CN was 48% or 49% of the control value in the presence of 2 or 5 mM 14C\textsuperscript{-}, respectively. Similarly, the internal pool was reduced to 45% or 46% of the control value, while 14C\textsuperscript{-}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 14C\textsuperscript{-}fixation declined by as much as 75% (Table 2).

Consequently, we estimated that 40% to 45% of the total acid-labile intracellular pool of 14C\textsuperscript{-} was comprised of N\textsuperscript{14}CO\textsuperscript{-}. 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 KOCN provided to the cells. Assuming a membrane potential of

### TABLE 1. Characterization of NCO\textsuperscript{-} uptake and metabolism in Synechococcus sp. strain UTEX 625

<table>
<thead>
<tr>
<th>Light conditions</th>
<th>N\textsuperscript{14}CO\textsuperscript{-} uptake (%)</th>
<th>14C\textsuperscript{-} fixation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light (control)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dark</td>
<td>6.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Light + 25 mM DCMU</td>
<td>7.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Light + 15 mM glyoxaldehyde</td>
<td>59.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Light + 15 mM oxalate</td>
<td>88</td>
<td>99.7</td>
</tr>
<tr>
<td>Light + 1 mM ethoxazolamide</td>
<td>39</td>
<td>32.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} NCO\textsuperscript{-} uptake and C\textsuperscript{-} fixation were measured by the silicone fluid filtering centrifugation technique using method A. Cells grown in standing culture were incubated with 1 mM KO\textsuperscript{14}CN for 2 min in BTP/PHCl buffer, pH 8.0, containing 25 mM NaCl at 30°C and illuminated at 300 \mu mol m\textsuperscript{-2} s\textsuperscript{-1}. Data are the average of 5 experiments in triplicate ±6.5%.
determine the effect of increasing [KOCN] on Na
independent HCO3
possibility that NCO
transport.

The presence of 100
active CO2 uptake activities (6, 9, 12, 31). These transport activ-
tions 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 NCO\textsuperscript{-} transport, then NCO\textsuperscript{-} must also be an inhibitor of C\textsubscript{i} transport activity. Figure 3A and B show that neither Na\textsuperscript{+}-independent HCO3\textsuperscript{-} transport nor Na\textsuperscript{+}-dependent HCO3\textsuperscript{-} transport was inhibited by up to a 24-fold excess of KOCN. However, in cells grown with low C\textsubscript{i} concentrations in standing culture and with high C\textsubscript{i} concentrations, CO2 transport was reduced by up to 50\% with 1 mM KOCN (Fig. 3C). Thus, NCO\textsuperscript{-} transport may occur through the CO2 transport system. The fact that NCO\textsuperscript{-} is an isoelec-
tronic structural analog of CO2 supports this view. However, although 14CO2 transport assays were conducted rapidly, the 12CO2 generated from the decomposition of cyanate (Fig. 4) may also have contributed to the apparent inhibition of 14CO2 transport. Thus, the involvement of the CO2 transport systems in NCO\textsuperscript{-} transport requires further analysis (see below).

The involvement of nitrate/nitrite transport systems in NCO\textsuperscript{-} transport was tested in a different way. The Synecho-
coccus strains grown under high-C\textsubscript{i} conditions have a greatly reduced capacity for HCO3\textsuperscript{-} transport and rely primarily on the low affinity CO2 uptake system for C\textsubscript{i} acquisition (5). Consequently, the contribution (if any) of the CO2 transport systems to NCO\textsuperscript{-} transport can be minimized. When cells grown with high C\textsubscript{i} concentrations were presented with H\textsuperscript{11002}\textsuperscript{14}CO\textsubscript{2}, a large and sustained rise in the extracellular 14CO2 concentration was observed that was light dependent (Fig. 4). The ad-

![FIG. 3. Effect of KOCN on HCO3\textsuperscript{-} and CO2 transport in Synecho-
coccus sp. strain UTEX 625 cells grown in standing culture. The initial rate (20 s) of HCO3\textsuperscript{-} transport was measured (method C) at substrate concentrations of 50 \muM (●) and 250 \muM (○) in pH 8 buffer to determine the effect of increasing [KOCN] on Na\textsuperscript{+}-independent HCO3\textsuperscript{-} transport in the presence of 100 \muM NaCl (A) or Na\textsuperscript{+}-dependent HCO3\textsuperscript{-} transport in the presence 30 mM NaCl (B). (C) The initial rate (10 s) of CO2 transport (■) was measured at 10 \muM in the presence of 100 \muM NaCl (method D). Also shown is the effect of KOCN on CO2 transport in cells grown under high-C\textsubscript{i} conditions (○). The data are the average of six determinations ± standard deviations.

![FIG. 4. KOCN-dependent CO2 efflux by cells grown under high-CO2 conditions. Illuminated wild-type (WT) S. elongatus PCC7942 cells were provided with 1 mM KO\textsuperscript{11002}\textsuperscript{14}CN, and the efflux of 14CO2 was followed by MS over time in the absence or presence of 25 mM NaNO3 or NaNO2. Bovine CA was added to the suspension to illustrate that CO2 was the C\textsubscript{i} species arising in the cell suspensions. Also shown are the CO2 fluxes arising from the cynA and cynS mutants and from Synechocystis sp. strain PCC6803 grown in high-CO2 concentrations.]

<table>
<thead>
<tr>
<th>Experimental condition (with 1 mM KO\textsuperscript{11002}\textsuperscript{14}CN)</th>
<th>% of control value at 20 s</th>
<th>% of control value at 2 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>H\textsuperscript{11002}\textsuperscript{14}CO\textsubscript{2} uptake</td>
<td>Pool</td>
<td>14C fixation</td>
</tr>
<tr>
<td>0 mM KHCO3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2 mM KHCO3</td>
<td>48.2 ± 9.3</td>
<td>45.4 ± 16.2</td>
</tr>
<tr>
<td>5 mM KHCO3</td>
<td>49.4 ± 7.3</td>
<td>46.4 ± 19.3</td>
</tr>
</tbody>
</table>

\( \text{Control value, 0.23 \mumol of C mg}^{-1} \text{ Chl.} \)

\( \text{Values were determined using method C in BTP-HCl buffer, pH 8, containing 25 mM NaCl. Light was supplied at 300 mol} \text{ of photons m}^{-2} \text{ s}^{-1}. \)

\( \text{Data are the average of seven experiments ± standard deviations.} \)

\( \text{Control value, 0.06 \mumol of C mg}^{-1} \text{ Chl.} \)

\( \text{Control value, 6.0 ± 2.0 mM.} \)

\( \text{Control value, 11.0 ± 7.0 mM.} \)

\( \text{Control value, 0.23 \mumol of C mg}^{-1} \text{ Chl.} \)
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 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 \textit{S. elongatus} strain PCC7942 was presented with KO\(^{13}\text{CN}\) in the presence of either 25 mM NaNO\(_3\) or 25 mM 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 NaCl (not shown). Thus, neither NO\(_3^-\) nor 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 NCO\(^-\). Similarly, neither phosphate nor sulfate affected the patterns of KOCN-dependent \(\text{CO}_2\) efflux (not shown).

In cells grown under low-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 NO\(_3^-\), NO\(_2^-\), Cl\(^-\), PO\(_4^{3-}\), and 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 NCO\(^-\).

\textit{cynS} is required but not sufficient to promote the metabolism of exogenous NCO\(^-\). Southern hybridization analysis of CleI-digested DNA from \textit{S. elongatus} strain PCC7942 wild-type and the \textit{cynS} mutant was carried out using a 187-bp EcoR1-BglII restriction fragment from \textit{cynS} as the probe. A single hybridization signal of approximately 7.0 kb was obtained from DNA from the \textit{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 SpTR cassette into \textit{cynS} and indicates that only a single disrupted copy of \textit{cynS} was present in the genome of the \textit{S. elongatus} strain PCC7942 \textit{cynS} mutant.

Previous studies have shown that targeted interruption of \textit{cynS} in \textit{S. elongatus} strain PCC7942 results in the loss of intracellular cyanase activity (19). Consistent with this is the observation that our \textit{cynS} mutant was unable to utilize exogenously supplied KOCN to support photosynthetic \(\text{O}_2\) evolution (Fig. 5A). The inability of the \textit{cynS} mutant to utilize 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-C\(_i\) conditions and responded in a comparable manner to wild-type cells when supplied with 25 \(\mu\text{M C}_i\). The \textit{cynS} mutant was also able to form and retain a large internal pool of \(\text{C}_i\) when provided with exogenous 25 \(\mu\text{M C}_i\) as judged by an increase in Chl \(a\) fluorescence quenching (Fig. 5B). The addition of KOCN also elicited substantial fluorescence quenching (43% of \(F_{\text{av}}\) 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 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 \textit{cynS} mutant (Fig. 5B), as was the rise in \(\text{CO}_2\) concentration following KOCN addition to cells grown under high-C\(_i\) conditions (Fig. 4). The disruption of \textit{cynS} thus led to the loss of intracellular cyanase activity (19) and to an inability to metabolize exogenous cyanate in vivo.

Although the cyanobacterium \textit{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, \textit{Synechocystis} sp. strain PCC6803 cells grown under high-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 \textit{cynS} are required to facilitate the metabolism of exogenous cyanate in the two \textit{Synechococcus} strains and that some or all of these components are lacking naturally or are not expressed in \textit{Synechocystis} sp. strain PCC6803.

\textit{cynA} is required for cyanate transport. Immediately upstream of \textit{cynS} in \textit{S. elongatus} strain PCC7942 and \textit{Synechococcus} sp. strain PCC6301, but not \textit{Synechocystis} sp. strain PCC6803, is a three-gene cluster (GenBank accession numbers AF001333 and U59481) initially designated as \textit{orf440}, \textit{orf263}, and \textit{orf289} and later renamed \textit{cynA}, \textit{cynB}, and \textit{cynD}, respectively (19, 20). The \textit{cyn} genes are all transcribed in the same direction (\textit{cynA} to \textit{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 \textit{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 NO3−. The periplasmic binding protein, has a high degree of amino acid similarity to its paralogs NtrA (46%) and CmpA (45%). The periplasmic binding protein, has a high degree of amino acid similarity to its paralogs NtrA (46%) and CmpA (45%).

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’ 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 O2 evolution or KOCN-dependent Chl a fluorescence quenching, although the mutant was capable of typical wild-type C1-dependent O2 evolution and C1-dependent Chl a fluorescence quenching (Fig. 5). Similarly, the cynA mutant cells grown with high C1 concentrations did not evolve CO2 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 N14CO− accumulation and the absence of sustained 14C 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 K14CN. This allowed the mutant sufficient time to fix contaminating 14C into acid-stable products, as judged by O2 evolution and fluorescence measurements. Consequently, direct measurements of the acid-labile intracellular N14CO− pool were possible (Fig. 2B). The average intracellular concentration of NCO− in the cynA mutant was 2.1 ± 1.1 mM (n = 6) after a 5-min exposure and 3.1 ± 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 ± 8.1 mM (n = 8) after 5 min and 37.1 ± 11.1 mM (n = 8) after 10 min. From these data, internal concentrations of NCO− of 12.2 and 16.7 mM, respectively, were calculated assuming that 45% of the measured pool in wild-type cells was free NCO−. Consequently, the level of 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 HOCN into the cells and its passive equilibration with NCO−. Thus, the ability of the cynA mutant to transport and concentrate NCO− is severely impaired.

Inhibition of CO2 transport by NCO−. Since the cynA mutant does not generate CO2 from NCO−, this mutant was used to further examine KOCN inhibition of CO2 transport (Fig. 3) using an MS assay (11). Pure CO2 was introduced into the reaction cuvette, and its disappearance was followed over time (Fig. 6). The differences 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 CO2 transport. The effect of 1, 3, 5, or 10 mM KOCN (B, C, D, and E) on CO2 transport are also shown.

The role of the CCM and the C1 transporters in cyanate metabolism. HCO3− 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 μmol mg−1 of Chl h−1. The role of the internal C1 pool in promoting cyanate metabolism was tested using the ΔchpX ΔchpY mutant of S. elongatus strain PCC7942 grown on 5% CO2. As this mutant lacks CO3 transport capabilities and HCO3− transport is repressed by growth under high-C1 conditions, it has little or no ability to concentrate C1 internally (52). Consequently, diffusion of C1 largely controls the size of the internal C1 pool in the ΔchpX ΔchpY mutant both in the light and dark. In these experiments (Fig. 7), we controlled the size of the C1 pool by imposing diffusion gradients between C1-free cells and the medium through the provi-
FIG. 7. Involvement of the cyanobacterial CCM in cyanate metabolism. (A) Time course of KOCN-dependent $^{13}$CO$_2$ efflux in the light (solid lines) and dark (dashed lines). S. elongatus strain PCC7942 ΔchpX ΔchpY cells grown on 5% CO$_2$ were suspended in 100 mM EPPS ([N-Hydroxyethyl]piperezine-N’-[3-propanesulfonic acid]) buffer, pH 8, and incubated in the light or dark for 5 min in the reaction cuvette, in the presence and absence of 10 mM NaH$^{13}$CO$_3$ ($^{13}$Ci). The experiment was started by the addition of 1 mM KOCN, and $^{13}$CO$_2$ efflux was measured over time by MS. (B) Dependence of $^{13}$C efflux rate on $^{13}$C$_i$ concentration. The average rate of $^{13}$C$_i$ efflux, at a constant cell density (7.5 $\times$ 10$^7$ cells/ml), was calculated from $^{13}$CO$_2$ efflux measurements over an 8-min interval as a function of $^{13}$C$_i$ concentration for ΔchpX ΔchpY (chpXY) cells in the light (■) and dark (○) provided with 1 mM KOCN. Also shown (dashed line and △) is the dependence on $^{13}$C$_i$ concentration of $^{13}$CO$_2$ efflux in S. elongatus PCC7942 wild-type (WT) cells grown under low-C$_i$ conditions.

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, Cyanothecae sp. strain PCC7780, Nostoc sp. strain PCC7120, Anabaena variabilis ATCC 29413, Gloeobacter violaceus PCC7421, Synechococcus sp. strain WH8102, and P. marinus CCMP1986. Other organisms are Rhodopseudomonas palustris strains CGA009, BisA53, and HaA2; Xanthobacter autotrophicus Py2; Bradyrhizobium japonicum USDA 110; Bradyrhizobium sp. strain BTAl; Roseovarius sp. strain R217; Alkalimicrobium ehrlich MLHE-1; Polokomomas sp. strain B6666; Rubrivivax gelatinosus PM1; Rhodofex ferrireducens T118; Methylbacillus flagellatus KT; and Pseudomonas aeruginosa PA2192. Organisms are represented on the figure generally by the strain designation or culture collection number.

sion of various concentrations of exogenous $^{13}$C$_i$. Following equilibration between the medium and the cells, we measured the average rate of decomposition of 1 mM KOCN as $^{13}$C$_i$ efflux over a 6- to 8-min period in the light and dark. A typical time course is shown for the ΔchpX ΔchpY mutant in the presence and absence of 10 mM NaH$^{13}$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 C$_i$ pool, approaching a maximum rate beyond 20 mM C$_i$ (Fig. 7B). In wild-type cells grown under low-C$_i$ conditions, a 20 mM C$_i$ pool is readily formed in the light when the external C$_i$ concentration is about 20 $\mu$M (6). Consequently, the C$_i$ that contaminated buffers and KOCN in our experiments was a sufficient source of substrate for the CCM to provide an internal HCO$_3^-$ concentration being at a near-saturating level for cyanase when the external C$_i$ concentration is extremely low.

There was no specific requirement for either the Na$^+$-dependent or Na$^-$-independent HCO$_3^-$ transport systems to form the C$_i$ pool as growth under high-C$_i$ conditions, which repressed these activities (5, 12), did not prevent cyanate uptake, decomposition (Fig. 4), or KOCN-dependent O$_2$ evolution. Similarly, ethoxylazole, a CO$_2$ transport inhibitor, did not completely prevent KO$_2$CN accumulation and $^{13}$C$_i$ fixation in cells grown under low-C$_i$ conditions (Table 1) or KOCN-dependent CO$_2$ efflux (not shown). We conclude that an internal 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 C$_i$), one or more of the C$_i$ transport systems is required to actively create an internal 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 \textit{S. elongatus} strain PCC7942 CynA as the query revealed that all 17 of the putative periplasmic substrate-binding proteins found encoded near \textit{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 \textit{S. elongatus} strain PCC7942 was the query, the permease components most similar to it were those that were encoded by permease components adjacent to \textit{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 \textit{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, \textit{cynABDS}-like operons were found in seven strains of the \textit{Alphaproteobacteria}, four strains of \textit{Betaproteobacteria}, and two strains of \textit{Gammaproteobacteria} (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 \textit{Synechococcus} depend upon the products of the \textit{cynABDS} operon and the biophysical activity of the CCM in a light-dependent manner. With the exception of \textit{cynS}, the molecular components required to support the transport and cellular decomposition of cyanate are distinct from the components encoded by the \textit{cynTSX} operon of \textit{E. coli} and thus constitute a novel mechanism that facilitates the assimilation of C and N from cyanate. Indeed, it is likely that an \textit{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 \textit{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 NCO\textsuperscript{-} to CO\textsubscript{2} and NH\textsubscript{3}. Similarly, inactivation of \textit{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 O\textsubscript{2} evolution and CO\textsubscript{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 C\textsubscript{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 concentration. The codependence of cyanate and C\textsubscript{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 C\textsubscript{i} transport is not a prerequisite for the activation of NCO\textsuperscript{-} transport as evidenced by the fact that the \textit{ΔcypX ΔcypY} mutant of \textit{S. elongatus} strain PCC7942 was able to decompose exogenous NCO\textsuperscript{-} when provided with an adequate supply of internal HCO\textsubscript{3}\textsuperscript{-}, which was acquired through CO\textsubscript{2} diffusion (Fig. 7).

**The role of the CCM in cyanate metabolism.** The rate of cyanate decomposition depends on the size of the internal HCO\textsubscript{3}\textsuperscript{-} 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 C\textsubscript{i} concentrations usually experienced during growth and points to the fact that CO\textsubscript{2} diffusion is normally inadequate to supply intracellular HCO\textsubscript{3}\textsuperscript{-} for cyanase. Coupling of cyanate decomposition to the CCM is a unique strategy for HCO\textsubscript{3}\textsuperscript{-} acquisition that distinguishes \textit{S. elongatus} strain PCC7942 from \textit{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 HCO\textsubscript{3}\textsuperscript{-} in the cytosol of \textit{E. coli}, is confined to carboxysomes in the \textit{Synechococcus} strains and functions in the opposite direction to supply CO\textsubscript{2} for photosynthetic fixation (5, 39). A direct role for cyanobacterial CA in the trapping of HCO\textsubscript{3}\textsuperscript{-} in the cytosol for cyanase is not indicated by virtue of the massive efflux of cyanate-derived CO\textsubscript{2} that occurs in cells grown under high-C\textsubscript{i} conditions. The high capacity of the cyanobacterial CCM to concentrate HCO\textsubscript{3}\textsuperscript{-} internally over a wide range of external C\textsubscript{i} concentrations is an important factor which circumvents the limitation imposed by external C\textsubscript{i} concentration and CO\textsubscript{2} diffusion on the cellular capacity for cyanate decomposition and is a key reason why \textit{Synechococcus} strains are able to have a 20-fold higher capacity for cyanate decomposition compared to \textit{E. coli} (30).

The dependence of cyanate decomposition on the activity of the CCM also facilitates the assimilation of cyanate-derived CO\textsubscript{2} through the photosynthetic carbon reduction cycle. This is most evident in cells grown in low C\textsubscript{i} concentrations where a high-affinity CCM is fully induced. In these cells, CO\textsubscript{2} efflux is substantially diminished (30), and this is reflected in a K\textsubscript{0.5} (NCO\textsuperscript{-}) value for photosynthetic cyanate utilization that is twofold lower compared to cells grown in high C\textsubscript{i} concentrations. The superior retention of cyanate-derived CO\textsubscript{2} by cells grown in low C\textsubscript{i} concentrations coincides with the up-regulation of the high-affinity CO\textsubscript{2} uptake system (51) that is predominantly localized to the thylakoid membranes. Presumably, CO\textsubscript{2} loss from the cytoplasm is curtailed through its conversion to HCO\textsubscript{3}\textsuperscript{-} by the light-driven action of the NdhF3/NdhD3/ChpX complex rather than carboxysome-localized CA. The trapped HCO\textsubscript{3}\textsuperscript{-} then contributes to the overall internal pool available for photosynthetic fixation. Cyanate-derived CO\textsubscript{2} that is lost from cells will undoubtedly be recovered by the C\textsubscript{i} transport systems and eventually fixed, as evidenced by the 1:1 stoichiometry between added NCO\textsuperscript{-} and CO\textsubscript{2} fixation (O\textsubscript{2} evolution). In the short term, the proportion of CO\textsubscript{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 CO₂ 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 NO₃⁻/NO₂⁻ transporter or HCO₃⁻ transporter in S. elongatus strain PCC7942 was investigated along with the hypothesis that native NO₃⁻/NO₂⁻ transporters or HCO₃⁻ transporters accept NCO⁻ as an alternative substrate. In competition assays (Fig. 4), however, neither excess NO₃⁻ nor NO₂⁻ (as well as Cl⁻, PO₄³⁻, or SO₄²⁻) impaired the release of cyanate-derived CO₂ from whole cells, indicating that these anions did not interfere with the initial uptake of NCO⁻, as would be expected for alternative substrates of a common transporter. It seems unlikely, therefore, that CynABD is an NO₃⁻/NO₂⁻ transporter that gratuitously transports NCO⁻. For the same reason, it is also unlikely that NCO⁻ transport in S. elongatus strain PCC7942 is mediated by the bona fide NO₃⁻/NO₂⁻ transporter, NrtABC, as suggested for Azotobacter chroococcum (33).

The potential role of the HCO₃⁻ and CO₂ transport systems in NCO⁻ transport proved to be more difficult to unravel due to the nonenzymatic interconversion between HCO₃⁻ and CO₂, the presence of multiple Cᵢ transporters, the contamination of labeled KOCN solutions with labeled Cᵢ, the slow but spontaneous hydrolysis of cyanate-forming Cᵢ, and the rapid enzymatic decomposition of NCO⁻ that yielded a large quantity of CO₂ within seconds of addition. ¹⁴C-bicarbonate transport assays (Fig. 3) revealed that NCO⁻ did not compete with H¹⁴CO₃⁻ for uptake, consistent with a lack of involvement of CmpABCD or SbtA in the transport of NCO⁻ by S. elongatus strain PCC7942. The roughly 50 to 60% reduction in ¹⁴C accumulation observed in N¹⁴CO⁻ transport assays (Table 2) when cells were supplied with excess ¹²Cᵢ can be ascribed to the competitive elimination by H¹²CO₃⁻ of the transport of H¹⁴CO₃⁻ that contaminates KOCN solutions rather than a direct inhibition of NCO⁻ transport by HCO₃⁻, since a reciprocal inhibition of HCO₃⁻ transport by NCO⁻ did not occur. The fact that the effect of “cold” HCO₃⁻ on N¹⁴CO⁻ transport saturated at 2 mM suggests that CynABD is not an HCO₃⁻ transporter since high HCO₃⁻ concentrations should eliminate NCO⁻ transport.

Inhibitor studies have provided evidence both for and against a role for the CO₂ transport system in NCO⁻ transport (Fig. 3 and 6). While 1 mM NCO⁻ appeared to inhibit ¹⁴CO₂ transport in wild-type S. elongatus strain PCC7942 cells, 1 mM NCO⁻ had no effect on CO₂ transport in the cynA mutant, as judged by MS. The most likely explanation for this apparent discrepancy is that cyanate-derived ¹³CO₂ released from wild-type cells during transport experiments competed with ¹⁴CO₂ for uptake, thereby reducing the internal concentration of ¹⁴C. Since the cynA mutant does not decompose cyanate, cyanate-derived ¹³CO₂ competition is not a complicating factor in determining whether or not cyanate inhibits CO₂ transport. Thus, these results lead us to conclude that cyanate is not a substrate of the CO₂ transport systems. This conclusion is also consistent with the observed inability of Synechocystis sp. strain PCC6803 to decompose externally supplied cyanate to CO₂ and NH₃ (Fig. 4), in spite of possessing a functional CO₂ transport system to potentially deliver NCO⁻ 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 ΔehpX ΔehpY mutant to decompose cyanate in the dark at high HCO₃⁻ 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⁻ 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⁻ 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⁻ from the flux and reflux of CO₂ and HCO₃⁻. It is clear, however, that a transient net flux of up to 230 µmol mg⁻¹ of Chl h⁻¹ is possible as this is the maximum steady-state rate of cyanate-dependent O₂ evolution. In our experiments, the ratio of [NCO⁻]₈/[NCO⁻]ₑₙₐ₅ was in the range of 8 to 10 at 1 mM NCO⁻. These estimates were complicated by the need to apply a substantial correction for ¹⁴Cₐ accumulation and subject to future refinement. Nevertheless, the data indicate that NCO⁻ 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 HCO₃⁻ required for NCO⁻ decomposition is provided by the CCM of these two organisms. A CCM has yet to be identified in autotrophic proteobacteria, such as Rhodo pseudomonas palustris and Bradyrhizobium sp. strain BTAl1, 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 HCO₃⁻ acquisition or utilize a cytosolic CA. Alternatively, NCO⁻ degradation may be restricted to naturally occurring high Cₑ environments where CO₂ diffusion could play a role. This latter route for HCO₃⁻ accumulation 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 CO₂ (Fig. 1 and 2) and NH₃ (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⁻ transport and decomposition may reflect a metabolic strategy for rapid utilization from environments where NCO⁻ is only sporadically available and has a short half-life. Utilization of NCO⁻ broadens the range of available C and N sources and may provide a small advantage over organisms that can only assimilate NH₃, particularly in oligotrophic environments where Cₑ and N sources limit growth (13, 14, 43). In other environments such as industrial waste waters, relatively high concentrations of NCO⁻ are available, pro-
duced by bacterial consortia (heterotrophs and autotrophs) that are actively degrading thiocyanate (41, 50). Here, the reduced by bacterial consortia (heterotrophs and autotrophs) that are actively degrading thiocyanate (41, 50). Here, the

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