

Regular paper

Characterization of two cytochrome oxidase operons in the marine cyanobacterium *Synechococcus* sp. PCC 7002: Inactivation of *ctaDI* affects the PS I:PS II ratio

Christopher T. Nomura^{1,2,*}, Søren Persson¹, Gaozhong Shen¹, Kaori Inoue-Sakamoto^{1,3} & Donald A. Bryant¹

¹Department of Biochemistry and Molecular Biology, Center for Biomolecular Structure and Function, The Pennsylvania State University, University Park, PA 16802, USA; ²Polymer Chemistry Laboratory, RIKEN Institute, 351-0198 Wako-shi, Saitama, Japan; ³Department of Bioengineering and Chemistry, Division of Chemistry, College of Environmental Engineering, Kanazawa Institute of Technology, Ohgigaoka, Nonoichi, Japan; *Author for correspondence (e-mail: cnomura@riken.jp; fax: 81-48-462-4667)

Received 19 February 2005; accepted in revised form 7 June 2005

Key words: Cyanobacteria, electron transport, heme-copper oxidases, oxygen consumption, oxygen evolution, photoinhibition

Abstract

Cyanobacteria have versatile electron transfer pathways and many of the proteins involved are functional in both respiratory and photosynthetic electron transport. Examples of such proteins include the cytochrome *b₆f* complex, NADH dehydrogenase and cytochrome oxidase complexes. In this study we have cloned and sequenced two gene clusters from the marine cyanobacterium *Synechococcus* sp. PCC 7002 that potentially encode heme-copper cytochrome oxidases. The *ctaCIDIEI* and *ctaCIIDIIEII* gene clusters are most similar to two related gene clusters found in the freshwater cyanobacterial strain *Synechocystis* sp. PCC 6803. Unlike *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002 does not have a *cydAB*-like gene cluster which encodes a quinol oxidase. The *ctaCIDIEI* and *ctaCIIDIIEII* gene clusters were transcribed polycistronically, although the levels of transcripts for the *ctaCIIDIIEII* gene cluster were lower than those of the *ctaCIDIEI* gene cluster. The *ctaDI* and *ctaDII* coding sequences were interrupted by interposon mutagenesis and full segregants were isolated and characterized for both single and double mutants. Growth rates, chlorophyll and carotenoid contents, oxygen consumption and oxygen evolution were examined in the wild type and mutant strains. Differences between the wild type and mutant strains observed in 77 K fluorescence spectra and in pulse-amplified modulated (PAM) fluorescence studies suggest that the cyanobacterial oxidases play a role in photoinhibition and high light tolerance in *Synechococcus* sp. PCC 7002.

Abbreviations: DCMU – 3-(3', 4'-dichlorophenyl)-1,1-dimethylurea; HEPES – N-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); PS I – Photosystem I; PS II – Photosystem II

Introduction

All aerobic bacterial species examined to date have multiple respiratory oxidases that allow them to

change their respiratory systems according to environmental challenges. These respiratory oxidases may fall into either the heme-copper respiratory oxidase super-family or into the unrelated

cytochrome *bd* oxidase family. Heme-copper respiratory oxidases can be further divided into two subgroups: heme-copper oxidases that are reduced by cytochrome *c*, which includes the mitochondrial cytochrome *c* oxidase, as well as bacterial oxidases of the *aa₃*, *ba₃*, *caa₃*, *cao₃*, and *bo₃* types and those heme-copper oxidases which are reduced by quinones. The mitochondrial heme-copper oxidase consists of 13 subunits, while most bacterial heme-copper respiratory oxidases have 3 to 4 subunits (Garcia-Horsman et al. 1994).

Cyanobacteria represent interesting organisms in which to examine electron transport proteins since they have respiratory electron transport proteins on both the cytoplasmic and thylakoid membranes. Thylakoids utilize both photosynthetic and respiratory electron transport proteins while the cytoplasmic membrane has only a respiratory electron transport chain. Although there have been many studies regarding cytochrome oxidases in cyanobacteria, little is known about their physiological role (Trnka and Peschek 1986; Peschek et al. 1989; Obinger et al. 1990; Tano et al. 1991; Alge and Peschek 1993a; Alge and Peschek 1993b; Sone et al. 1993; Schmetterer et al. 1994; Howitt and Vermass 1998). Previous biochemical studies examining P700⁺ redox kinetics in cyanobacteria such as *Synechococcus* sp. PCC 7002 (Yu et al. 1993) and *Fremyella diplosiphon* (Schubert et al. 1995) indicate that these organisms may use cytochrome oxidases as a sink for excess electron throughput not accounted for by photosystem I activity. *Synechococcus* sp. PCC 7002 is a marine cyanobacterial strain that is related to *Synechocystis* sp. PCC 6803 but has some differences in its electron transport chain composition. Unlike most other cyanobacteria, *Synechococcus* sp. PCC 7002 appears to have only one functional mobile electron transport protein (cytochrome *c₆*) between cytochrome *b₆f* and either photosystem I or cytochrome oxidase (Nomura and Bryant 1997). Results from this study reveal that unlike *Synechocystis* sp. PCC 6803, which has gene sets for three functional oxidases, *Synechococcus* sp. PCC 7002 has only the *ctaCIDI* and the *ctaCIIDIIEII* gene clusters which encode two putative heme-copper respiratory oxidases.

In order to examine the roles of cytochrome oxidases in *Synechococcus* sp. PCC 7002, interposon mutagenesis was used to create strains lacking the genes (*ctaDI*, *ctaDII*) encoding the large

subunits of the cytochrome oxidases in *Synechococcus* sp. PCC 7002. These mutant strains were grown under various conditions and the role of the cytochrome oxidases in oxygen consumption, oxygen evolution, and high light tolerance was examined. The results of this study suggest that the *Synechococcus* sp. PCC 7002 cytochrome oxidases are involved in countering photoinhibition.

Materials and methods

Bacterial strains and culture conditions

Table 1 describes the strains, plasmids and oligonucleotides used in this study. The PR6000 strain of the marine cyanobacterium, *Synechococcus* sp. strain PCC 7002 was maintained in liquid culture and on 1.5% agar plates in medium A under continuous light (250 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 38 °C as previously described (Stevens et al. 1973) supplemented with 1 g l⁻¹ NaNO₃ (referred to as A⁺ medium). The following antibiotic concentrations were added to A⁺ when appropriate for *Synechococcus* sp. PCC 7002 cytochrome oxidase mutant strains: kanamycin (100 $\mu\text{g ml}^{-1}$) and spectinomycin (100 $\mu\text{g ml}^{-1}$). Cells were also grown under several light intensities as described in the results and growth rates were monitored by the increase of light scattering of liquid cultures by measuring the optical density at 550 nm with a Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY) as described previously (Sakamoto et al. 1998). *E. coli* DH5 α was used for all recombinant DNA manipulations and grown on LB media as described previously (Ausubel et al. 1987) with the following antibiotic concentrations when appropriate: ampicillin (100 $\mu\text{g ml}^{-1}$), kanamycin (30 $\mu\text{g ml}^{-1}$), and spectinomycin (50 $\mu\text{g ml}^{-1}$).

Isolation, analysis, and manipulation of nucleic acids

Alkaline lysis plasmid isolations were performed as described previously (Birnboim and Doly 1979). Cyanobacterial chromosomal DNA isolation was performed as previously described by Ghassemian et al. (1994). RNA isolation was performed as described by Golden et al. (1987). Dye terminator DNA sequencing was done by

Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study

Strains, plasmids, and oligonucleotides	Relevant characteristics	Source or reference
<i>Bacterial strains</i>		
<i>Synechococcus</i> sp. PCC 7002, PR6000	wild type	Pasteur Culture Collection
<i>E. coli</i> DH5 α	F ⁻ , <i>endA</i> , <i>hsdR17</i> , <i>supE44</i> , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>argF</i>	Bethesda Research Laboratories
<i>Plasmids</i>		
pBluescript SK +	Amp ^r , <i>colE1</i> , <i>lac</i>	Stratagene
pBCTA1	pBluescript SK + derivation; <i>Synechococcus</i> sp. PCC 7002 <i>ctaCIDIEI</i>	This study
pBCTA2	pBluescript SK + derivation; <i>Synechococcus</i> sp. PCC 7002 <i>ctaCIIDIIEII</i>	This study
pBCTA1pD	pBluescript SK + derivation; <i>Synechococcus</i> sp. PCC 7002, <i>ctaDI::aphII</i> , Km ^r ; parallel orientation	This study
pBCTA1aD	pBluescript SK + derivation; <i>Synechococcus</i> sp. PCC 7002, <i>ctaDI::aphII</i> , Km ^r ; anti-parallel orientation	This study
pBCTA2D	pBluescript SK + derivation; <i>Synechococcus</i> sp. PCC 7002, <i>ctaDII::</i> □, Spc ^r	This study
<i>Oligonucleotides</i>		
ctaCI.1	5'-GTG AAT ATT CCC AAT AGC ATC-3'	This study
ctaCI.2	5'-CCC CAT CTC CTC GGC GTA GGC-3'	This study
ctaDI.II	5'-ATG AGT GAC GCG ACA ATA CAC-3'	This study
ctaDI.I2	5'-TAG GTG TCA TGG ACA TGG-3'	This study
ctaEI.1	5'-TAC GGC GAT CGC CAC CGA-3'	This study
ctaEI.2	5'-CAA GGC CGA ACA GGA TAA TTC-3'	This study
ctaCII.1	5'-CAC TTT CGG CGA TCG CCC TAC TTT TGG GGG-3'	This study
ctaCII.2	5'-GGG ATG ATA ATT CAC CAC-3'	This study
ctaDII.1	5'-CCA TGA CCC AAG CTC CC -3'	This study
ctaDII.2	5'-CGG GAA CCA GTG GTA CAC CGC-3'	This study
ctaEII.1	5'-GAC TGC CAT CAA TGA AAC C-3'	This study
ctaEI.II	5'-ATT GCC AGA GAT AAA TCA GCC-3'	This study

the Nucleic Acid Facility (The Pennsylvania State University).

Cloning, transformation and nucleic acid hybridization procedures

Restriction endonucleases were obtained from Promega labs (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA) and used according to the manufacturers' recommendations. DNA fragments were isolated with GenE-luteTM Agarose Spin Columns according to the instructions of the manufacturer (SIGMA, St. Louis, MO, USA). Routine DNA manipulations were performed as previously described (Sambrook et al., 1989) and were carried out in *E. coli* strain DH5 α . Transformations of *E. coli* were done using a BTX Transporator Plus from

Harward Apparatus (Holliston, MA, USA). Transformations of *Synechococcus* sp. strain PCC 7002 were performed as described previously (Buzby et al., 1983). The *Synechococcus* sp. PCC 7002 *ctaDII* gene cluster was found by screening a genomic library. This library was constructed by inserting random *EcoRI* *Synechococcus* sp. PCC 7002 DNA fragments of 30–40 kb into the SuperCos (Stratagene, La Jolla, CA, USA) cosmid vector. Southern hybridization was performed as described previously (Bryant and Tandeau de Marsac 1988). Northern hybridization was performed as described previously (Sakamoto and Bryant 1997). For the phenotypic analysis studies, the *ctaDI* (1.1)-(parallel) orientation of the insertional mutant was used and is denoted as *ctaDI*. The other mutant strains were denoted as follows: *ctaDII* the mutant strain homozygous for the

interruption of the *ctaDII* locus, and *ctaDI ctaDII* for the mutant strain homozygous for the interruption of both loci.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Primers used for RT-PCR are described in Table 1. Separate reactions for each *ctaII* gene were carried out using 1 µg of total RNA and 20 µM of the primer located 3' to the start of each specific *ctaII* gene. The samples were incubated at 70 °C for 5 min to denature secondary structures followed by a brief incubation on ice. A total of 5 µl of 5×M-MLV reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT), 1 µl of 25 mM dNTPs, 0.625 µl RNasin from Promega (Madison, WI, USA), and 1.5 µl of M-MLV reverse transcriptase from Promega (Madison, WI, USA) was added to each sample and the reaction mixtures were incubated at 42 °C for 1 h. The reactions were stopped by incubation for 5 min at 70 °C and treatment with 5 U of RNase H from Promega (Madison, WI, USA) and 5 U of RNase A from Promega (Madison, WI, USA) for 20 min at 37 °C. All individual mixtures were brought up to a volume of 200 µl with TE and concentrated using Pall Nanosep 100 spin columns from VWR International (West Chester, PA, USA). The three different cDNAs were collected from the membrane by washing with 20 µl of TE and 4 µl of each reaction were used for second strand synthesis by PCR.

Determination of chlorophyll a and carotenoid contents

Chlorophyll *a* and total carotenoid concentrations were determined as described previously (Sakamoto and Bryant, 1998). The cells were grown into exponential phase after which, 1 ml of cells were harvested by centrifugation in 15 ml COREX™ tubes at 10,000×g at room temperature for 5 min. The supernatant was removed and chlorophyll *a* and carotenoids were extracted with dimethylformamide for 15 min at room temperature. The concentrations of chlorophyll *a* and carotenoids in the dimethylformamide extract were calculated from the following equations (A_{750} was subtracted to correct for light scattering):

$$[\text{Chlorophyll } a \text{ (}\mu\text{g ml}^{-1}\text{)}] = (A_{664} - A_{750}) \times 11.92$$

$$[\text{Carotenoids (}\mu\text{g ml}^{-1}\text{)}] = \{(A_{461} - A_{750}) - 0.046 \times (A_{664} - A_{750})\} \times 4$$

Oxygen evolution and consumption rates

Oxygen evolution and consumption rates were determined using a Clark-type electrode from Hansatech, Inc (Norfolk, UK). Cells were harvested by centrifugation, washed with fresh A⁺ media, centrifuged again and resuspended to either a final equal OD₅₅₀ per ml for oxygen evolution measurements or 50–100 µg of chlorophyll *a* per ml for oxygen consumption measurements. The cells were kept in the dark for 10–15 min and then agitated to saturate the samples with air levels of oxygen prior to addition to the electrode chamber. A total of 1 ml of cells was added to the electrode chamber for each experiment. The electrode chamber temperature was maintained at 38 °C with a circulating water bath and the chamber contents were continuously stirred. Measurements of oxygen evolution were determined after two min in the dark by stimulation with saturating amounts of light (2.5 mE m⁻² s⁻¹). Oxygen consumption rates were determined in complete darkness.

P700⁺ kinetic measurements

The photoinduced absorption change attributable to P700 can be monitored with a single beam spectrophotometer (Maxwell and Biggins 1976). Thus, the reduction kinetics of P700⁺ in whole cells were measured using a detection system similar to the one described by Yu et al. (1993) and originally by Maxwell and Biggins (1976). Cells were grown under standard growth conditions (38 °C, 250 µE m⁻² s⁻¹, 1.5% CO₂/air) and harvested by centrifugation. The cells were resuspended in 1 ml saturated Ficoll (200 mg ml⁻¹), 25 mM Tris-HCl, pH 8.3 and adjusted to a chlorophyll *a* content of 40 µg ml⁻¹. The reduction kinetics of whole cells were determined as described in (Yu et al. 1993) with some modifications. The cells were illuminated for 22 s with a saturating light

followed by a dark incubation for 15 s. The data were transferred to and analyzed with IGORPRO v. 3.5 as described previously (Yu et al. 1993).

77 K fluorescent measurements

Cyanobacterial cells in mid-exponential phase, which had been grown under various light intensities (see results), were harvested by centrifugation at room temperature at $8000\times g$ for 5 min and resuspended in 200 μ l of 60% glycerol, 50 mM HEPES, pH 7.0. Samples were adjusted to a final OD₇₃₀ of 1.0 in 1.0 ml of 60% glycerol, 50 mM HEPES, pH 7.0. Measurement of 77 K fluorescence was determined with an SLM-Aminco 8000 C spectrofluorometer (Spectronic Instruments Inc, Rochester, NY, USA); the excitation wavelength was 440 nm. Absorption spectra were obtained with a Cary-14R spectrophotometer modified for computerized operation, data collection and data analysis by On-Line Instruments Systems (Bogart, GA, USA) as described previously (Sakamoto and Bryant, 1998).

Pulse amplitude modulated fluorescence measurements (PAM)

A total of 25 ml of exponentially growing cyanobacterial cells grown under standard growth conditions (38°C , $250\ \mu\text{E m}^{-2}\text{ s}^{-1}$, 1.5% CO_2) were harvested by centrifugation at room temperature at $8000\times g$ for 5 min. The cell pellet was resuspended and washed with 25 mM HEPES-NaOH buffer, pH 7.0. The cells were collected again by centrifugation and resuspended to a final OD₅₅₀ of 1. Between 1–3 ml of cells at a chlorophyll concentration of $5\ \mu\text{g ml}^{-1}$ in 25 mM HEPES, pH 7.0, 1 mM bicarbonate was added to the cuvette. The cells were incubated for 2 min in complete darkness before establishing F_0 . F_M' was obtained with a saturating light pulse for 1 second. Fluorometer settings were changed to 100 Hz from 1600 Hz to obtain F_V' . After establishing F_M' , DCMU was added to $1\ \mu\text{M}$ in order to obtain F_M by closing the PS II reaction centers completely.

Results

Screening and cloning of the *ctaI* and *ctaII* gene clusters from *Synechococcus* sp. PCC 7002

A *Bam*HI fragment of 4.5 kb was isolated from a partial genomic library of *Synechococcus* sp. PCC 7002 DNA by cross-hybridization with a *Synechocystis* sp. PCC 6803 *ctaDI* probe and contained the full nucleotide sequences of the *ctaCI*, *ctaDI*, and *ctaEI* genes. The *Synechococcus* sp. PCC 7002 *ctaCIIDIIEII* gene cluster was found by screening a cosmid genomic library with a *Synechocystis* sp. PCC 6803 *slr2082* reading frame encoding the *ctaDII* probe (Kaneko et al. 1996). Cosmid 2B1, containing a 40 kb insert of *Synechococcus* sp. PCC 7002 DNA was identified as a positive clone and a 3.5 kb *Hinc*II fragment was subcloned from this construct. Sequence analysis of this 3.5 kb *Hinc*II fragment insert revealed two open reading frames with homology to *ctaCII* and *ctaDII* from *Synechocystis* sp. PCC 6803 as well as two open reading frames with high sequence homology to *sll1485* and *sll1486* of *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996). Primers were designed to sequence cosmid 2B1 outside of the 3.5 kb *Hinc*II fragment. Sequencing downstream from *ctaDII*, we were able to identify and sequence the *ctaEII* reading frame from the 2B1 cosmid. Nucleotide sequences for the *ctaCIDIEI* and *ctaCIIDIIEII* operons were deposited into the GenBank database (accession numbers AF3810848 and AF381049, respectively). A *Synechocystis* sp. PCC 6803 *cydA* probe, was also used to screen the *Synechococcus* sp. PCC 7002 genomic library for the presence of quinol oxidase. No cross-hybridizing DNA fragments corresponding to the *cydAB* genes from *Synechocystis* sp. PCC 6803 were found in *Synechococcus* sp. PCC 7002 genomic DNA Southern blot hybridizations (data not shown). Furthermore, blastn and tblastn searches using either the *cydAB* nucleotide or amino acid sequences against the draft sequence of *Synechococcus* sp. PCC 7002 genome failed to reveal the presence of a quinol oxidase, suggesting that *Synechococcus* sp. PCC 7002 has only the heme-copper cytochrome oxidase gene clusters, *ctaCI-DIEI* and *ctaCIIDIIEII*.

Insertional mutagenesis of *ctaDI* and *ctaDII* from *Synechococcus* sp. PCC 7002

In order to characterize the role of the cytochrome oxidase gene clusters in electron transport pathways, the *ctaDI* and *ctaDII* genes were disrupted by interposon mutagenesis. A unique *Bgl*II site within *ctaDI* was used to introduce a 1.3 kb *Bam*HI fragment harboring the *aphII* gene that confers kanamycin resistance. Two constructs named pBCTAD1pD for the parallel orientation of the *aphII* gene and pBCTAD1aD for the anti-parallel orientation of the *aphII* gene were used to independently transform wild type *Synechococcus* sp. PCC 7002 cells and *ctaDII* strains. Total genomic DNA was isolated from the wild type and *ctaDI* strains and homozygosity of the insertion of the *aphII* gene was assayed by PCR using primers ctaDI.I1 and primer ctaDI.I2 (Table 1). Figure 1 shows that all transformants were homozygous for the *ctaDI* locus, indicating that this gene was not necessary under standard growth conditions for *Synechococcus* sp. PCC 7002.

The *ctaDII* gene was interrupted by insertion of a 2 kb *Sma*I Ω fragment which confers spectinomycin resistance (Prentki and Krisch, 1984) into a unique *Eco*RV site within the *ctaDII* coding sequence. The resulting construct, pBCTAD2D was used to transform wild type *Synechococcus* sp. PCC 7002 as well as the *ctaDI* strains of *Synechococcus* sp. PCC 7002. Southern blot analysis (Figure 2) shows that this locus was also completely segregated in all backgrounds that were transformed with pBCTAD2D. These results indicate that both the *ctaDI* and *ctaDII* genes were successfully interrupted and that neither is essential under standard growth conditions for *Synechococcus* sp. PCC 7002.

Expression of the *cta* gene clusters in *Synechococcus* sp. PCC 7002

The level of mRNA and the size of the transcripts of the wild type *Synechococcus* sp. PCC 7002 *ctaI* and *ctaII* gene clusters were analyzed by RNA blot analysis. Specific DNA probes were made for all cloned and sequenced *cta* genes using primers from Table 1. A 3.5 knt RNA fragment was detected by all of the *ctaDI* specific probes on the total RNA blot (Figure 3). This indicates that the *ctaI* gene cluster is transcribed as a polycistronic mRNA

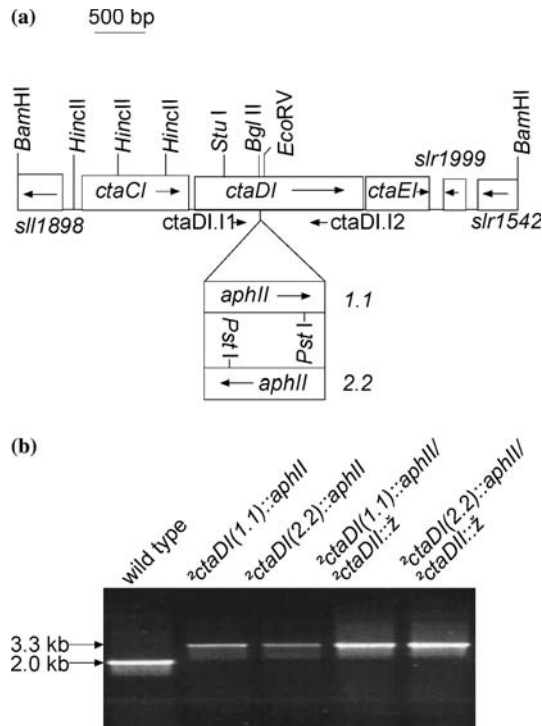


Figure 1. Interposon mutagenesis of the *ctaDI* gene from *Synechococcus* sp. PCC 7002. (a) Physical map of the 4.5-kb *Bam*HI fragment encoding the *ctaCIDIEI* gene cluster of *Synechococcus* sp. strain PCC 7002 and disruption of the *ctaDI* locus by interposon mutagenesis. Arrows indicate the direction of transcription. The *aphII* gene, which encodes aminoglycoside 3'-phosphotransferase II and confers kanamycin resistance, was inserted into a *Bgl*II site within the coding region of *ctaDI* in both orientations to create the constructs pBCTA1pD (parallel) and pBCTA1aD (anti-parallel). This construct was used to transform the wild type and Δ *ctaDII* strains of *Synechococcus* sp. PCC 7002. (b) PCR analysis of the *ctaDI* locus. PCR analysis was performed on genomic DNA isolated from the wild type and mutant strains of *Synechococcus* sp. PCC 7002 using the primers ctaDI.I1 and ctaDI.I2 as indicated in (a). The sizes of the PCR products are indicated on the left.

with a size of 3.5 knt. No hybridization signal was detected by the *ctaII* gene specific probes on RNA blots from wild type *Synechococcus* sp. PCC 7002, indicating that the level of mRNA transcript accumulation for *ctaII* is lower than the detection limit of RNA blot analysis (data not shown).

Because the *ctaII* mRNA transcripts were not detected by RNA blot analysis, the transcription unit of the *ctaII* genes was analyzed by RT-PCR using *ctaCIIDIEI* gene specific primers and RNA template isolated from wild type *Synechococcus* sp. PCC 7002 cells. Three different primers, ctaEII.2, ctaDII.2, and ctaCII.2 as indicated in

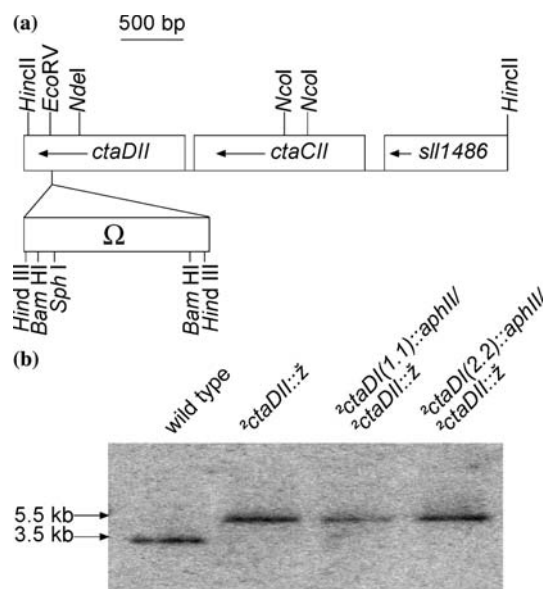


Figure 2. Interposon mutagenesis of the *ctaDII* gene from *Synechococcus* sp. PCC 7002. (a) Physical map of the 3.5-kb *HincII* fragment encoding *ctaCIIDII* and disruption of the *ctaDII* locus by interposon mutagenesis. Arrows indicate the direction of transcription. The Ω fragment, conferring spectinomycin resistance was inserted into an *EcoRV* site within the coding region of *ctaDII*. The construct pDCTADII was used to transform the wild type and *ctaDI* strains of *Synechococcus* sp. PCC 7002. Transformants were selected on A^{+} spectinomycin plates as described in Materials and Methods. (b) Southern blot analysis of the Δ *ctaDII* locus. Genomic DNA was digested with *HincII* and probed with a ^{32}P -labeled PCR product for *ctaDII*. The sizes of the hybridizing bands are indicated on the left.

Table 1 and Figure 4, were used for the RT reaction to synthesize three cDNA templates; as a control, total RNA templates were treated with RNase prior to the RT reactions. In all cases, no RT-PCR product was detected in any of the individual samples, indicating that all PCR amplification products were derived from RNA templates rather than from contaminating genomic DNA (data not shown).

Primers corresponding to the 5' ends of the *ctaCIIDII* genes were used in conjunction with the 3' primers *ctaEII.2*, *ctaDII.2*, and *ctaEII.2* to amplify the regions corresponding the specific *ctaII* mRNAs (Figure 4). The template synthesized using the *ctaEII.2* primer was used to assay for the transcripts of the *ctaCIIDII* coding regions by amplifying the individual *ctaCII*, *ctaDII*, and *ctaEII* regions. Amplification of these mRNA regions with the proper primer sets revealed that all three genes were present on the single RT

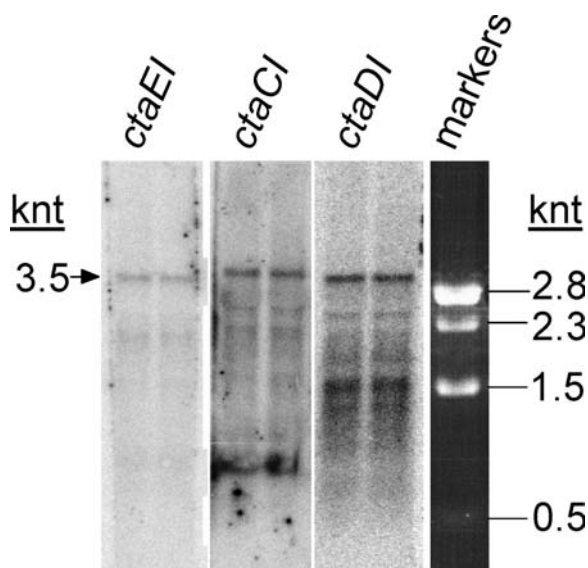


Figure 3. Northern blot analysis of the *ctaI* gene cluster of *Synechococcus* sp. PCC 7002. PCR products specific for *ctaEI*, *ctaDI* and *ctaCI* were made and used as probes for Northern blot analysis. 20 μ g of total RNA was electrophoresed per lane of the gel. Size of the major hybridizing RNA species is indicated on the left. Sizes of the ribosomal RNA are indicated on the right.

product synthesized from *ctaEII.2* primer which is located at the furthest point on the 3' end (Figure 4). Attempts at amplifying the *ctaEII* region from the shorter RT product using *ctaDII.2* failed. Furthermore, attempts to amplify either the *ctaEII* or *ctaDII* regions from the RT product using *ctaCII.2* primer also failed (data not shown), indicating that the *ctaII* gene cluster, like the *ctaI* gene cluster, is transcribed as a polycistronic mRNA. However, the inability to detect an mRNA signal by RNA blot analysis indicates that the amount of these transcripts is very low in *Synechococcus* sp. PCC 7002.

Growth analysis of the of wild type and cta deficient Synechococcus sp. 7002

The growth rates of the mutant strains and wild type strain of *Synechococcus* sp. PCC 7002 were analyzed for cells grown under different light intensities and the results are summarized in Table 2. All cells were grown under constant temperature (38 °C) with 1.5% CO_2 /air constantly bubbling through the liquid cultures. Under low and normal light intensities (150 and 250 $\mu E m^{-2} s^{-1}$) and under moderate light stress

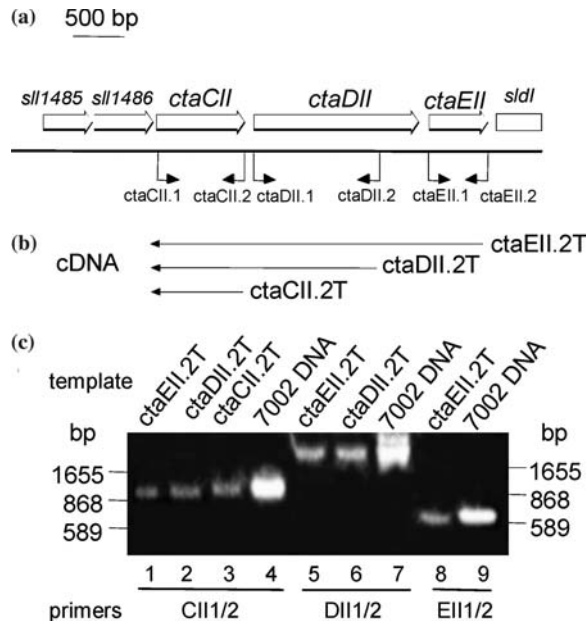


Figure 4. RT-PCR analysis of the *ctaII* gene cluster of *Synechococcus* sp. PCC 7002. (a) Gene arrangement of the *ctaII* gene cluster. The small numbered arrows below the physical map represent primers used for PCR amplification. Primer sequences are given in Table 1. (b) Products from the reverse transcriptase reactions using total RNA from wild type of *Synechococcus* sp. PCC 7002. Three primers *ctaEII.2*, *ctaDII.2* and *ctaCII.2* were used to make the specific cDNA products derived from mRNA. These RT products were used as templates for PCR to amplified the individual *ctaCII*, *ctaDII*, and *ctaEII* regions using the specific primers. (c) PCR analysis of the cDNA products. Templates for the PCR analysis are listed above the lanes. Primers *ctaCII.1* and *ctaCII.2* were used for lanes 1–4. Primers *ctaDII.1* and *ctaDII.2* were used for lanes 5 to 7. Primers *ctaEII.1* and *ctaEII.2* were used for lanes 8 and 9.

(700 $\mu\text{E m}^{-2} \text{s}^{-1}$), the doubling times of the mutant strains were nearly indistinguishable from the doubling time of the wild type strain (Table 2). This indicates that under these conditions, cytochrome oxidase activity does not significantly contribute to the growth of the cells.

Respiratory activity and oxygen evolution activity of wild type and cta deficient Synechococcus sp. 7002

Table 3 shows respiratory rates of whole cells of the wild type and mutant strains grown under normal light intensity (250 $\mu\text{E m}^{-2} \text{s}^{-1}$). The *ctaDI* strains displayed a decrease of 66% in oxygen uptake compared to the wild type rate of oxygen uptake, while the *ctaDII* strain had an oxygen uptake rate nearly identical to the wild type strain.

The *ctaDI ctaDII* double mutant was similar to the *ctaDI* single mutant strain in that it exhibited a decrease in respiratory rate of 70% when compared to wild type cells. All strains were sensitive to the addition of KCN to a final concentration of 1 mM. Very low rates of oxygen uptake were seen in the presence of KCN. This level of oxygen uptake can be attributed to the level of oxygen consumed by the electrode during the assay. Oxygen evolution activity was virtually unchanged between mutant and wild type strains under a variety of conditions, indicating that disruption of the *cta* genes has no effect on oxygen evolution under standard growth conditions (Table 2).

Chlorophyll and carotenoid contents of wild type and cta deficient Synechococcus sp. 7002

Table 2 also shows chlorophyll *a* and carotenoid contents of wild type and mutant strains grown under different light intensities. In cells grown under normal light intensity and moderate light stress (250 and 700 $\mu\text{E m}^{-2} \text{s}^{-1}$, respectively) strains that are *ctaDI*-deficient exhibit a slight but consistent decrease in the chlorophyll content compared to the wild type strain. This is consistent with the 77 K fluorescence data and indicates that cytochrome oxidases may be important for countering photoinhibition (see below).

77 K fluorescence spectroscopy of wild type and cta deficient Synechococcus sp. PCC 7002

77 K fluorescence spectroscopy was performed on mutant and wild type strains in order to detect possible differences in PS I and PS II levels. Figure 5 shows the 77 K fluorescence emission spectra of whole cells from the wild type strain, *ctaDI*, *ctaDII*, and *ctaDI ctaDII* strains for cells grown at 100, 400, and 1500 $\mu\text{E m}^{-2} \text{s}^{-1}$. Cells grown under low light (100 $\mu\text{E m}^{-2} \text{s}^{-1}$) exhibit little or no difference in fluorescence emission (Figure 5a). Cells grown under moderate light stress (400 $\mu\text{E m}^{-2} \text{s}^{-1}$) have a decreased level of PS II content in the *ctaDI* and *ctaDII* single mutant strains and a greater effect in the *ctaDI ctaDII* double mutant strain when compared to the wild type strain. This is easily visualized by examining the decrease in fluorescence emission at 685 nm and 695 nm respectively of the mutant strains compared to the wild type strains

Table 2. Chlorophyll *a* concentrations, carotenoid concentrations, oxygen evolution rates and F_v'/F_m' for strains of *Synechococcus* sp. PCC 7002. All values shown represent the averages $\pm$ SD of five separate experiments

Strain	Light Intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)	Doubling time (h)	Chlorophyll <i>a</i> ($\mu\text{g ml}^{-1} \text{OD}_{550 \text{ nm}}^{-1}$)	Carotenoids ($\mu\text{g ml}^{-1} \text{OD}_{550 \text{ nm}}^{-1}$)	Oxygen evolution ($\mu\text{mol of O}_2(\text{mg of Chl})^{-1} \text{h}^{-1}$)	F_v'/F_m'
wild type	150	4.0 ± 0.4	3.7 ± 0.4	0.8 ± 0.2	410 ± 20	ND
<i>ctaDI</i>	150	4.2 ± 0.4	3.7 ± 0.3	0.8 ± 0.1	400 ± 10	ND
<i>ctaDII</i>	150	4.1 ± 0.4	3.6 ± 0.6	0.8 ± 0.1	430 ± 20	ND
<i>ctaDI ctaDII</i>	150	3.9 ± 0.4	3.3 ± 0.3	0.8 ± 0.1	430 ± 30	ND
wild type	250	3.6 ± 0.4	3.4 ± 0.6	0.8 ± 0.1	420 ± 20	0.47 ± 0.1
<i>ctaDI</i>	250	4.0 ± 0.4	3.0 ± 0.5	0.8 ± 0.1	470 ± 20	0.38 ± 0.1
<i>ctaDII</i>	250	3.8 ± 0.4	3.4 ± 0.5	0.8 ± 0.2	450 ± 40	0.46 ± 0.1
<i>ctaDI ctaDII</i>	250	4.1 ± 0.4	3.2 ± 0.7	0.9 ± 0.1	380 ± 40	0.33 ± 0.1
wild type	700	4.0 ± 0.4	1.5 ± 0.1	0.7 ± 0.3	240 ± 10	ND
<i>ctaDI</i>	700	4.0 ± 0.4	1.3 ± 0.1	0.7 ± 0.1	270 ± 10	ND
<i>ctaDII</i>	700	4.0 ± 0.4	1.5 ± 0.4	0.7 ± 0.3	250 ± 20	ND
<i>ctaDI ctaDII</i>	700	4.0 ± 0.4	1.4 ± 0.6	0.7 ± 0.2	210 ± 40	ND

ND-not determined.

(Figure 5b). This phenomenon is exacerbated when all the strains are grown under higher light intensities ($1500 \mu\text{E m}^{-2} \text{s}^{-1}$), but is especially evident in the mutant strains (Figure 5c). These results indicate that the ratio of photosystems changes when the cells are grown under various light intensities and the absence of functional cytochrome oxidases intensifies the effects of high light intensity.

P700 redox kinetics of wild type and cta deficient Synechococcus sp. 7002

To determine the effect that the cytochrome oxidases have on photosynthetic electron transport, we examined P700 redox kinetics. In order to obtain full reduction of P700 in this experiment, 15 s dark incubation times were used followed by a 22 s light exposure to induce oxidation of P700.

The results are tabulated in Table 4. In the absence of any inhibitors, the *ctaDI* strain is 1.7-fold faster in its reduction of P700 versus the wild type reduction rate and the reduction of *ctaDII* has a 1.4-fold shorter half-life versus the wild type strain. The *ctaDI ctaDII* strain has a 2.2-fold shorter half-life versus the wild type strain. The addition of DCMU inhibits electron flow from PS II; therefore most of the electron flow must be derived predominantly from the NADH dehydrogenase and from cyclic electron flow around PS I (Yu et al. 1993). In the case of the wild type, the addition of DCMU to a final concentration of 10 mM increases the half-time of P700^+ to 330 ms as opposed to 120 ms without DCMU. The mutant strains all had faster reduction kinetics compared to wild type in the presence of DCMU. In the presence of DCMU, *ctaDI* has a 2.5-fold shorter half-time compared to the wild type strain

Table 3. Oxygen consumption rates of photoautotrophically grown *Synechococcus* sp. PCC 7002 strains in darkness. Values shown represent the averages $\pm$ SD of five separate experiments

Strain	Oxygen uptake ($\mu\text{mol of O}_2 (\text{mg of chl})^{-1} \text{h}^{-1}$) no additions	Oxygen uptake ($\mu\text{mol of O}_2 (\text{mg of chl})^{-1} \text{h}^{-1}$) + 1 mM KCN	Oxygen uptake ($\mu\text{mol of O}_2 (\text{mg of chl})^{-1} \text{h}^{-1}$) KCN sensitive activity
Wild type	24 ± 6	2.5 ± 0.2	22 ± 6
<i>ctaDI</i>	8 ± 2	2.5 ± 0.4	6 ± 2
<i>ctaDII</i>	21 ± 6	2.4 ± 0.2	19 ± 6
<i>ctaDI ctaDII</i>	7 ± 1	2.5 ± 0.1	5 ± 1

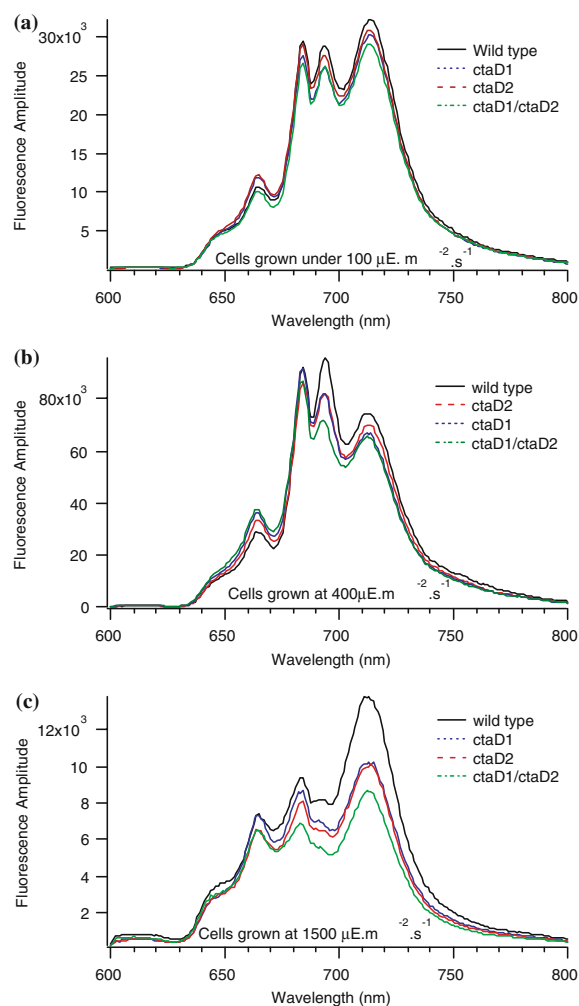


Figure 5. Fluorescence emission spectra at 77 K of whole cells of *Synechococcus* sp. PCC 7002 wild type and cytochrome oxidase mutant strains. Strains used are indicated in figure. (a) Cells grown at $100 \mu\text{E m}^{-2} \text{s}^{-1}$. (b) Cells grown at $400 \mu\text{E m}^{-2} \text{s}^{-1}$. (c) Cells grown at $1500 \mu\text{E m}^{-2} \text{s}^{-1}$.

under the same conditions, the *ctaDII* deficient strain has a 1.7-fold shorter half-time compared to the wild type strain in the presence of DCMU and *ctaDI ctaDII* deficient strain is 2.6-fold shorter than the wild type strain. The addition of KCN to a final concentration of 1 mM in addition to the DCMU caused a decrease in the half-time of 1.4-fold in the wild type strain. The *ctaDI* and *ctaDI ctaDII* mutant strains saw no decrease in their half-times, but the *ctaDII* strain saw a decrease in half-time of 1.3-fold. These results indicate that the presence or absence of cytochrome oxidase affects the reduction times of the active photosystem I centers.

Pulse amplitude modulated (PAM) fluorescence measurements of wild type and *cta* deficient *Synechococcus* sp. 7002

PAM fluorescence measurements use a constant, weak ($1\text{--}2 \mu\text{mol m}^{-2} \text{s}^{-1}$) modulated light source with a system that can accurately detect the modulated signal despite large variations in background irradiance (Schreiber et al., 1986). Since the modulated source is constant, it can be used to measure the modulated fluorescence signal once the background irradiance is high enough to close all of the reaction centers (Geider and Osborne 1992), and enables the determination of the number of functional PS II complexes in whole cells. In order to examine the electron flow around PS II, we conducted room temperature PAM fluorescence studies to determine the total number of active PS II sites compared to total chlorophyll content. Results are shown in Table 2 and indicate that there is a decrease in this ratio for the *ctaDI* mutants; thus, the presence or absence of cytochrome oxidase has dramatic effects on electron flow around PS II. Changes in the F_V'/F_M' ratios among the *ctaDI* strains and wild type strain of *Synechococcus* sp. PCC 7002 clearly show that electron flow around PS II has been modified in the *ctaDI* strains. However, this ratio is virtually unchanged between *ctaDII* and wild type strains. F_V'/F_M' reflects the photochemical efficiency of open PS II centers in higher plants under a given light acclimation status and represents the combined regulation of PS II via both reversible non-photochemical quenching and photoinhibitory inactivation of PS II. In cyanobacteria, however, changes in F_V'/F_M' also combine non-photochemical influences on PS II function as well as photoinhibitory inactivation of PS II. Cyanobacteria have a very high capacity to remove electrons from PS II with oxygen as a terminal acceptor for electron flow from water, forming an effective buffer against excess excitation of PS II by removing electrons as required (Campbell et al. 1998). Thus, from the evidence presented here, it is logical to conclude that the primary oxidase, CtaCIDIEI serves as a buffer from excess excitation of PS II by removing electrons as necessary in *Synechococcus* sp. PCC 7002. These conclusions are in complete agreement with our physiological results with the *ctaDI* mutants and are reflected in the ratio of F_V'/F_M' .

Table 4. Half-times (ms) for P700 reduction in *Synechococcus* sp. PCC 7002. All values shown represent the averages $\pm$ SD of five separate experiments

Strain	No inhibitors	10 mM DCMU	10 mM DCMU, 1 mM KCN
Wild type	120 $\pm$ 8	330 $\pm$ 10	237 $\pm$ 10
<i>ctaDI</i>	70 $\pm$ 5	130 $\pm$ 10	146 $\pm$ 10
<i>ctaDII</i>	87 $\pm$ 7	262 $\pm$ 10	203 $\pm$ 10
<i>ctaDI ctaDII</i>	53 $\pm$ 4	127 $\pm$ 10	160 $\pm$ 10

Discussion

Synechococcus sp. PCC 7002 has two cytochrome oxidases

Many cyanobacteria examined to date have genes encoding cytochrome oxidases and there have been many studies to determine the physiological role of these complexes. The *ctaCIDIEI* operon encoding the primary *aa*₃-type cytochrome heme-copper oxidase was cloned from *Synechocystis* sp. PCC 6803 (Alge and Peschek 1993a; Alge and Peschek 1993b) and a similar gene cluster was cloned from *Synechococcus vulcanus* (Sone et al. 1993). A quinol oxidase of the *bd*-type and a second *bo*-type oxidase were identified and characterized in *Synechocystis* sp. PCC 6803 (Howitt and

Vermaas 1998). Examination of mutant backgrounds in each of the individual oxidase operons as well as double and triple mutants of the different oxidase operons, allowed researchers to conclude that *ctaCIDIEI* and *cydAB* encode functional oxidases in *Synechocystis* sp. PCC 6803 (Howitt and Vermaas 1998). It has been shown that in the presence of glucose or increased light intensity that the Cyd activity increases and it has been suggested that the *cyd-bd* complex provides an electron sink in *Synechocystis* sp. PCC 6803 (Berry et al. 2002). In addition, in *Anabaena variabilis* strain ATCC 29413, the equivalent of the *ctaCIDIEI* operon (*coxCAB*) is necessary for heterotrophic growth, indicating that respiratory oxidases allow metabolic and carbon use flexibility for cyanobacteria (Schmetterer et al. 2001).

(a) Alignment of the Mg²⁺ binding motif from CtaD

CtaDI 7002	368	APFDIHV	HD TYFVVGHFHYV
CtaDI 6803	371	VPFDIHV	HD TYFVVGHFHYV
CtaDII 7002	382	VPVDIHV	NN TYFVVGHFHYV
CtaDII 6803	361	APFDLHV	NN TYFVVGHFHYV
<i>Bos taurus</i>	361	SSLDIVL	HD TYYVVAHFHYV
<i>S. cerevisiae</i>	361	ASLDVAF	HD TYYVVGHFHYV
<i>P. denitrificans</i>	396	GSLDRVY	HD TYYIVAHFHYV
<i>B. subtilis</i>	368	AAADYQF	HD TYFVVAHFHYV

(b) Alignment of the Cu_A binding motif from CtaC

CtaCI 7002	274	PVI	CAEL CGSY HGG MKTTMTVETAEGYDQW
CtaCI 6803	245	PVI	CAEL CGAY HGG MKSVFYAHTPEEYDDW
CtaCII 7002	211	RIR	DSQF SGTY FAAM QADV VVESQEDYQTW
CtaCII 6803	230	KLH	DSQF SGTY FAV MTAPVVVQSLSDYQAW
<i>Bos taurus</i>	193	YGQ	CSEI CGSN HSF MPIVLELVPLKYFEKW
<i>S. cerevisiae</i>	221	YGA	CSEL CGTG HAN MPIKIEAVSLPKFLEW
<i>P. denitrificans</i>	213	FGQ	CSEL CGIN HAY MPIVVKAVSQEKYEAW
<i>B. subtilis</i>	214	FGK	CAEL CGPS HAL MDFKVKTMSAKEFQGW

Figure 6. Oxidase Mg²⁺ and Cu_A binding motif alignments. (a) Alignments of the heme-copper oxidase Mg²⁺ binding region in the cyanobacterial CtaD subunit with the large subunits of other cytochrome oxidases. Putative binding ligands are in bold. In the cyanobacterial CtaDII subunits, the conserved histidine and aspartic acid have been replaced by asparagines. (b) Alignments of the heme copper oxidase Cu_A binding region in the cyanobacterial CtaC subunit with subunit II from other cytochrome oxidases. Putative binding ligands are in bold. In the cyanobacterial CtaCII subunits, the conserved cysteine is replaced by aspartic acid. The conserved glutamic acid is replaced by glutamine. The conserved histidine is replaced by phenylalanine. A single methionine is conserved all CtaC subunits of heme copper oxidases.

Table 5. (A) Sequence Identity (%) of the Putative CtaCI, CtaDI, and CtaEI Polypeptides from *Synechococcus* sp. PCC 7002 with other Heme-Copper Oxidase Homologs from Other Organisms and CtaCII, CtaDII, and CtaEII from *Synechococcus* sp. PCC 7002. (B) Sequence Identity (%) of the Putative CtaCII, CtaDII, and CtaEII Polypeptides from *Synechococcus* sp. PCC 7002 with other Heme-Copper Oxidase Homologs from Other Organisms and CtaCI, CtaDI, and CtaEI from *Synechococcus* sp. PCC 7002

Subunit from <i>Synechococcus</i> sp. PCC 7002 (% Identity)				
(A)				
Organism	Subunit	CtaCI	CtaDI	CtaEI
<i>Synechococcus</i> sp. PCC 7002	CtaII	24	53	36
<i>Synechocystis</i> sp. PCC 6803	CtaI	61	80	68
<i>Synechocystis</i> sp. PCC 6803	CtaII	32	57	39
<i>Anabaena</i>	Cta	51	64	57
<i>Bacillis subtilis</i>	Cta	17	33	26
(B)				
Organism	Subunit	CtaCII	CtaDII	CtaEII
<i>Synechococcus</i> sp. PCC 7002	CtaI	24	53	36
<i>Synechocystis</i> sp. PCC 6803	CtaI	31	54	41
<i>Synechocystis</i> sp. PCC 6803	CtaII	41	64	56
<i>Anabaena</i>	Cta	31	54	45
<i>Bacillis subtilis</i>	Cta	21	31	23

Unlike *Synechocystis* sp. PCC 6803, which has the *cydAB* genes, *Synechococcus* sp. PCC 7002 has operons for only the two heme-copper oxidase operons. The CtaD protein is the largest subunit of the bacterial cytochrome oxidase and *Synechococcus* sp. PCC 7002 has two genes that potentially encode CtaD subunits. The *ctaDI* gene is predicted to encode a 549 amino acid protein that would have a molecular mass of 60.9 kDa and an estimated pI of 5.62. The *ctaDII* gene is predicted to encode a 551 amino acid protein with a molecular mass of 61 kDa and an estimated pI of 6.37. Alignments in the region of the Mg^{2+} binding site show a very high degree of identity with their counterparts from *Synechocystis* sp. PCC 6803 (Figure 6), and as in *Synechocystis* sp. PCC 6803, the CtaDII subunit of *Synechococcus* sp. PCC 7002 is divergent in the region of the Mg^{2+} binding site.

Synechococcus sp. PCC 7002 also has two genes that potentially encode CtaC subunits, containing the putative Cu_A binding motif. The *ctaCI* gene is predicted to encode a 362 amino acid protein that would have a molecular mass of 39.6 kDa and an estimated pI of 4.55 and the *ctaCII* gene is predicted to encode a 298 amino acid protein with a molecular mass of 33.8 kDa and an estimated pI of 6.50. Figure 6 also shows an alignment of the Cu_A binding motifs and shows that the CtaCII subunits from both *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 are similarly divergent from the CtaCI subunits.

From these observations, it may be concluded that, as in *Synechocystis* sp. PCC 6803, the CtaDII and CtaCII subunits are similar to *bo*-type quinol oxidase subunits, while the counterparts CtaDI and CtaCI are similar to the subunits of the *aa₃* type cytochrome oxidase (Howitt and Vermass 1998). Because previous studies using *Synechocystis* sp. PCC 6803 show that the CydAB-oxidase is important for mixotrophic growth (Berry et al. 2002), it would be interesting to see if either the CtaI or CtaII oxidases are important for mixotrophic growth conditions in *Synechococcus* sp. PCC 7002.

Both the *ctaDI* and *ctaDII* genes of *Synechococcus* sp. PCC 7002 are dispensable for growth under normal to moderately high and stressful light conditions. As in *Synechocystis* sp. PCC 6803, the primary cytochrome oxidase is encoded by the *ctaCIDIEI* gene cluster in *Synechococcus* sp. PCC 7002, while the secondary cytochrome oxidase, encoded by the *ctaCIIDIEII* gene cluster, seems to contribute very little to respiratory processes under the conditions tested (Table 3). In *Synechocystis* sp. PCC 6803, mutagenesis of either *cydAB* or *ctaDIEI* singly had no effect on respiratory rates when compared to *Synechocystis* sp. PCC 6803 wild type respiratory rates, while a double mutation in these loci had a dramatic effect on oxygen consumption (Howitt and Vermass 1998). In marked contrast, the single mutant, *ctaDI* in *Synechococcus* sp. PCC 7002 drastically

reduces the rates of oxygen consumption when compared to the *Synechococcus* sp. PCC 7002 wild-type strain. This is further functional evidence that *Synechococcus* sp. PCC 7002 lacks a *cydAB*-type quinol oxidase.

Although background levels of cyanide-insensitive oxygen uptake were observed in all of the mutant strains, this activity is not likely from a *CydAB*-like quinol oxidase. Chlororespiration studies indicate that a sequence of reactions catalyzed by NADH dehydrogenase, peroxidase, superoxide dismutase, and the non-enzymic electron transfer from Fe-sulfur protein to O_2 , can lead to a net consumption of oxygen in the dark (Casano et al. 2000), and the cyanide-sensitive activity observed in the current study may be attributed to inhibition of non-photosynthetic chlororespiration.

Photoinhibition occurs under conditions of excessive light intensities or electron flow limitation due to environmental stresses such as water loss, or chilling (Savitch et al. 1996). In this study, the absence of *CtaDI* caused changes in the electron flow around the photosystems that were similar to those observed in cells grown under photoinhibitory conditions. It is clear from the changes observed in the F_V'/F_M' ratio between the *ctaDI* mutants and wild type strain of *Synechococcus* sp. PCC 7002 that electron flow around PS II was modified in *ctaDI* deficient strains. Under low to moderate light stress conditions, the mutations in the cytochrome oxidase loci caused little or no physiological duress (Table 2). However, the $P700^+$ reduction kinetics of the mutants were severely affected, with half times decreasing dramatically suggesting that cytochrome oxidase may be necessary for proper poisoning of electrons in the electron transport chain for PS I. There is also a decrease in the total amount of chlorophyll in the cells of the *ctaDI* mutants when compared to the chlorophyll levels in wild type cells under many different light conditions, suggesting that the lack of a functional cytochrome oxidase causes photoinhibition in *Synechococcus* sp. PCC 7002 (Tables 2, 5). The overall reduction in the number of functional PS II centers in the mutant cells at higher light intensities may be due to oxidative damage to PS II centers or inactivation due to photoinhibition. The inability of the cells to deal with an increased amount of reductive stress becomes more evident when the cells are exposed

to increasing light intensities. As the light intensity increases, the cells lower the number of active PS II complexes as reflected by a decrease in the 77 K fluorescence peak at 685 nm and 695 nm respectively of the mutant strains compared to the wild type strains (Figure 5). Thus, cyanobacteria have evolved to account for possible excessive reductive stress by placing both cytochrome oxidase and photosystem complexes within the same membrane.

In summary, this study has shown that cytochrome oxidases play a dual role in *Synechococcus* sp. PCC 7002 by assuring proper redox poise of electron flow around the photosystems and minimizing oxidative damage to the photosynthetic proteins by consuming oxygen produced by photosynthesis.

Acknowledgements

This work was supported by grant NSF MCB-0077586 to D.A. Bryant. The authors would like to thank Dr. I. Vasiliev and Dr. J.H. Golbeck for technical assistance in the $P700^+$ measurements and Dr. T. Sakamoto for helpful commentary and critical reading of this manuscript.

References

- Alge D, Peschek GA (1993a) Characterization of a *cta/CDE* operon-like genomic region encoding subunits I–III of the cytochrome *c* oxidase of the cyanobacterium *Synechocystis* PCC 6803. *Biochem Mol Biol Int* 29: 511–525
- Alge D, Peschek GA (1993b) Identification and characterization of the *ctaC* (*coxB*) gene as part of an operon encoding subunits I, II, and III of the cytochrome *c* oxidase (cytochrome *aa₃*) in the cyanobacterium *Synechocystis* PCC 6803. *Biochem Biophys Res Commun* 191: 9–17
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) *Current Protocols in Molecular Biology*, Wiley, New York
- Berry S, Schneider D, Vermaas WF, Rogner M (2002) Electron transport routes in whole cells of *Synechocystis* sp. strain PCC 6803: the role of the cytochrome *bd*-type oxidase. *Biochemistry* 41: 3422–3429
- Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7: 1513–1523
- Bryant DA, de Tandeau Marsac N (1988) Isolation of genes encoding components of the photosynthetic apparatus. *Methods Enzymol* 167: 755–765
- Buzby JS, Porter RD, Stevens JSE (1983) Plasmid transformation in *Agmenellum quadruplicatum* PR-6: construction of biphasic plasmids and characterization of their transformation properties. *J Bacteriol* 154: 1446–1450

- Campbell D, Hurry V, Clarke AK, Gustafsson P, Öquist G (1998) Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. *Microbiol Mol Biol Rev* 62: 667–683
- Casano LM, Zapata JM, Martin M, Sabater B (2000) Chlororespiration and poisoning of cyclic electron transport. Plastocyanin as electron transporter between thylakoid NADH dehydrogenase and peroxidase. *J Biol Chem* 275: 942–948
- Garcia-Horsman JA, Barquera B, Rumbley J, Ma J, Gennis RB (1994) The superfamily of heme-copper respiratory oxidases. *J Bacteriol* 176: 5587–5600
- Geider RJ, Osborne BA (1992) *Algal Photosynthesis*, Chapman and Hall, New York
- Ghassemian M, Wong B, Ferriera F, Markley JL, Straus NA (1994) Cloning, sequencing and transcriptional studies of the genes for cytochrome *c*-553 and plastocyanin from *Anabaena* sp. PCC 7120. *Microbiology* 140: 1151–1159
- Golden SS, Brusslan J, Haselkorn R (1987) Genetic engineering of the cyanobacterial chromosome. *Methods Enzymol* 153: 215–231
- Howitt CA, Vermaas WFJ (1998) Quinol and cytochrome oxidases in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochemistry* 37: 17944–17951
- Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirose M, Sugiura M, Sasamoto S (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* 3: 109–136
- Maxwell PC, Biggins J (1976) Role of cyclic electron transport in photosynthesis as measured by the photoinduced turnover of P700 in vivo. *Biochemistry* 15: 3975–3981
- Nomura C, Bryant DA (1997) Cytochrome *c*₆ from *Synechococcus* sp. PCC 7002. In: Peschek GP, Löffelhardt W and Schmetterer G (eds) *The Phototrophic Prokaryotes*, pp 269–274. Kluwer Academic/Plenum Publishers, New York, USA
- Obinger C, Knepper JC, Zimmermann U, Peschek GA (1990) Identification of a periplasmic c-type cytochrome as electron donor to the plasma membrane-bound cytochrome oxidase of the cyanobacterium *Nostoc mac.* *Biochem Biophys Res Commun* 169: 492–501
- Peschek GA, Wastyn M, Trnka M, Molitor V, Fry IV, Packer L (1989) Characterization of the cytochrome *c* oxidase in isolated and purified plasma membranes from the cyanobacterium *Anacystis nidulans*. *Biochemistry* 28: 3057–3063
- Prentki P, Krisch HM (1984) In vitro insertional mutagenesis with a selectable DNA fragment. *Gene* 29: 303–313
- Sakamoto T, Bryant DA (1997) Temperature-regulated mRNA accumulation and stabilization for fatty acid desaturase genes in the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Mol Microbiol* 23: 1281–1292
- Sakamoto T, Bryant DA (1998) Growth at low temperature causes nitrogen limitation in the cyanobacterium *Synechococcus* sp. PCC 7002. *Arch Microbiol* 169: 10–19
- Sakamoto T, Delgazio VB, Bryant DA (1998) Growth on urea can trigger death and peroxidation of the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Appl Environ Microbiol* 64: 2361–2366
- Sambrook J, Fritsch E, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Savitch LV, Maxwell DP, Huner NPA (1996) Photosystem II excitation pressure and photosynthetic carbon metabolism in *Chlorella vulgaris*. *Plant Physiol* 111: 127–136
- Schmetterer G, Alge D, Gregor W (1994) Deletion of cytochrome *c* oxidase genes from the cyanobacterium *Synechocystis* sp. PCC6803: Evidence for alternative respiratory pathways. *Photosynth Res* 42: 43–50
- Schmetterer G, Valladares A, Pils D, Steinbach S, Pacher M, Muro-Pastor AM, Flores E, Herrero A (2001) The *coxBAC* operon encodes a cytochrome *c* oxidase required for heterotrophic growth in the cyanobacterium *Anabaena variabilis* strain ATCC 29413. *J Bacteriol* 183: 6429–6434
- Schreiber U, Schliwa U, Bilger W (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynth Res* 10: 51–62
- Schubert H, Matthijs HCP, Mur LR (1995) *In vivo* assay of P700 redox changes in the cyanobacterium *Fremyella diplosiphon* and the role of cytochrome *c* oxidase in regulation of photosynthetic electron transfer. *Photosynthetica* 31: 517–527
- Sone N, Tano H and Ishizuka M (1993) The genes in the thermophilic cyanobacterium *Synechococcus vulcanus* encoding cytochrome *c* oxidase [published erratum appears in *Biochim Biophys Acta* 1994 Apr 28; 1188 (2):255]. *Biochim Biophys Acta* 1183: 130–138
- Stevens JSE, Patterson COP, Myers J (1973) The production of hydrogen peroxide by blue green algae: a survey. *J Phycol* 9: 427–430
- Tano H, Ishizuka M, Sone N (1991) The cytochrome *c* oxidase genes in blue-green algae and characteristics of the deduced protein sequence for subunit II of the thermophilic cyanobacterium *Synechococcus vulcanus*. *Biochem Biophys Res Commun* 181: 437–442
- Trnka M, Peschek GA (1986) Immunological identification of aa3-type cytochrome oxidase in membrane preparations of the cyanobacterium *Anacystis nidulans*. *Biochem Biophys Res Commun* 136: 235–241
- Yu L, Zhao J, Mühlenhoff U, Bryant DA and Golbeck JH (1993) *PsaE* is required for in vivo cyclic electron flow around photosystem I in the cyanobacterium *Synechococcus* sp. PCC 7002. *Plant Physiol* 103