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Roles for heme–copper oxidases in extreme high-light and oxidative stress response in the cyanobacterium *Synechococcus* sp. PCC 7002

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Abstract The *ctaCIDIEI* and *ctaCIIDIIEII* gene clusters that encode heme–copper cytochrome oxidases have been characterized in the marine cyanobacterium *Synechococcus* sp. PCC 7002 and the inactivation of *ctaDI* was shown to affect high-light adaptation. In this study, *Synechococcus* sp. PCC 7002 wild-type, *ctaDI*, *ctaDII*, and *ctaDI–ctaDII* double mutants were grown under extreme high-light and oxidative stress to further assess the roles of cytochrome oxidases in cyanobacteria. Cells of the *ctaDI* mutant strain barely grew under extreme high-light illumination of $4.5 \text{ mE m}^{-2} \text{ s}^{-1}$, suggesting that CtaDI is required for high-light acclimation in *Synechococcus* sp. PCC 7002. The *ctaDI–ctaDII* double mutant cells unexpectedly tolerated extreme high-light intensity, indicating that the disruption of *ctaDII* gene suppresses the high-light sensitivity phenotype of the *ctaDI* single mutant. The *ctaDII* mutant cells also exhibited higher tolerance to the oxidative stress compound, methyl viologen, in the growth media. The *ctaDII* mutant and the *ctaDI–ctaDII* double mutant cells had approximately twofold higher levels of superoxide dismutase (SOD) activity, indicating that the disruption of *ctaDII* gene increased the capacity to decompose active oxygen species. These results suggest that the CtaII

cytochrome oxidase may be involved with the oxidative stress response, including the control of SOD expression.

Keywords Cyanobacteria · Heme–copper oxidases · High-light stress · Oxidative stress

Abbreviations MV: Methyl viologen · HEPES: *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) · SOD: Super-oxide dismutase · BHT: Butylated hydroxytoluene · Q: Ubiquinone · QH₂: Ubiquinol

Introduction

Cyanobacteria are photosynthetic, oxygen-evolving prokaryotes that share some electron transport proteins for both photosynthesis and respiration, and have adapted to a wide range of ecological niches (Stanier and Cohen-Bazire 1977). Some of the common stresses encountered by cyanobacteria in their natural environments include changes in temperature, varying light intensities, and exposure to oxidative stress. Under changing oxidative conditions, the cells must balance different needs in order to optimize their energetics. One way that cyanobacteria counter these effects is by adjusting the types of proteins present in the electron transfer chains. A protein that is present in both the respiratory and photosynthetic electron transport chains in cyanobacteria is cytochrome oxidase. Terminal oxidases may be used to generate a maximal H⁺/e⁻ gradient (Puustinen et al. 1991), to remove excess reducing equivalents, and to consume oxygen to maintain anaerobicity or to lower the oxygen concentration for the cell (Kelly et al. 1990). Previous biochemical studies examining P700⁺ reduction kinetics in cyanobacteria such as *Synechococcus* sp. PCC 7002 (Nomura et al. 2006; Yu et al. 1993) and *Fremyella diplosiphon* indicate that these organisms may use cytochrome oxidases as a

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sink for removing excess electrons not accounted for by PS I activity (Schubert et al. 1995). Additional studies in cyanobacteria point towards the importance of respiratory oxidases for heterotrophic (Schmetterer et al. 2001) and photoheterotrophic growth (Berry et al. 2002).

Previously, two cytochrome oxidase operons were cloned and characterized from the marine cyanobacterium, *Synechococcus* sp. PCC 7002, and it was shown that mutations in the *ctaD* loci affected electron flow around PSI and PSII as well as the ratio of the photosystems (Nomura et al. 2006). To further assess the roles of cytochrome oxidases during high-light stress responses in cyanobacteria, wild-type and *ctaD* *Synechococcus* sp. PCC 7002 mutant strains were grown under extreme high-light conditions or under oxidative stress conditions caused by the addition of the herbicide, methyl viologen (MV). This report presents evidence that both the CtaI and CtaII complexes are important for extreme high-light tolerance and that the CtaII complex may play a role in redox stress management in *Synechococcus* sp. PCC 7002, suggesting that there is a complex mechanism in cyanobacteria to balance the electron transport chain and acclimate cells to environmentally stressful redox conditions.

Materials and methods

Bacterial strains and culture conditions

The PR6000 (wild-type) and *ctaD* mutant strains of the marine cyanobacterium, *Synechococcus* sp. strain PCC 7002 were maintained in liquid culture and on 1.5% agar plates in medium A⁺ under continuous light (250 μE m⁻² s⁻¹) at 38 °C. The *ctaDI* and *ctaDII* genes were disrupted by interposon mutagenesis. The *aphII* gene, which confers kanamycin resistance, was inserted into the *ctaDI* gene and the *ctaDII* gene was interrupted by insertion of a 2 kb *SmaI* Ω fragment with strong transcription terminators flanking both ends of the gene and confers spectinomycin resistance (Nomura et al. 2006). These mutant strains were examined after experimentation by Southern hybridization and found to be homozygous (data not shown). Kanamycin (100 μg/ml) and spectinomycin (100 μg/ml) were used for the selection of mutants. 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). For oxidative stress growth conditions, MV was added to a final concentration of 50 μM when appropriate. The activities from whole cells were normalized by OD₅₅₀, where a cell suspension with OD₅₅₀ = 1.0 contains (1.0 ± 0.2) × 10⁸ cells in 1 ml by microscopic count and (4.7 ± 0.6) × 10⁷ colony-forming units (CFUs) in 1 ml (Sakamoto and Bryant 1998, 2002). The plating efficiency is estimated and may vary in a range of 50–80% for growth conditions on plates.

High-light treatment and oxygen evolution

For high-light treatment, cells were illuminated with 150 W halogen bulbs and light intensities were measured using a model QSL-100 quantum scalar irradiance meter (Biospherical Instruments, Inc., San Diego, CA) (Sakamoto and Bryant 2002). The temperature was maintained by using a refrigerated water circulator. Oxygen evolution assays were performed as previously described (Nomura et al. 2006). Briefly, cells were incubated at various times with either 4.5 mE m⁻² s⁻¹ or 250 μE m⁻² s⁻¹ followed by an incubation in the dark for 2 min after which oxygen evolution was stimulated with saturating amounts of light (2.5 mE m⁻² s⁻¹).

Cell viability

Exponentially growing cells (OD₅₅₀ = 0.25) from all strains of *Synechococcus* sp. strain PCC 7002 were divided into four culture tubes. Duplicate cultures of each strain were incubated under standard growth conditions with or without the addition of 50 μM MV. The second set of duplicates for each strain were incubated in the dark at 37°C with or without the addition of 50 μM MV for 4 h. The cells were harvested by centrifugation and washed with fresh A⁺ liquid media, and resuspended in A⁺ media to give a final OD₅₅₀ = 1. From these cultures, 10 μl aliquots with a final OD₅₅₀ = 0.01 were spread on an appropriate A⁺ plate. The cells were grown on plates for 2 days prior to the determination of CFUs.

Superoxide dismutase activity measurements

Superoxide dismutase (SOD) is an enzyme that catalyzes the reaction: O₂⁻ + O₂⁻ + 2H⁺ → H₂O₂ + O₂. SOD activity in soluble and membrane fractions of *Synechococcus* sp. PCC 7002 was measured by monitoring the reduction of an artificial substrate, nitro-blue tetrazolium, as previously described (Winterbourn et al. 1975). The exponentially growing cyanobacterial cells were collected by centrifugation and washed with 50 mM potassium phosphate buffer, pH 7.8, and resuspended in 50 mM potassium phosphate buffer followed by breakage with an SLM-AMINCO French Press. After centrifugation at 4°C, 5,000g for 5 min to remove unbroken cells, the whole cell extract was subjected to a high-speed centrifugation (45,000g for 1 h) at 4°C to separate the soluble and membrane fractions. The soluble fraction was collected and immediately used in SOD assays and the membrane fraction was collected and resuspended in a minimal amount of 50 mM potassium phosphate buffer, pH 7.8 and used for SOD assays. Protein concentrations were determined by Bradford assay.

Catalase activity

Catalase is an enzyme that catalyzes the reaction: 2H₂O₂ → 2H₂O + O₂. Catalase activity in whole

cells of *Synechococcus* sp. PCC 7002 was measured by monitoring the rate of H₂O₂ decomposition at 240 nm by using the extinction coefficient for H₂O₂ of 43.6 M⁻¹ cm⁻¹ (Beers and Sizer 1952). Cells grown under standard growth conditions (38°C, 250 μE m⁻² s⁻¹, 1.5% CO₂) were harvested by centrifugation at room temperature at 8,000g for 5 min and the cell pellets were resuspended and washed with 50 mM potassium phosphate buffer, pH 7.0. The washed cells were added to the reaction mixture (3 ml) to a final OD₅₅₀ = 1.0.

Peroxidase activity

Peroxidase activity was measured by the following reaction: H₂O₂ + peroxidase + oxygen acceptor (colorless) → H₂O + oxidized acceptor (colored) as previously described, where the artificial oxygen acceptor, phenol was used in the presence of 4-aminoantipyrene (Trinder 1969). Cells grown under standard growth conditions (38°C, 250 μE m⁻² s⁻¹, 1.5% CO₂) were harvested by centrifugation at room temperature at 8,000g for 5 min and the cell pellets were resuspended and washed with 20 mM potassium phosphate buffer, pH 7.0. The washed cells were added to the reaction mixture (3 ml) to give a final OD₅₅₀ = 2.0. Peroxidase activity was measured by monitoring the change in the color of phenol at 510 nm (Trinder 1969).

Detection of hydroperoxides

A modified ferrous oxidation/xylenol orange assay was used to determine the levels of hydroperoxides in cyanobacterial cells (Sakamoto et al. 1998). Exponentially growing cells were first diluted to an OD₅₅₀ of 0.2 and were incubated under standard growth conditions (250 μE m⁻² s⁻¹ at 38°C) or in darkness for 4 h in the presence or absence of 50 μM MV. Cells were harvested by centrifugation at 5,000g for 10 min. The cell pellets were resuspended in 0.8 ml of methanol/0.01% butylated hydroxytoluene (BHT), 0.1 ml of Reagent A (2.5 mM ammonium iron (II) sulfate/0.25 M sulfuric acid) and 0.1 ml of Reagent B (40 mM BHT, 1.25 mM xylenol orange in methanol). The mixture was incubated for 30 min at room temperature and then centrifuged at 10,000g to remove any cell debris. The absorbance at 560 nm was determined for the reaction mixtures and the concentration of hydroperoxides was determined by using the extinction coefficient ($E_{560} = 4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (Sakamoto et al. 1998).

Results

Effects of extreme high-light stress on cell growth of *Synechococcus* sp. PCC 7002 and *ctaD* strains

It was previously shown that under normal light intensity and moderate light stress, the wild-type and *ctaD*

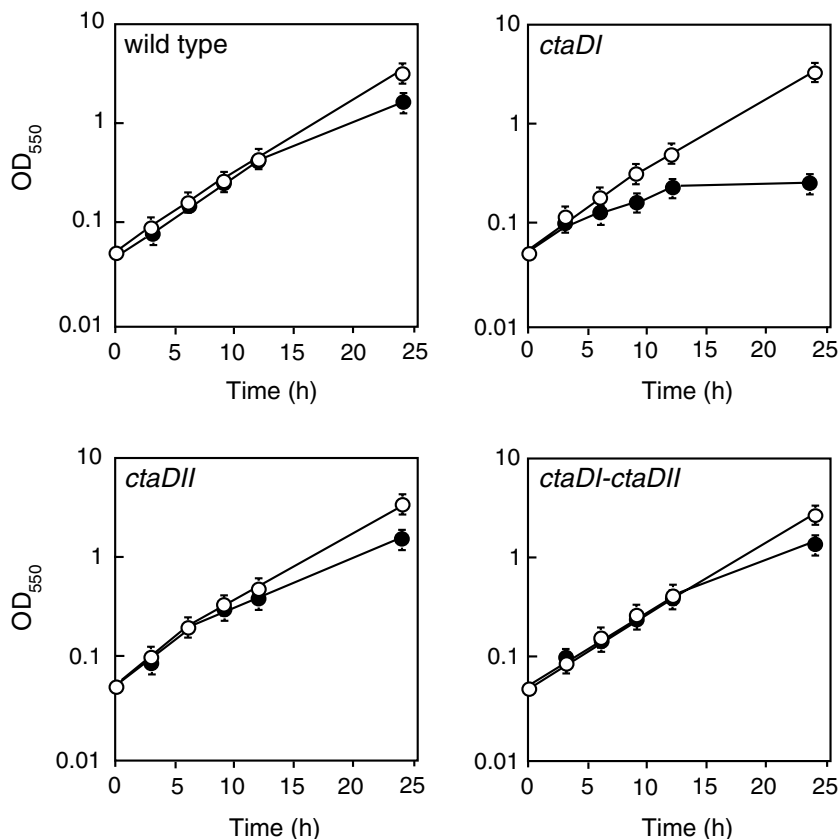
strains of *Synechococcus* sp. PCC 7002 had virtually identical growth rates, indicating that under these conditions, cytochrome oxidase activity does not significantly contribute to the growth physiology and energetics of the cell (Nomura et al. 2006). However, the electron flow around the photosystems and photosystem ratios were somewhat changed in the mutant strains compared to the wild-type strain indicating that cytochrome oxidase may combat oxidative and reductive stress under more extreme conditions (Nomura et al. 2006). To test this hypothesis in *Synechococcus* sp. PCC 7002 strains, the cells were grown under extreme high-light intensity (4.5 mE m⁻² s⁻¹) while the temperature and CO₂ concentration were held constant (38°C and 1.5% (v/v) CO₂, respectively).

Growth curves for wild-type and *ctaD* strains grown under standard and extreme high-light intensity conditions are shown in Fig. 1 and clearly demonstrate that the growth rate of the *ctaDI* single mutant was affected when compared to the wild-type strain when grown under extreme high-light conditions. These results suggest that under extreme high-light conditions, the absence of *ctaDI* causes the cells to enter the stationary phase or death phase prematurely. Although *ctaDI* is dispensable for growth of cells grown under a range of light from 150 to 700 μE m⁻² s⁻¹ (Nomura et al. 2006), the results of this study show that it is important when the cells are grown under extreme high-light intensity. However, unlike the *ctaDI* strain, the *ctaDII* and *ctaDI-ctaDII* strains had growth rates similar to the wild-type strain when grown under extreme high-light intensity (Fig. 1). These results suggest that the absence of *ctaDII* allows the cells to grow at 4.5 mE m⁻² s⁻¹ even in the absence of *ctaDI*. Thus, the absence of *ctaDII* suppresses the phenotype observed when *ctaDI* is inactivated.

Effect of *ctaD* mutations on chlorophyll content and high-light tolerance of photosynthesis

The chlorophyll contents of the mutant and wild-type *Synechococcus* sp. PCC 7002 strains grown under standard (250 μE m⁻² s⁻¹, 38°C, 1.5% (v/v) CO₂) and extreme high-light intensity conditions are summarized in Table 1. These results, in addition to those previously reported (Nomura et al. 2006), show that chlorophyll contents for all strains decrease with an increase in light intensity. Chlorophyll contents were nearly identical for all strains grown under standard conditions. The wild-type strain maintained the highest chlorophyll content (1.2 ± 0.1 μg ml⁻¹ OD_{550 nm}⁻¹) when grown under extreme high-light intensity. The *ctaDI* strain did not grow under extreme high-light conditions and thus the chlorophyll content was not measured. The *ctaDII* strains were able to grow under extreme high-light conditions although their chlorophyll contents (0.7 ± 0.1 μg ml⁻¹ OD_{550 nm}⁻¹) were slightly lower than the wild-type strain.

Fig. 1 Effect of high-light intensity on cell growth of wild-type, *ctaDI* and *ctaDII* strains of *Synechococcus* sp. PCC 7002. Exponentially growing cells under standard conditions ($250 \mu\text{E m}^{-2} \text{s}^{-1}$, 1% CO_2 , 38°C) were diluted to an OD_{550} of 0.05 and grown at either $250 \mu\text{E m}^{-2} \text{s}^{-1}$ or $4.5 \text{mE m}^{-2} \text{s}^{-1}$ constant illumination. Open circles represent cells grown under $250 \mu\text{E m}^{-2} \text{s}^{-1}$. Filled circles represent cells grown under $4.5 \text{mE m}^{-2} \text{s}^{-1}$. Results shown are typical of one of seven independent growth experiments. Standard deviations are indicated as error bars on the graph



To assess the effects of growth under various light intensities on photosynthesis in the *Synechococcus* sp. PCC 7002 wild-type and *ctaD*-deficient strains, high-light tolerance of the capacity of photosynthetic electron transport activity was examined by incubation of the different strains under extreme high-light conditions ($4.5 \text{mE m}^{-2} \text{s}^{-1}$, 38°C) using a saturating level of NaHCO_3 (10 mM) as a final electron acceptor (Fig. 2). For the first 40 min of incubation under extreme high-light conditions, there was little effect on the oxygen evolving capacity in the wild-type strain of *Synechococcus* sp. PCC 7002. After 1 h of extreme high-light

treatment, O_2 evolution in the wild-type cells was 40% of the initial level. The photosynthetic oxygen evolving activity of the *ctaDII* strain was unexpectedly tolerant to incubation under extreme high-light intensity, displaying nearly the same level of oxygen evolving activity during extreme high-light treatment of $4.5 \text{mE m}^{-2} \text{s}^{-1}$ for up to 1 h (Fig. 2, open circles). The *ctaDI-ctaDII* strain also showed little effect for the first 40 min of high-light treatment but exhibited a 30% decrease in oxygen evolving activity after incubation for 1 h under high-light conditions, similar to the wild-type strain (compare open diamonds and inverted triangles in Fig. 2). Unlike all other strains tested, the *ctaDI* strain displayed a consistent decrease in oxygen evolving capacity after exposure to high light (Fig. 2, filled circles), indicating that the *ctaDI* strain is sensitive to high-light conditions. The results shown in Fig. 2 demonstrate that the absence of the *ctaDII* gene product compensates for the absence of the *ctaDI* gene product and allowed cells to tolerate high-light conditions that rapidly induce damage to the photosynthetic electron transport chain.

Table 1 Chlorophyll contents of cells grown under extreme high-light intensity

Strain	Light intensity ($\mu\text{E m}^{-2} \text{s}^{-1}$)	Chlorophyll <i>a</i> ($\mu\text{g ml}^{-1} \text{OD}_{550}^{-1}$)
Wild type	250	3.4 ± 0.6
<i>ctaDI</i>	250	3.0 ± 0.5
<i>ctaDII</i>	250	3.4 ± 0.5
<i>ctaDI-ctaDII</i>	250	3.2 ± 0.6
Wild type	4,500	1.2 ± 0.2
<i>ctaDI</i>	4,500	ND ^a
<i>ctaDII</i>	4,500	0.7 ± 0.2
<i>ctaDI-ctaDII</i>	4,500	0.7 ± 0.2

Values shown represent the average \pm SD of five independent experiments

^aNot determined because the strain could not grow under extreme high-light conditions

Oxidative stress responses of *Synechococcus* sp. PCC 7002 wild-type and *ctaD* strains

MV is an herbicide that forms the toxic superoxide radical anion in cyanobacterial cells. In order to examine the effects of oxidative stress on *Synechococcus* sp. PCC

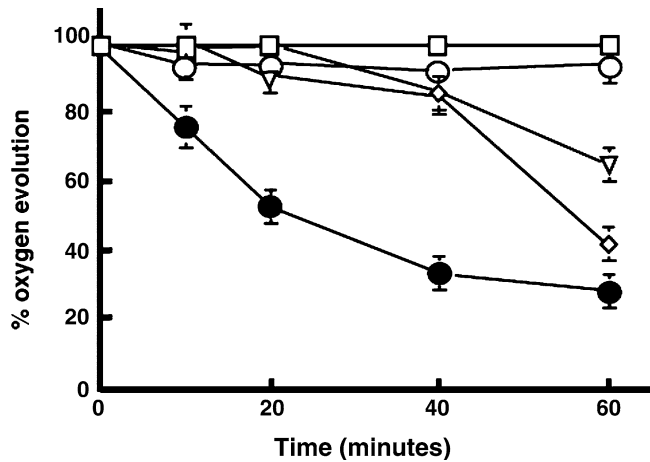
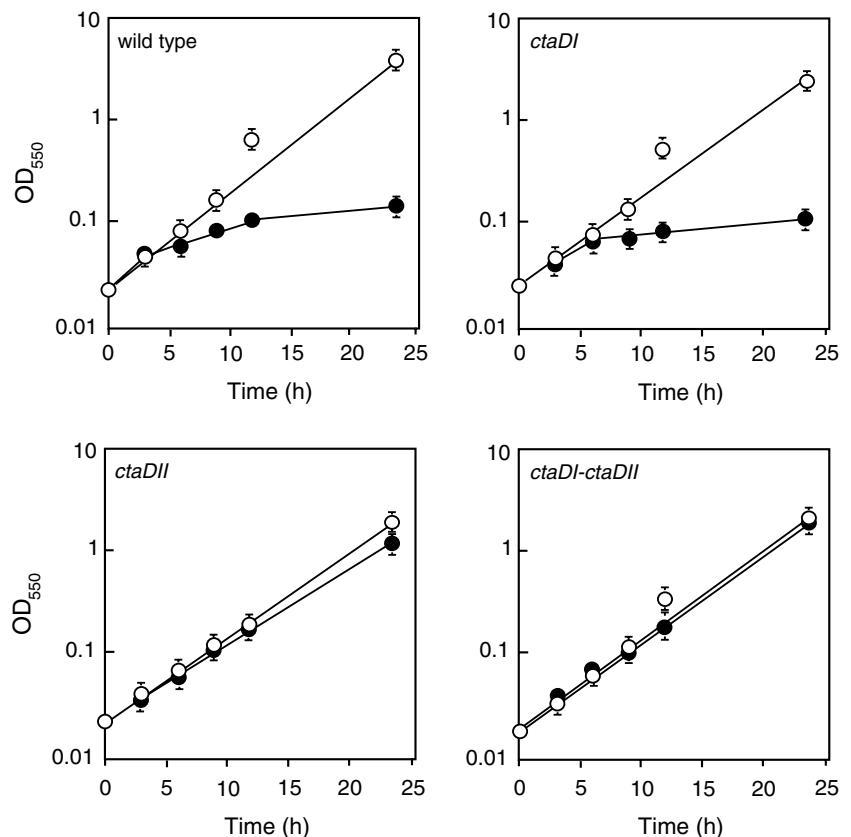


Fig. 2 Effects of high-light treatment on photosynthetic O_2 evolution in wild-type, *ctaDI* and *ctaDII* strains of *Synechococcus* sp. PCC 7002. Cells in the exponential phase of growth were supplemented with 25 mM HEPES-NaOH (pH 7.0), 10 mM $NaHCO_3$ and were incubated at $38^\circ C$ with either a high-light intensity of $4.5 \text{ mE m}^{-2} \text{ s}^{-1}$ [wild-type (open diamonds), *ctaDII* (open circles), *ctaDI-ctaDII* (inverted triangles), *ctaDI* (filled circles)], or normal light intensity of $250 \text{ } \mu\text{E m}^{-2} \text{ s}^{-1}$ [representative of all strains (open squares)] before measurement of oxygen evolving activity. The remaining activity of photosynthetic oxygen evolution from whole cells was measured at $38^\circ C$ after incubation for 2 min in the dark. The data shown are the average of five independent experiments. Standard deviations are indicated as error bars on the graph

Fig. 3 Effects of methyl viologen (MV) on cell growth of wild-type, *ctaDI* and *ctaDII* strains of *Synechococcus* sp. PCC 7002. The exponentially growing cells under standard conditions ($250 \text{ } \mu\text{E m}^{-2} \text{ s}^{-1}$, 1% CO_2 , $38^\circ C$) were diluted to an OD_{550} of 0.05 and grown in the presence or absence of $50 \text{ } \mu\text{M}$ MV. Open circles represent cells grown without $50 \text{ } \mu\text{M}$ MV. Filled circles represent cells grown in the presence of $50 \text{ } \mu\text{M}$ MV. Results shown are typical of one of seven independent growth experiments. Standard deviations are indicated as error bars on the graph



7002, wild-type and cytochrome oxidase mutant strains were grown in the presence and absence of MV and their growth rates were compared. Figure 3 shows the growth curves of the *Synechococcus* sp. PCC 7002 wild-type and mutant strains grown in the presence and absence of $50 \text{ } \mu\text{M}$ MV. Both the *ctaDII* and *ctaDI-ctaDII* strains were able to grow in the presence of $50 \text{ } \mu\text{M}$ MV at rates nearly equal to cultures grown without MV in the media; in contrast, the wild-type and *ctaDI* strains failed to grow in the presence of $50 \text{ } \mu\text{M}$ MV. These results indicate that strains harboring the *ctaDII* mutation must have a mechanism to counteract the increase in superoxide generated by MV. In addition, the *ctaDII* strains grown in the presence of MV had lower chlorophyll content ($2.5 \pm 0.6 \text{ } \mu\text{g ml}^{-1} OD_{550}^{-1}$, $n = 5$) compared to cells grown without MV ($3.4 \pm 0.5 \text{ } \mu\text{g ml}^{-1} OD_{550}^{-1}$, $n = 5$).

The tolerance towards MV shown by the *ctaD* mutant strains of *Synechococcus* sp. PCC 7002 was further characterized by cell viability counts (Table 2). When cells of wild-type and *ctaDI* mutant strains were incubated for 4 h under standard conditions ($250 \text{ } \mu\text{E m}^{-2} \text{ s}^{-1}$, $38^\circ C$, 1.5% (v/v) CO_2 /air) in the presence of $50 \text{ } \mu\text{M}$ MV, approximately 40% of cells were still viable. However, there was no discernable difference in the number of viable cells for *ctaDII* strains incubated with or without $50 \text{ } \mu\text{M}$ MV under standard growth conditions (Table 2). The total number of viable cells apparently decreased for all strains during dark

Table 2 Cell viability after incubation with or without 50 μM methyl viologen (MV), in either light or darkness

Treatment	Strains of <i>Synechococcus</i> sp. PCC 7002			
	Wild type ($\times 10^7$)	<i>ctaDI</i> ($\times 10^7$)	<i>ctaDII</i> ($\times 10^7$)	<i>ctaDI-ctaDII</i> ($\times 10^7$)
Light	8.0 \pm 0.3	5.0 \pm 0.3	7.4 \pm 0.3	5.8 \pm 0.5
Light + 50 μM MV	3.6 \pm 0.1	2.0 \pm 0.1	7.7 \pm 0.4	6.0 \pm 0.4
Dark	4.8 \pm 0.4	4.6 \pm 0.3	6.6 \pm 0.2	4.0 \pm 0.3
Dark + 50 μM MV	4.4 \pm 0.4	4.6 \pm 0.3	6.8 \pm 0.2	3.7 \pm 0.4

Conditions are described in [Materials and methods](#). Results are given as the number of CFUs in 1 ml of cell suspension, $\text{OD}_{550} = 1.0$. Values are shown as average \pm SD for three independent experiments

incubation, but there were no differences between the number of viable cells incubated in the presence or absence of MV in the dark (Table 2), indicating that MV most likely forms the cation radical during photosynthesis as described previously (Epel and Neumann 1973), and that the toxicity of MV on the viability of cells is enhanced by light. Consistent with the data from the growth experiments (Fig. 3), the *ctaDII* mutants were tolerant to MV in the viable cell counting experiment. One possible explanation for the tolerance toward MV observed in the *ctaDII* strains is that they may have increased levels of enzymes (SOD, peroxidase, and catalase) related to the alleviation of oxidative stress. In order to test this hypothesis, the levels of these enzymes were measured to characterize the capacity of the wild-type and *ctaD* mutant strains of *Synechococcus* sp. PCC 7002 for decomposing active oxygen.

Superoxide dismutase activity

Both soluble and membrane fractions derived from the *ctaDII* strain of *Synechococcus* sp. PCC 7002 have an increased level of SOD activity compared to the fractions from the wild type (Table 3). The SOD activity of the membrane fractions for the *ctaDII* strain is higher than for the wild type (> twofold difference). These results suggest that the *ctaDII* locus is somehow involved in regulating the amount of membrane bound SOD activity in *Synechococcus* sp. PCC 7002 and in the absence of *ctaDII*, there are greater amounts of func-

Table 3 Superoxide dismutase activity for *Synechococcus* sp. PCC 7002

Strain	Localization	Units of SOD activity (mg^{-1} protein)	Percentage activity relative to wild type
Wild type	Soluble	34 \pm 6	100
<i>ctaDI</i>	Soluble	35 \pm 7	100
<i>ctaDII</i>	Soluble	39 \pm 4	116
<i>ctaDI-ctaDII</i>	Soluble	40 \pm 5	118
Wild type	Membranes	12 \pm 4	100
<i>ctaDI</i>	Membranes	12 \pm 6	100
<i>ctaDII</i>	Membranes	24 \pm 9	207
<i>ctaDI-ctaDII</i>	Membranes	27 \pm 5	232

Values shown represent the average \pm SD of five independent experiments

tional, membrane-bound SOD. The activity of the two fractions (soluble and membrane) together represents 100% of the total SOD activity assayed from the cells. Therefore, the total SOD activity is 1.7-fold higher in the *ctaDII* strains compared to the wild-type strain of *Synechococcus* sp. PCC 7002.

Catalase and peroxidase activities

All of the strains had a low level of catalase activity, but the results indicate that there were only slight differences in catalase activity when the wild-type, *ctaDI* and *ctaDII* strains of *Synechococcus* sp. PCC 7002 were compared ($50 \pm 10 \text{ mU ml}^{-1} \text{ OD}_{550}^{-1}$, $n = 5$ for wild-type and *ctaDI* strains and $60 \pm 20 \text{ mU ml}^{-1} \text{ OD}_{550}^{-1}$, $n = 5$ for *ctaDII* strains). Peroxidase activity ($50 \pm 10 \text{ mU ml}^{-1} \text{ OD}_{550}^{-1}$, $n = 5$ for all strains) was nearly identical in all the strains. These results support the observation that although interruption of *ctaDII* affects the levels of SOD activity, there are only slight increases in the levels of catalase or peroxidase activity compared to the wild-type strain and relative to SOD activity. This observation is also reflected in the elevated hydroperoxide levels detected in the *ctaDII* strains (Table 4) and implies that the interruption of *ctaDII* only affects SOD activity in these cells.

Hydroperoxide levels

The total level of hydroperoxides was measured in both wild-type and mutant strains grown under standard

Table 4 Hydroperoxide levels in *Synechococcus* sp. PCC 7002 in the presence and absence of MV

Strain	50 μM MV treatment	Peroxides ($\text{nmol ml}^{-1} \text{ OD}_{550}^{-1}$)
Wild type	–	58 \pm 3
<i>ctaDI</i>	–	60 \pm 9
<i>ctaDII</i>	–	76 \pm 9
<i>ctaDI-ctaDII</i>	–	74 \pm 1
Wild type	+	54 \pm 9
<i>ctaDI</i>	+	54 \pm 6
<i>ctaDII</i>	+	67 \pm 2
<i>ctaDI-ctaDII</i>	+	70 \pm 4

Values shown represent the average \pm SD of five independent experiments

–, no MV added to cultures; +, 50 μM MV added to the cultures

conditions in the presence and absence of 50 μM MV and the results are summarized in Table 4. The results indicate that the wild-type and *ctaDI* strains have similar levels of hydroperoxides both in the presence and absence of MV (Table 4). The *ctaDII* strain has a 31% higher level of hydroperoxides compared to the wild-type strain in the absence of MV and a 24% higher level of hydroperoxides when treated with MV. The *ctaDI-ctaDII* double mutant had similar higher levels of hydroperoxides compared to the wild-type strain. H_2O_2 is a direct by product of SOD activity (Fridovich and Hassan 1979), and the presence of higher concentrations of hydroperoxides in the *ctaDII* mutant strains implies that there is an increase in the level of activity of SOD (Table 3).

Discussion

Cytochrome oxidases have been found in most aerobic bacteria examined to date, allowing them to change their respiratory systems according to environmental challenges. As oxygen-evolving, photosynthetic bacteria, it is a conundrum as to why respiratory oxidases are found in cyanobacteria. Many studies have been conducted to assess the role of respiratory oxidases in cyanobacteria and it has been shown that the enzymes contribute to the poisoning of electrons in the electron transport chains and the maintenance of the photosystems (Berry et al. 2002; Nomura et al. 2006) as well as for heterotrophic growth (Schmetterer et al. 2001) and photoheterotrophic growth (Berry et al. 2002). This study defines an expanded role for respiratory oxidases in cyanobacteria. Results of whole chain electron transport analysis from this study showed that *ctaDI* strain was more sensitive to photoinhibition and extreme high-light stress compared to the wild-type strain, while the oxygen evolving capacity of strains harboring the *ctaDII* mutation were more tolerant to high-light treatment than either the wild-type or *ctaDI* strain. Also in stark contrast to the *ctaDI* strain of *Synechococcus* sp. PCC 7002, the *ctaDII* strain is able to grow as well as the wild-type strain at $4.5 \text{ mE m}^{-2} \text{ s}^{-1}$, suggesting that the CtaII complex is not playing the same role as the CtaI complex in *Synechococcus* sp. PCC 7002. Interestingly, this tolerance to extreme light intensity is observed even in the *ctaDI-ctaDII* double mutant strain, despite the sensitivity of the *ctaDI* strain to high-light, indicating that the *ctaDII* strain plays a dominant role in the ability of cells to adapt to high-light stress.

Previously, it was shown that *ctaDI*-deficient strains had much lower oxygen uptake capacities [$6 \pm 2 \mu\text{mol of O}_2 \text{ (mg of chl)}^{-1} \text{ h}^{-1}$ and $5 \pm 1 \mu\text{mol of O}_2 \text{ (mg of chl)}^{-1} \text{ h}^{-1}$ KCN sensitive activity for the *ctaDI* and *ctaDI-ctaDII* strains, respectively] compared to the wild-type and *ctaDII* single mutant strain [$22 \pm 6 \mu\text{mol of O}_2 \text{ (mg of chl)}^{-1} \text{ h}^{-1}$ and $19 \pm 6 \mu\text{mol of O}_2 \text{ (mg of chl)}^{-1} \text{ h}^{-1}$ KCN sensitive activity for the wild-type and *ctaDII* strains, respectively], indicating that *ctaDI* encoded the subunit for the main, active heme-copper

oxidase (Nomura et al. 2006). However, the *ctaDI-ctaDII* strain, which is severely affected in its ability to take up oxygen, has a similar level of tolerance towards high-light stress as the *ctaDII* single mutant strain's (Fig. 1). Therefore, it is not simply an increased level of respiratory activity and electron flow shunting that accounts for the increased tolerance to light stress observed in the *ctaDII* mutant strains.

The unusual observation of the *ctaDII* strains ability to grow under high-light intensity led us to examine possible mechanisms for this tolerance. It was proposed that the mechanisms to tolerate high-light stress might allow the cells to tolerate oxidative stress conditions. By adding MV to the growth medium, we were able to generate oxidative stress in the cyanobacterial strains and determine if any enzymes involved in oxidative stress response could be involved in the observed tolerance. It was found that the *ctaDII* strains could tolerate the addition of high concentrations of MV to the growth medium. MV causes oxidative stress in cells by increasing the amount of superoxide (O_2^-) and this study showed that strains harboring mutations in the *ctaDII* locus are resistant to high levels (50 μM) of MV in the growth medium, while wild-type and *ctaDI* cells are sensitive to the addition of MV to the growth medium. Because the *ctaDI-ctaDII* double mutant cells, which were shown previously to be severely affected in their ability to uptake oxygen (Nomura et al. 2006), have a similar level of tolerance to MV as the *ctaDII* single mutants (Fig. 3), as was the case for the extreme high-light tolerance exhibited by the *ctaDII* strains, it is not simply an increased level of respiratory activity that accounts for the tolerance of the *ctaDII* mutant strains to MV.

Although cells were incubated with MV for 4 h under standard growth conditions, the cells surprisingly had relatively similar levels of hydroperoxides (Table 4). One possibility for this result is that the superoxide anion generated by MV was not immediately converted to hydroperoxide within the cells during the incubation period tested. However, cells treated with MV were monitored for growth after the treatment and those strains that were not *ctaDII*-deficient also ceased growing in a manner similar to that presented in Fig. 3 (data not shown) indicating the likelihood that the SOD activity (Table 3) was responsible for the production of hydroperoxides prior to MV addition.

Examination of the activities associated with the oxidative stress enzymes, SOD, peroxidase, and catalase revealed that *ctaDII* strains had elevated levels of SOD compared to the wild-type and *ctaDI* strain. SOD catalyzes the destruction of the superoxide (O_2^-) radical and has been widely implicated in protecting cells against the harmful effects of active oxygen (Asada 1999; Storz and Imlay 1999). There are three types of SOD enzymes that can be distinguished by their metal co-factors at the active site: iron (Fe-SOD), manganese (Mn-SOD), and copper/zinc (Cu/Zn-SOD) (Asada 1999). Two of the enzymes have been found in cyanobacteria: the iron

(Fe-SOD) enzyme and the manganese (Mn-SOD) enzyme (Herbert et al. 1992; Li et al. 2002; Okada et al. 1979). The SOD activity can be divided into a soluble activity that most likely represents the constitutively expressed Fe-SOD enzyme activity and a membrane fraction containing the Mn-SOD enzyme (Okada et al. 1979). The genes for both the Fe-SOD and Mn-SOD have been identified in the sequencing project for the *Synechococcus* sp. PCC 7002 genome (data not shown). In this study, the enzymatic activities of both soluble and membrane-bound SOD activities were measured (Table 3). The SOD activity assays indicate that there are higher levels of SOD activity in the *ctaDII* strains of *Synechococcus* sp. PCC 7002. Higher SOD activity would allow cells to tolerate the excess superoxide anion generated from the MV added to the media. Thus, higher levels of SOD activity partially explain the tolerance of the *ctaDII* strains exhibit in the presence of MV.

The phenotype of oxidative stress tolerance seen in the *ctaDII* strains may be due to an interruption in a signaling pathway in which the secondary cytochrome oxidase is involved. If the CtaDII enzyme is somehow involved in the down-regulation of SOD, the absence of CtaDII in the *ctaDII* strains could result in cells that contain higher basal levels of the SOD responsible for dealing with oxidative stress. It has been shown here that the *ctaDII* strains have increased levels of membrane bound SOD activity, but that the levels of other enzymes associated with oxidative stress (catalase and peroxidase) were relatively unaffected by this mutation. The use of an oxidase as a signaling transducer is not unprecedented in bacteria. An aerotaxis transducer has been found in archaeobacteria (Brooun et al. 1998), and in *R. sphaeroides*, the *ccb₃*-type cytochrome is responsible for the repression of photosynthesis gene expression in the presence of oxygen (O'gara et al. 1998; O'gara and Kaplan 1997) and is believed to play a role in aerotaxis signaling (Armitage et al. 1985). It will be interesting to see if there is a similar regulatory pathway regulating the levels of membrane bound SOD activity in *Synechococcus* sp. PCC 7002 and it will also be interesting to examine further the regulation of the soluble SOD activity of *Synechococcus* sp. PCC 7002.

In addition to SOD, catalases, and peroxidases, it was shown that glutathione and glutaredoxins play an important role in *Rhodobacter capsulatus* during the oxidative stress response (Li et al. 2004). It has also been recently shown that peroxiredoxins play an important role in the oxidative stress response in cyanobacteria (Hosoya-Matsuda et al. 2005). Thus, there is a possibility that the observed phenotype in the *ctaDII* strains is caused by changes in the expression of multiple proteins that additively allow survival of oxidative stress. Thus, it remains to be determined whether there are other enzymes involved in the increased survival rate shown by the *ctaDII* strains.

Further physiological characterization of the heme-copper oxidase mutant strains will reveal additional clues as to the functions of these enzymes in vivo and will help

to better define the roles of the heme-copper oxidases in *Synechococcus* sp. PCC 7002. Previous studies using *Chlorella* have suggested that low temperature growth poses similar stresses as growth under high-light stress (Savitch et al. 1996), and it would be interesting to examine the *cta*-deficient strains grown under low temperature conditions. If the CtaCIIDIIIEII enzyme is acting as a redox response regulator, it would be very interesting to find out what its redox partners are and how it regulates the expression of other genes within cyanobacteria. It has been shown that aerobically grown ubiquinol-deficient (Q-deficient) yeast is hypersensitive to the addition of linolenic acid and that ubiquinone (QH₂) may inhibit oxidative damage (Do et al. 1996). However, strains lacking SOD and Q were no more sensitive than strains that were Q-deficient alone (Do et al. 1996). Perhaps cyanobacteria are more reliant on SOD for dealing with oxidative damage. Future studies to examine oxidative stress in cyanobacteria are underway and will allow us to better characterize the high-light and oxidative stress responses of cyanobacterial cells.

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