Quinol and Cytochrome Oxidases in the Cyanobacterium *Synechocystis* sp. PCC 6803[†]

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ABSTRACT: The genome of *Synechocystis* sp. PCC 6803 contains three sets of genes for terminal respiratory oxidases: the previously identified cytochrome aa_3-type cytochrome *c* oxidase (CtaI), a second putative oxidase (CtaII) that we interpret to be a cytochrome *bo*-type quinol oxidase, and a putative cytochrome *bd* quinol oxidase (Cyd). Genes for the two putative oxidases were cloned, and deletion constructs were made. Strains that lack one, two, or all three of the oxidases were generated. Deletion of the respiratory oxidases had no effect on photoautotrophic or photomixotrophic growth. Strains that lack one oxidase respire at near-wild-type rates, whereas those that lack both CtaI and Cyd do not respire. Thus, CtaII does not play a significant role in cellular metabolism under the conditions tested. An expression construct containing *cydAB* from *Synechocystis* sp. PCC 6803 was able to restore aerobic growth in a strain of *Escherichia coli* that lacks the cytochrome *bo* oxidase and the cytochrome *bd* oxidase encoded by *cydAB*. These results show that the *cydAB* operon from *Synechocystis* sp. PCC 6803 encodes a functional quinol oxidase. Deletion of Cyd and/or CtaII in strains lacking photosystem I did not change the fluorescence decay kinetics after illumination, and therefore, these oxidases do not significantly utilize reducing equivalents in the thylakoid membrane. This, combined with our inability to delete CtaI from strains lacking photosystem I, suggests that CtaI is the major oxidase on the thylakoid membrane and that Cyd is localized mostly on the cytoplasmic membrane. Transcripts for *ctaDI* were detected under all growth conditions tested, while transcripts for *cydA* and *ctaEII* could only be detected in cells grown at low light intensity (5 μE m^{-2} s^{-1}).

Cyanobacteria are photosynthetic prokaryotes that contain complete electron transport chains on both the thylakoid and cytoplasmic membranes. The thylakoid membrane is utilized for both photosynthetic and respiratory electron transport, while the cytoplasmic membrane only contains a respiratory electron transport chain but no photosynthetic reaction centers. On the thylakoid membrane, the photosynthetic electron transport chain and respiratory electron transport to the aa_3-type cytochrome *c* oxidase share the plastoquinone pool, the cytochrome b_6f complex, and the soluble electron carriers (see ref *1* for a review).

All aerobic bacterial species examined in detail have multiple respiratory oxidases (*2*, *3*) allowing the cells to customize their respiratory needs to meet changing environmental conditions. Initial spectroscopic (*4*), inhibitor (*5*), and EPR studies (*6*) in cyanobacteria indicated that their respiratory chains contained an aa_3-type cytochrome *c* oxidase. Furthermore, immunological studies have shown that the cyanobacteria contain polypeptides that cross-react with antibodies raised against subunits I and II of the cytochrome aa_3-type cytochrome *c* oxidase from *Paracoccus denitrificans* (*5*, *7*−*9*). A gene cluster encoding subunits I, II, and III of the aa_3-type cytochrome *c* oxidase has been cloned from *Synechocystis* sp. PCC 6803 (*10*, *11*). The gene cluster consists of *coxBAC* (*slr1136*, *slr1137*, and *slr1138* according to CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html) numbering of *Synechocystis* sp. PCC 6803 open reading frames), encoding subunits II, I, and III of the oxidase, respectively. The gene cluster has since been renamed *ctaCIDIEI* to conform with standard nomenclature (see ref *12* for a review). A gene cluster encoding these genes in the same order has also been cloned from *Synechococcus vulcanus* (*13*).

Schmetterer and co-workers (*11*) deleted most of *ctaDI* and about half of *ctaEI* to produce a strain of *Synechocystis* sp. PCC 6803 that was homozygous for the deletion. The resultant strain was viable under both photoautotrophic and photomixotrophic conditions. The strain respired at near wild-type rates, and this respiration was cyanide sensitive (*11*). Isolated membranes from the mutant were unable to oxidize reduced horse heart cytochrome *c*, suggesting that the respiration was not caused by an aa_3-type cytochrome *c* oxidase (*11*). This was the first conclusive evidence for a second cyanide-sensitive terminal respiratory oxidase in cyanobacteria.

The entire genome of *Synechocystis* sp. PCC 6803 has been sequenced (*14*). As will be presented in the Results section, analysis of the sequence indicated genes for two additional putative terminal respiratory oxidases, a quinol oxidase of the cytochrome *bd*-type (Cyd) and an oxidase that most resembles a second cytochrome aa_3-type cytochrome *c* oxidase (CtaII), in addition to the known aa_3-type cyto-

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Table 1: Sequence Identity (%) of CydA and CydB from *Synechocystis* sp. PCC 6803 with Homologs from Other Organisms

organism	subunit	
	CydA	CydB
Escherichia coli	35	27
Bacillus subtilis	37	29
Salmonella typhimurium	33	27
Azobacter vinelandii	33	26

chrome *c* oxidase gene cluster (*14*). In this paper the expression of these putative genes and the apparent activity of the two alternate oxidase complexes is investigated through the use of deletion mutagenesis, and the identity of CtaII is evaluated.

EXPERIMENTAL PROCEDURES

Synechocystis sp. PCC 6803 was cultivated at 30 °C in modified BG-11 medium buffered with 10 mM TES−NaOH (pH 8.0) (*15*). The BG-11 modification consisted of partial substitution of $NaNO_3$ with an equal concentration of NH_4NO_3 (final concentration of ammonia was 4.5 mM). For photomixotrophic growth the medium was supplemented with 5 mM glucose. For growth on plates, 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate were added, and BG-11 was supplemented with antibiotics appropriate for the particular strain (25 μg mL^{-1} kanamycin, 25 μg mL^{-1} erythromycin, 25 μg mL^{-1} spectinomycin, and/or 35 μg mL^{-1} chloramphenicol). Cultures were grown under normal illumination (50 μE m^{-2} s^{-1}). To obtain low-light cultures, cells were grown photomixotrophically at normal illumination (50 μE m^{-2} s^{-1}) in the presence of 15 mM glucose until midexponential phase was reached ($OD_{730} \sim 0.6$) and then diluted to an OD_{730} of approximately 0.2 and transferred to 5 μE m^{-2} s^{-1} of illumination for 48 h before cells were harvested. Light-activated heterotrophic growth (LAHG)[1] was performed according to ref *16*, with the cultures grown up photomixotrophically (with 15 mM glucose) at 50 μE m^{-2} s^{-1} before dilution followed by transfer to LAHG conditions for 48 h (15 min of light per day at 20 μE m^{-2} s^{-1}, dark otherwise) prior to harvesting.

DNA from *Synechocystis* sp. PCC 6803 was prepared essentially as described in ref *17*. After restriction digestion of genomic DNA the fragments were separated by agarose gel electrophoresis and transferred to GeneScreen Plus (Du Pont NEN) according to the manufacturer's instructions. RNA was isolated according to ref *18*. The RNA was separated by electrophoresis on formaldehyde gels containing 1.2% agarose and transferred to GeneScreen Plus according to the manufacturer's instructions. Probes for both Northern and Southern blots were prepared by hot PCR with ^{32}P dATP using the cloned genes as the template. For Northern analysis the same membrane was probed with all four probes used. Between each hybridization the membrane was stripped by incubating in a solution of boiling 1% SDS and was then verified to be nonradioactive before reuse with another probe. RT PCR was performed using a Gene Amp EZ *rTth* RNA PCR kit from Perkin-Elmer. A 0.5 μg amount of total RNA was used as the template for each reaction. Reverse transcription was performed according to the manufacturer's instructions.

Table 2: Sequence Identity (%) of the Putative CtaCII, CtaDII, and CtaEII Polypeptides from *Synechocystis* sp. PCC 6803 with Appropriate Counterparts from Known Cytochrome *aa*$_3$-Type Cytochrome Oxidases (Cta) and a Cytochrome *bo*-Type Quinol Oxidase (Cyo)

organism	subunit		
	CtaCII	CtaDII	CtaEII
Synechocystis sp. PCC 6803, CtaI	31	54	39
Paracoccus denitrificans, Cta	25	41	33
Saccharomyces cerevisiae, Cta	24	39	27
Bacillus subtilis, Cta	27	43	36
Escherichia coli, Cyo	21	38	32

Oxygen consumption measurements were carried out in a manner similar to that described previously for oxygen uptake (*19*). Chlorophyll *a* concentrations were determined according to ref *20*. Fluorescence decay measurements were performed as described in ref *19*.

RESULTS

Search for Oxidase-Type ORFs in the Synechocystis sp. PCC 6803 Genome. When searching the *Synechocystis* sp. PCC 6803 genome sequence for sequences similar to those of known quinol or cytochrome oxidase genes, we identified three sets of genes. One was the known *ctaCIDIEI* cluster, and the other two appeared to code for other oxidases. One of these, composed of adjacent open reading frames *slr1379* and *slr1380*, is most similar to *cydA* and *cydB* genes, respectively, encoding cytochrome *bd*-type quinol oxidases in a variety of prokaryotes (Table 1). The third set of genes consists of three open reading frames, of which two (*ctaDII* and *ctaEII*; *slr2082* and *slr2083*)) are clustered and the third (*ctaCII*; *sll0813*) is encoded elsewhere in the genome. Inhibitor studies on mutants of *Synechocystis* sp. PCC 6803 lacking soluble electron carriers and/or the *aa*$_3$-type cytochrome *c* oxidase have indicated that these two additional putative oxidases may be functional (*21*). The proteins encoded by the *ctaII* genes are most similar to subunits of *aa*$_3$-type cytochrome *c* oxidases from other organisms, with CtaCII, CtaDII, and CtaEII being most similar to subunits II, I, and III, respectively (Table 2). However, as will be argued in the Discussion section, critical residues that are involved in binding Mg^{2+} and Cu_A in cytochrome *c* oxidases are absent in this cluster. A strong similarity is found as well between the CtaII components and cytochrome *bo*-type quinol oxidases, in which the Mg^{2+} and Cu_A sites are missing. Therefore, as will be presented in more detail in the Discussion section, we view *slr2082*, *slr2083*, and *sll0813* to code for a quinol oxidase.

Insertional Deletion of the Genes for the Putative Oxidases. The putative *cydAB* operon was amplified using the polymerase chain reaction (PCR) and cloned in pUC18 using restriction sites that had been engineered into the primers. The primers 5′ cydAB and 3′ cydAB are shown in Table 3. A 1.9 kb *Dra*I- *Avr*II fragment from the *cydAB* coding region (nucleotides numbered 673 845−675 736 according to

[1] Abbreviations: DBMIB, dibromothymoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HQNO, 2-heptyl-4-hydroxyquinoline; LAHG, light-activated heterotrophic growth; LL, photomixotrophic growth at low light; PA, photoautotrophic growth; PM, photomixotrophic growth; PS I, photosystem I; PS II, photosystem II.

Table 3: Sequence of Primers Used in This Study, with Their Relative Positions within the Genome of *Synechocystis* sp. PCC 6803[a]

primer	sequence	position
5′ cydAB	5′ TCA ATC CAG AAt tcG TTT ACG ATC GCC 3′	673 503−673 529
3′ cydAB	5′ TAT CGC TGT Tgc aTG CAA CGA ACT CCC 3′	676 354−676 380
5′ cydAP	5′ GTC TCA GCC AGC ATG GGG CGG 3′	673 652−673 672
3′ cydAP/RP	5′ CCA GTT GGG GTT TAA GTT CCA AAA TG 3′	674 782−674 807
5′ cydAPR	5′ TTA CAG ACC CCC GCT GGG GGC 3′	674 351−674 371
5′ cydex	5′ TAC AGA TGC AGa ATT cTT TGA GTA ACA CAG 3′	673 884−673 913
5′ ctaDEII	5′ CTA TCG TCg GtA CCT GGA TCA GC 3′	1 540 405−1 540 427
3′ ctaDEII	5′ TAG CAG GAA gCT tGC TCC ACT CCC 3′	1 543 335−1 543 358
5′ ctaDIIP	5′ CCC TTT CAA CGC CCC TGG CAC G 3′	1 540 677−1 540 698
3′ ctaDIIP	5′ GGT GTT ATT GAC GTG CAA ATC GAA GG 3′	1 541 776−1 541 801
5′ ctaEIIPR	5′ GCC GAC GGA AGA ACA GGA ACC GG 3′	1 542 373−1 542 395
3′ ctaEIIPR	5′ GTG GGG CTG GAG GGA GCG CC 3′	1 542 807−1 542 826
5′ ctaDIPR	5′ CCG GCA GGG GCG GCC TTT GCC 3′	792 064−792 084
3′ ctaDIPR	5′ ACC ACC GGG TCT CCC CCC CCC 3′	792 507−792 527
5′ rps1PR	5′ GCT CTG ATT GAC ATT GGG GCG 3′	1 097 212−1 097 232
3′ rps1PR	5′ ATG TGG TCG TGG GAA ATC TCA G 3′	1 097 750−1 097 771

[a] Nucleotides in lower case represent changes made to the sequence to introduce restriction sites used for cloning. Numbering for the position of the primers is according to CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html). Primers whose names end in P were used to make probes for Southern analysis, while those whose names end in PR were used to make probes for Northern analysis. P/PR indicates the primer was used for both Northern and Southern analysis probes. ex indicates the primer was used to make an expression construct.

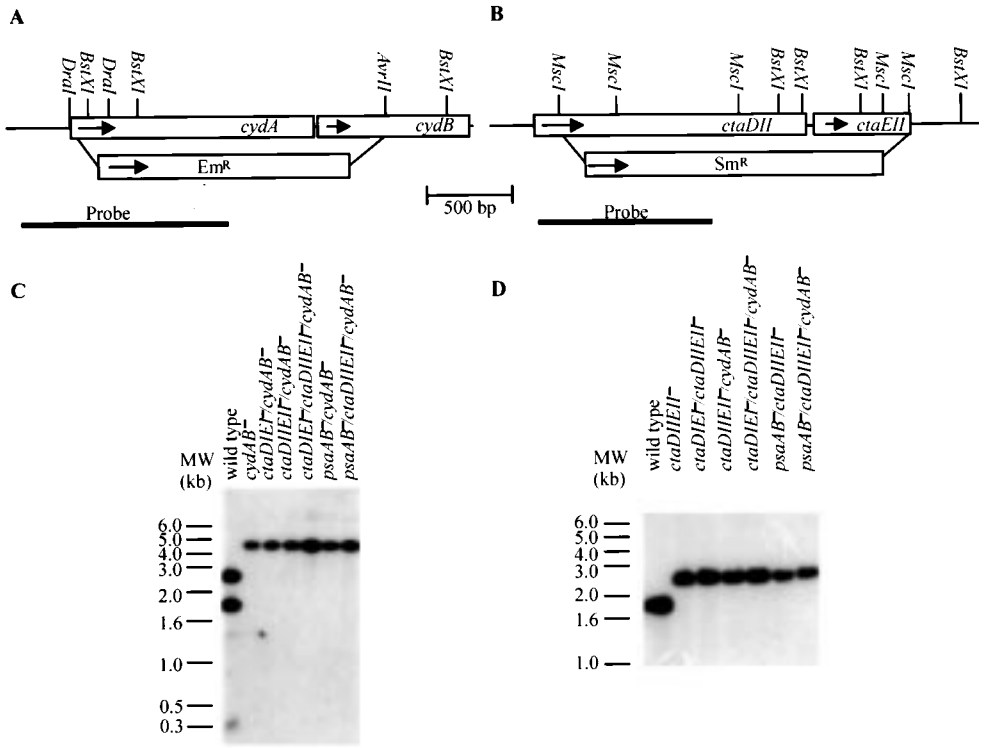

FIGURE 1: Southern blot analysis of the *cydAB*$^-$ and *ctaDIIEII*$^-$ strains. Upper part: Schematic representation of the *cydAB* (A) and *ctaDIIEII* (B) clones and deletion constructs. *Bst*XI sites 1100 bp to the 5′ end of the *cydAB* clone and 90 bp to the 5′ end of the *ctaDIIEII* clone are not shown. Arrows indicate the direction of transcription of the genes. Em^R = erythromycin resistance gene; Sm^R = spectinomycin resistance gene. The scale for (A) and (B) is identical. Lower part: Southern blot analysis of the *cydAB*$^-$ (C) and *ctaDIIEII*$^-$ (D) strains. Genomic DNA was digested with *Bst*XI and probed with the ^{32}P labeled fragment shown in (A) and (B), respectively. Lanes were loaded with genomic DNA from strains as indicated in the figure.

CyanoBase) was deleted and replaced by an erythromycin resistance cassette from pRL425 (*22*) (Figure 1a). Wild type *Synechocystis* sp. PCC 6803 and strains lacking either *ctaDI* and half of *ctaEI* (coding for components of the aa_3-type cytochrome oxidase) (*11*) or part of the *psaAB* operon coding for the photosystem I (PS I) reaction center (*23*) were transformed with this construct, and transformants were selected for on plates containing erythromycin. Transformants were subcultured at increasing erythromycin concentrations to allow segregation of wild type and mutant genome copies to occur and thus to obtain a homozygous genotype, resulting in strains that lacked the putative cytochrome *bd* oxidase (designated *cydAB*$^-$).

PCR was also used to amplify a putative *ctaDIIEII* operon, encoding two of the subunits of the second putative terminal respiratory oxidase. The PCR product was cloned into pUC19 using restriction sites that had been engineered into the primers. The primers 5′ ctaDEII and 3′ ctaDEII are shown in Table 3. A 2.1 kb *Msc*I-*Msc*I fragment (nucleotides 1 540 788−1 542 925; see CyanoBase) from the putative coding region was deleted and replaced by a spectinomycin resistance cassette from pHP45Ω (*24*) (Figure 1b). Wild type,

Table 4: Oxygen Consumption of Photomixotrophically Grown *Synechocystis* sp. PCC 6803 Strains in Darkness[a]

strain	oxygen uptake (μmol of O_2 (mg of chl)$^{-1}$ h^{-1})	
	no additions	+KCN
wild type	38 ± 9.0	2.5 ± 1.6
ctaDIEI$^-$	44 ± 21	2.2 ± 2.4
ctaDIIEII$^-$	42 ± 6.6	0.5 ± 0.7
cydAB$^-$	36 ± 14	1.1 ± 1.3
ctaDIEI$^-$/*ctaDIIEII*$^-$	37 ± 14	0.5 ± 1.0
ctaDIEI$^-$/*cydAB*$^-$	6.5 ± 2.8	5.0 ± 3.1
ctaDIIEII$^-$/*cydAB*$^-$	24 ± 12	0.3 + 0.6
ctaDIEI$^-$/*ctaDIIEII*$^-$/*cydAB*$^-$	8.7 ± 3.0	6.7 ± 2.6

[a] Values given are the averages from 3 separate determinations. Respiratory rates after harvesting were measured in darkness using an oxygraph. Intact cells that were resuspended in 10 mM HEPES−NaOH (pH 7.4) were used for this assay. Where indicated, KCN was added to a final concentration of 1 mM.

CtaI-less and PS I-less strains of *Synechocystis* sp. PCC 6803 were transformed with this construct, as were strains that had previously been transformed with the *cydAB*$^-$ construct, to give the appropriate double and triple oxidase mutants. Transformants were selected by means of spectinomycin resistance and subcultured to allow segregation to occur. The resultant strains were designated *ctaDIIEII*$^-$.

Southern analysis was used to determine if the strains created were homozygous for the *cydAB* and *ctaDIIEII* deletions. As shown in Figure 1c, all *cydAB*$^-$ strains were homozygous for the deletion, with the probe hybridizing to 2.6, 1.7, and 0.3 kb *Bst*XI fragments in wild type (Figure 1c) and only to a 4.3 kb *Bst*XI fragment in all of the *cydAB*$^-$ strains (Figure 1c). Southern analysis also showed that all the *ctaDIIEII*$^-$ strains were homozygous for the deletion, with the probe hybridizing to a 1.8 kb *Bst*XI fragment in wild type (Figure 1d) and to a 2.5 kb *Bst*XI fragment in all of the *ctaDIIEII*$^-$ strains (Figure 1d).

Growth Analysis of the Oxidase Minus Strains. Under both photoautotrophic and photomixotrophic conditions the doubling times of the mutant strains that had been created were essentially indistinguishable from their respective background strains. In the wild type background doubling times were between 9 and 10 h when grown under both photoautotrophic and photomixotrophic conditions (data not shown). In the PS I-less background the doubling times of the strains created were between 18 and 19 h (data not shown). This indicates that under these conditions respiratory activity does not add significantly to the growth rate of the cells.

Table 4 shows the respiratory rates of whole cells of wild type and the mutant strains when grown under normal illumination (50 μE m^{-2} s^{-1}). Strains in which either CtaI or only one of the putative oxidases had been deleted respired at near wild type rates. In these strains, oxygen uptake was almost completely blocked by KCN addition, as is the case in wild type. The *ctaDIEI*$^-$/*ctaDIIEII*$^-$ and *ctaDIIEII*$^-$/*cydAB*$^-$ strains also respired at essentially wild-type rates, and this respiration was KCN sensitive as well. In contrast, the *ctaDIEI*$^-$/*cydAB*$^-$ and *ctaDIEI*$^-$/*ctaDIIEII*$^-$/*cydAB*$^-$ strains showed very low rates of oxygen uptake which were not sensitive to KCN, suggesting that this oxygen uptake is not due to a respiratory process.

The apparent lack of respiration in the *ctaDIEI*$^-$/*cydAB*$^-$ strain suggests that under the growth conditions used CtaII is either not expressed or not functional. Therefore, rates of oxygen uptake in this strain were determined under different growth conditions and compared to the corresponding rates in wild type and the *ctaDIEI*$^-$/*ctaDIIEII*$^-$/*cydAB*$^-$ strains. When grown at low light intensity (5 μE m^{-2} s^{-1}) or LAHG conditions, all three strains consumed oxygen at rates similar to those seen when the respective strains were grown at normal illumination intensity (data not shown). In addition, under both of these conditions the remaining, very small rate of oxygen uptake in the *ctaDIEI*$^-$/*cydAB*$^-$ and *ctaDIEI*$^-$/*ctaDIIEII*$^-$/*cydAB*$^-$ strains was KCN-insensitive (data not shown), again suggesting that CtaII activity is very low or nonexistent in *Synechocystis* sp. PCC 6803.

Variable Fluorescence Decay. The data presented above suggest that the *cydAB* cluster encodes a functional oxidase complex supporting a significant rate of respiration, at least in the absence of CtaI. An important question is where this oxidase may be located. If present in thylakoids, one expects that it will be able to accept electrons from photosystem II (PS II). If present exclusively in the cytoplasmic membrane, this will not be the case. The oxidation state of Q_A, the first electron-accepting plastoquinone in PS II, can be monitored through chlorophyll fluorescence levels; Q_A quenches chlorophyll fluorescence, whereas the reduced Q_A^- does not. By this method it has been shown that in the absence of PS I a KCN-sensitive pathway exists to accept electrons from PS II (*19*). If Cyd is present in the thylakoid membrane, the decay of chlorophyll fluorescence measured after illumination of PS I-less strains is expected to be dependent on the presence of *cydAB*. For this reason, Q_A^- decay was measured after a second of illumination. The half-time of fluorescence decay in PS I-less strains is approximately 40 ms regardless of the presence of Cyd (data not shown). In the presence of 1 mM KCN the half-time is 1 order of magnitude slower, approximately 550 ms in all strains (data not shown), and reflects the kinetics of charge recombination between Q_A^- and the water splitting system of photosystem II, indicating a full reduction of the quinone pool and therefore a full inhibition of the oxidase. Half-times for the PS I-less *cydAB*$^-$/*ctaDIIEII*$^-$ strain are similar to those of the PS I-less strain indicating that Cyd and CtaII are either not present on the thylakoid membrane or do not play a significant role in accepting reducing equivalents from electron transport chains in thylakoids.

In line with the reasoning that the *cyd* gene products are not involved in electron-transfer processes in the thylakoid membrane, we have been unable to delete the *ctaDIEI* genes from PS I-less strains: the transformants did not segregate (not shown), suggesting that the simultaneous deletion of PS I and CtaI is lethal. This implies that CtaI is the major respiratory oxidase on the thylakoid membrane and that in the absence of PS I this oxidase is the sole sink for electrons generated by PS II. If both PS I and CtaI would be lacking, the plastoquinone pool would be greatly overreduced, and this is thought to be a lethal situation (see refs *19* and *23*). As Cyd activity is considerable (Table 4), this implies that Cyd is not located in the thylakoids.

Functional Complementation of Escherichia coli with Cyd. To investigate whether *cydAB* of *Synechocystis* sp. PCC 6803 indeed coded for a quinol oxidase, an expression construct was created by amplifying the *cydAB* operon by PCR using the primers 5′cydex and 3′cydAB (Table 3). The PCR

product was cloned into pUC18 using the restriction sites engineered into the primers. The *Eco*RI site in 5′ cydex was designed such that the reading frame of *cydA* was in frame with that of *lacZ*. This resulted in the replacement of the first three amino acids of CydA (MQD) with the sequence MTMITN. The resulting plasmid was transformed into *E. coli* strain GO105 (*cyo*, Δ*cyd*, *recA)* (*25*), which lacks the cytochrome *bo* oxidase as well as the cytochrome *bd* oxidase encoded by *cydAB*. Strain GO105 is unable to grow aerobically (*25*). As controls, the GO105 strain was also transformed with pUC18 and with the clone of the *cydAB* operon containing the upstream region (371 bp upstream of the start of *cydA*) from *Synechocystis* sp. PCC 6803 (described above). Ampicillin-resistant transformants were selected under anaerobic conditions, and plasmid minipreps were performed to verify that the strains contained the correct plasmid. Samples from each of the three strains were grown to midexponential phase under anaerobic conditions and then plated and incubated aerobically at 37 °C. Only the cell strain that contained the expression construct was able to grow aerobically whereas control strains did not (data not shown). These results show that the *cydAB* operon from *Synechocystis* sp. PCC 6803 encodes a functional quinol oxidase. As *E. coli* lacks plastoquinone and contains ubiquinone as its major quinone component, these data also show that Cyd from *Synechocystis* sp. PCC 6803, which organism lacks ubiquinone and has plastoquinone as its major quinone component, is able to utilize other quinones besides plastoquinone as its electron donor as *E. coli* contains ubiquinone and menaquinone but not plastoquinone (*2*).

Analysis of the Expression Patterns of the Oxidases. To help understand the regulation of the three oxidases, the expression patterns of representative genes were investigated under different growth conditions in the various strains that had been created. Probes were made to regions of *ctaDI*, *ctaEII*, and *cydA* that were entirely within the region that was deleted in the corresponding deletion strains. As the level of rRNA may not accurately reflect the level of total mRNA under many different conditions, a control blot was performed using *rps1* as the probe. The *rps1* gene, encoding subunit one of the small ribosomal subunit, is expressed at moderate levels and is a good indicator of the amount of mRNA (*26*). As shown in Figure 2, the RNA was stable and does not show signs of degradation, except in some of the LAHG samples, particularly that of the *ctaDIEI*$^-$*/cydAB*$^-$ strain. This is understandable as strains that lack CtaI are unable to grow under LAHG conditions (*11*), and these strains therefore are very much stressed under these conditions. However, even though upon loading of similar RNA concentrations the level of rRNA is fairly constant for the different strains, the level of *rps1* transcript is variable depending on the strains and the growth conditions. In particular in *ctaDIEI*$^-$ strains *rps1* transcript levels are low indicating that these cells are stressed. This control blot indicates that total RNA and rRNA levels may not accurately reflect total transcript levels.

Transcripts that hybridized to the *ctaDI* probe were detected in all *ctaI* containing strains, grown under all four conditions tested. Under all conditions the pattern of hybridization resembled that seen when the cells were grown photomixotrophically at low light intensity (Figure 3, panel A), with a transcript of 2.7 kb in all CtaI-containing strains.

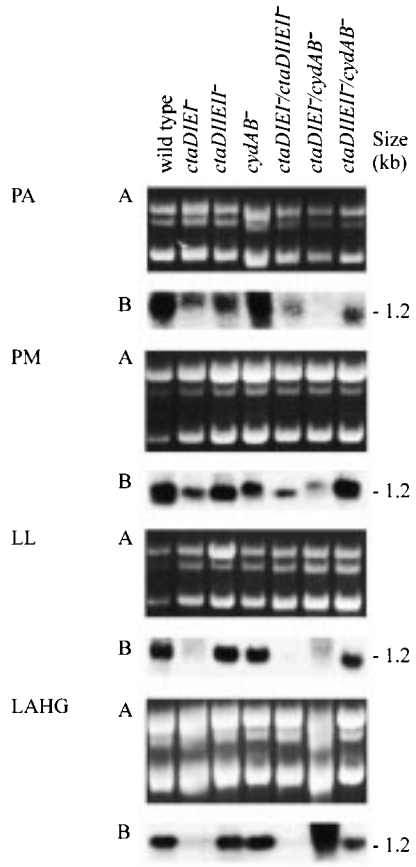

FIGURE 2: Total RNA and *rps1* transcript analysis. Total RNA was isolated from each strain grown under the four different growth conditions (PA, photoautotrophic; PM, photomixotrophic; LL, photomixotrophically at low light intensity (5 μE m^{-2} s^{-1}); LAHG, light-activated heterotrophic growth). Cells were grown as described in the Experimental Procedures. All cultures from the same growth condition were grown in parallel, subcultured to the same OD_{730}, and harvested at the same time. An amount of 5 μg of total RNA was loaded in each lane. rRNA is shown in panel A for each growth condition. The RNA was transferred to GeneScreen Plus membranes and then probed with *rps1* for each growth condition (panel B).

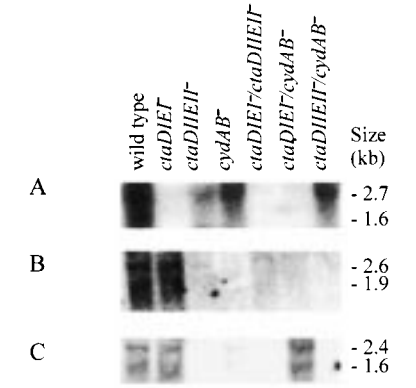

FIGURE 3: Transcript analysis of *ctaDI*, *cydA*, and *ctaEII* in low-light grown cultures. The membrane probed with *rps1* (Figure 2) was stripped and then reprobed with *ctaDI* (panel A), *cydA* (panel B), and *ctaEII* (panel C), respectively. The membrane was verified to be nonradioactive between two subsequent probings.

When grown under low light an apparent transcript of 1.6 kb was also seen in wild type (Figure 3, panel A). Transcripts for *cydA* (2.6 and 1.9 kb) were present in wild type and the *ctaDIEI*$^-$ strains and at barely detectable levels (which could not be reproduced on the photograph) in the *ctaDIIEII*$^-$ and *ctaDIEI*$^-$*/ctaDIIEII*$^-$ strains when grown at low light inten-

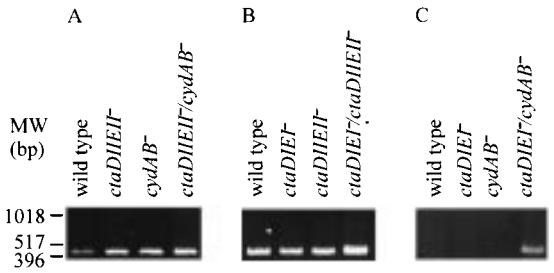

FIGURE 4: RT PCR of *ctaDI*, *cydA*, and *ctaEII* transcripts isolated from low-light grown cultures. An amount of 0.5 μg of total RNA was used as the template. RT PCR for *ctaDI*, *cydA*, and *ctaEII* are shown in panels A−C, respectively. The scales for panels A−C are identical.

sity (Figure 3, panel B). Probing with *ctaEII* showed transcripts with apparent sizes of 2.4 and 1.6 kb that were present in all *ctaDIIEII*-containing strains when grown at low light intensity (Figure 3, panel C). No transcripts for *cydA* or *ctaEII* could be detected by Northern analysis in cells grown photoautotrophically, photomixotrophically, or under LAHG conditions (data not shown).

To confirm that the putative transcripts seen were not due to nonspecific hybridization of the probes, RT PCR was performed on RNA isolated from cultures grown at low light. The same primers that were used to make the probes for Northern analysis were used for the RT PCR. Prior to performance of the RT PCR, all RNA samples were treated with DNase, and then standard PCR was performed at least twice on all samples to confirm that they were DNA free. No PCR products were seen in these controls indicating that the samples were free of DNA (data not shown). As can be seen in Figure 4 transcripts for *cydA* and *ctaDI* were present in all *cydA*- and *ctaDI*-containing strains. However, a transcript for *ctaEII* was only present in the *ctaDIEI*$^-$/*cydAB*$^-$ and not in strains which contained one or both of the putative oxidases encoded by these genes.

DISCUSSION

Terminal Oxidases Functionally Present in Synechocystis sp. PCC 6803. Synechocystis sp. PCC 6803 has been shown to respire at wild-type rates in the absence of the aa_3-type cytochrome *c* oxidase, which indicated the presence of another respiratory oxidase (*11*). Deletion of *cydAB* in the *ctaDIEI*$^-$ strain resulted in a strain that did not respire when grown under any of the conditions tested, and deletion of *ctaDIIEII* in the *ctaDIEI*$^-$ strain had no effect on respiration (Table 4). These data indicate that the respiration seen in the *ctaDIEI*$^-$ strains occurred via Cyd and suggest that CtaII did not accumulate or was inactive under the conditions tested. Therefore, *Synechocystis* sp. PCC 6803 appears to have only two functional terminal oxidases under laboratory conditions, CtaI and Cyd, and both are dispensable for growth in the light, regardless of the presence of a fixed-carbon source.

cydAB Encodes a Quinol Oxidase on the Cytoplasmic Membrane. Alignment of CydA and CydB from *Synechocystis* sp. PCC 6803 with the respective subunits of the cytochrome *bd*-containing quinol oxidase from other organisms revealed that the three putative heme ligands (His19, His186, and Met393 in CydA from *E. coli*) (see ref *27* for a review) and nine of the eleven residues of the putative quinone binding domain (Q-loop) in CydA from *E. coli* (*28*) are conserved. The two differences in the Q-loop from *Synechocystis* sp. PCC 6803 as compared to *E. coli's* are also not conserved in CydA from other organisms. Interestingly, CydA from *Synechocystis* sp. PCC 6803 has a deletion of 61 amino acid residues in the loop between transmembrane helices V and VI when compared to CydA from *E. coli*. A similar deletion is also present in the putative CydA from *Bacillus subtilis*.

An expression construct containing the *cydA* gene in frame with *lacZ* from pUC18 followed by *cydB* was able to restore aerobic growth of *E. coli* strain GO105, which lacks the cytochrome *bo* oxidase and the cytochrome *bd* oxidase encoded by *cydAB*. It is unknown if strain GO105 contains a functional *app* locus which encodes a second cytochrome *bd* oxidase (*29*, *30*). However, the negative controls for the functional complementation did not show any aerobic growth in *E. coli* strain GO105. This indicates that the restoration of aerobic growth in the strain containing the expression construct was due to the presence of the expression construct and not to activity related to the *app* locus. This, combined with (1) the observation that the respiratory rates of the *ctaDIEI*$^-$/*ctaDIIEII*$^-$ strain are virtually identical to that of the wild type and (2) the striking similarity of *cydAB* of *Synechocystis* sp. PCC 6803 with that of cytochrome *bd*-containing quinol oxidases from other organisms confirms that *Synechocystis* sp. PCC 6803 contains a functional quinol oxidase probably of the cytochrome *bd* type.

Cyd appears to be localized predominantly on the cytoplasmic membrane of *Synechocystis* sp. PCC 6803: (1) Deletion of *cydAB* from a PS I-less strain did not change the reoxidation kinetics of Q_A^-, as measured by chlorophyll fluorescence, as compared to the PS I-less control strain. This indicates that Cyd does not contribute significantly to oxidation of plastoquinone in the thylakoid membrane. (2) In a PS I-less strain, *cydAB* is easily deleted without an apparent phenotype, whereas *ctaI* genes cannot be deleted in this background. Therefore, Cyd cannot effectively utilize reducing equivalents in the thylakoid membrane, and we propose that Cyd is located on the cytoplasmic membrane. A proposed electron transport scheme for the cytoplasmic and thylakoid membranes is shown in Figure 5.

Our conclusion that Cyd is absent from the thylakoid membrane appears to contradict a recent proposal that respiration on the thylakoid membrane in photoautotrophically grown *Synechocystis* sp. PCC 6803 does not involve the cytochrome b_6f complex (*31*). However, the interpretation in ref *31* was based on the fact that dibromothymoquinone (DBMIB), an inhibitor of the cytochrome b_6f complex, inhibited photosynthesis but not respiration. However, the respiratory rates reported in ref *31* were 1 order of magnitude lower than the ones in our study and, therefore, were not limited by the amount of cytochrome b_6f. The inhibition of photosynthetic electron transport by DBMIB reported by Endo (*31*) was not complete, and the remaining flux through the cytochrome b_6f complex to CtaI is expected to be more than sufficient to account for the respiratory rates observed in that study.

Activity and Role of CtaII. Surprisingly, we found no evidence for the functional presence of CtaII under the conditions of our experiments. The global sequence analysis of CtaII suggested this to be a cytochrome aa_3-type cytochrome *c* oxidase, as such oxidases scored best in sequence

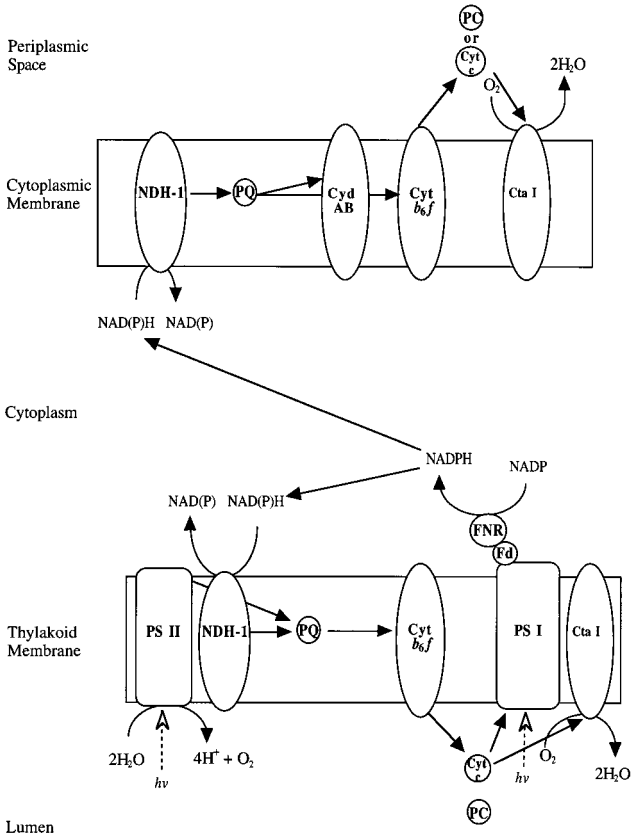

FIGURE 5: Proposed electron transport chains in the thylakoid and cytoplasmic membranes of *Synechocystis* sp. PCC 6803. Abbreviations: Cta I, cytochrome aa_3 oxidase; CydAB, proposed cytochrome *bd* oxidase; Cyt b_6f, cytochrome b_6f complex; Cyt c, cytochrome *c*; Fd, ferredoxin; FNR, Ferredoxin NADP reductase; NDH-1, type-1 NAD(P)H dehydrogenase; PC, plastocyanin; PS I, photosystem I; PS II, photosystem II.

similarity searches. However, closer analysis of the primary structure of CtaCII, CtaDII, and CtaEII reveals that these subunits are more likely to be part of a quinol oxidase, which is a member of the same heme copper oxidase super family (*3*, *32*) but lacks a Cu_A and Mg^{2+} binding site. As can be seen in Figure 6a, in CtaCII four of five residues that serve as ligands for Cu_A are not conserved. The sixth ligand to Cu_A is a histidine located 35 amino acids upstream of the first conserved cysteine and is conserved in CtaCII. The three residues thought to bind Mg^{2+}, the residue marked with an asterisk in Figure 6a and the two boxed residues in Figure 6b, are not conserved in CtaII.

Alignment of CtaCII, CtaDII, and CtaEII with the proteins encoded by the *cco* operon from *Paracoccus denitrificans* (encoding this organism's cytochrome cbb_3-containing cytochrome *c* oxidase) shows 23% identity between CtaDII and CcoN. However, CtaCII and CtaEII are not similar to any of the other Cco subunits, and therefore, it is unlikely that CtaII is a cytochrome cbb_3-containing cytochrome *c* oxidase. However, when aligned with the proteins encoded by the *cyo* operon from *E. coli* (encoding this organism's cytochrome *bo*-containing quinol oxidase), CtaCII, CtaDII, and CtaEII showed significant similarity to the corresponding Cyo subunits, CyoA, CyoB, and CyoC (Table 2). No homologues of *E. coli's* CyoD are found in the genome of *Synechocystis* sp. PCC 6803. However, CyoD is only required for assembly of the complex and is not required for its function in vitro (see ref *33* and references therein).

A. Alignment of the Cu_A binding motif from CtaC

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                    *
CtaCI    245 PVICAELCGA YHGGMKSVFY AHTPEEYDDW
CtaCII   230 KLHDSQFSGT YFAVMTAPVV VQSLSDYQAW
B.t.     193 YGQCSEICGS NHSFMPIVLE LVPLKYFEKW
H.s.     193 YGQCSEICGA NHSFMPIVLE LIPLKIFEMG
G.g.     192 YGQCSEICGA NHSYMPIVVE STPLKHFEAW
S.c.     221 YGACSELCGT GHANMPIKIE AVSLPKFLEW
P.d.     213 FGQCSELCGI NHAYMPIVVK AVSQEKYEAW
B.s.     214 FGKCAELCGP SHALMDFKVK TMSAKEFQGW
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B. Alignment of the Mg^{2+} binding motif from CtaD

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CtaDI     371 VPFDIHVHDT YFVVGHFHYV
CtaDII    375 APFDLHVNNT YFVVGHFHYV
B.t.      361 SSLDIVLHDT YYVVAHFHYV
H.s.      361 SSLDIVLHDT YYVVAHFHYV
G.g.      361 SSLDIALHDT YYVVAHFHYV
S.c.      361 ASLDVAFHDT YYVVGHFHYV
P.d.      396 GSLDRVYHDT YYIVAHFHYV
B.s.      368 AAADYQFHDT YFVVAHFHYV
```

FIGURE 6: Alignment of the Cu_A (A) and Mg^{2+} (B) motifs in aa_3-type cytochrome *c* oxidases. Residues serving as ligands to Cu_A and thought to be important for Mg^{2+} binding are boxed. CtaCI and CtaDI are subunits of the functional cytochrome aa_3 oxidase, whereas CtaCII and CtaDII represent subunits of the putative cytochrome *bo* oxidase. *B.t.* = *Bos taurus*, *H.s.* = *Homo sapiens*, *G.g.* = *Gallus gallus; S.c* = *Saccharomyces cerevisiae*, P.d. = *Paracoccus denitrificans*, and B.s. = *Bacillus subtilis*.

Thus, it is possible that in *Synechocystis* sp. PCC 6803 another protein may fulfill this assembly role.

The apparent inactivity of CtaII in this study contrasts with the interpretation of Pils et al. (*21*), who reported functional evidence for all three terminal respiratory oxidases in *Synechocystis* sp. PCC 6803 on the basis of inhibitor studies using strains in which *ctaCIDIEI*, *petE*, *petJ*, and *cytM* (encoding CtaI, plastocyanin, cytochrome c_{553}, and cytochrome c_M, respectively) had been deleted singly or in various combinations. Respiration in these strains was inhibited 100% by KCN while HQNO, an inhibitor of quinol oxidases (*34*), only inhibited 72% of the respiratory rate in the CtaI-less strain. Pils et al. (*21*) reported that HQNO completely inhibited respiration in the CtaI-less/cytochrome c_{553}-less strain, suggesting that the HQNO insensitive respiration in the CtaI-less strain occurred via a pathway involving cytochrome c_{553}. However, this proposed pathway cannot explain the almost complete inhibition of respiration by HQNO in the CtaI-less/plastocyanin-less and CtaI-less/cytochrome c_M-less strains (90% and 97% inhibition, respectively) seen by Pils et al. (*21*). One possible explanation is that HQNO does not completely inhibit quinol oxidases in all cases. This is supported by the finding that the 50 μM concentration of HQNO used *(21)* was insufficient to completely inhibit the cytochrome *bd* oxidase from *E. coli (34)*.

Transcripts. The presence of transcripts for *ctaDI*, *ctaEII*, and *cydA* (Figures 3 and 4) is the first evidence for transcripts for respiratory oxidases in cyanobacteria. The presence of transcripts of 2.7 and 1.6 kb for *ctaDI* in wild-type grown under low light (Figure 3, panel A) suggests that the *ctaI* operon is processed as the 2.7 kb transcript is the expected size for either *ctaCI* and *ctaDI* together, or *ctaDI*, *ctaEI*, and *trxA*, a small open reading frame downstream from *ctaEI*. The size of the 1.6 kb transcript corresponds to that expected for *ctaDI* alone.

When probing with *cydA*, we detected two transcripts (2.6 and 1.9 kb) (Figure 3, panel B). The 2.6 kb transcript is close

to the expected size for a transcript containing both *cydA* and *cydB*, and the 1.9 kb transcript is close to the size expected for *cydA* alone (the coding region is 1.5 kb). The RT PCR only detected a transcript for *ctaEII* in the *ctaDIEI*$^-$/*cydAB*$^-$ strain (Figure 4, panel C) suggesting that the *ctaEII* transcripts seen in Northern analysis (Figure 3, panel C) were due to nonspecific hybridization of the probe.

The *ctaDI* transcript was detected by Northern analysis under all conditions tested, whereas *cydA* transcripts were detected only when cells were grown at low light intensity. These observations suggest that CtaI is the major oxidase in *Synechocystis* sp. PCC 6803 under all growth conditions. This has been confirmed for growth under LAHG conditions, as *ctaDIEI*$^-$ strains are unable to grow under such conditions (*11*). The presence of transcripts for *cydA* under conditions of low light, but not under LAHG conditions, indicates that it may play a role in the change from photosynthetic growth to respiratory growth during light to dark transitions.

The presence of a transcript for *ctaEII* in the *ctaDIEI*$^-$/*cydAB*$^-$ strain when grown under low light intensity (Figure 4, panel C) indicates that CtaII may play a role in cellular metabolism under conditions when CtaI and Cyd are not active. However, respiratory assays in the *ctaDIEI*$^-$/*cydAB*$^-$ strain grown at low light intensity provided no evidence for respiration suggesting that CtaII is not active even at low light. One possible role for CtaII is in growth under anaerobic or microaerobic conditions, as the putative *ctaDIIEII* operon has a structural motif (TTGATCCAGAACAA) centered 61 bp upstream from the putative start codon that only differs by one bp from the consensus sequence of the binding site for the FNR protein (TTGATN$_4$ATCAA). This is an oxygen-responsive transcriptional regulator required for the switch from aerobic to anaerobic metabolism (*35*, *36*). A possible role of CtaII under anaerobic/microaerobic conditions is supported by the finding that cytochrome *o* is present in *Synechocystis* sp. PCC 6803 when grown under microaerobic conditions (1% (v/v) O_2 in N_2) (*37*).

Therefore, even though CtaI is likely to be the major terminal oxidase in *Synechocystis* sp. PCC 6803, this organism contains a quinol oxidase, probably of the cytochrome *bd* type on the basis of homology with other cytochrome *bd* oxidases. This oxidase is functional at least in the absence CtaI and appears to be located predominantly in the cytoplasmic membrane. Even though a transcript for CtaII, presumably a quinol oxidase, was detected under low-light conditions in the *ctaDIEI*$^-$/*cydAB*$^-$ strain, we do not find functional evidence for this oxidase. The function of Cyd and CtaII in vivo remains to be elucidated.

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