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Sll1717 Affects the Redox State of the Plastoquinone Pool by Modulating Quinol Oxidase Activity in Thylakoids

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A *Synechocystis* sp. strain PCC 6803 mutant lacking CtaI, a main subunit of cytochrome *c* oxidase, is not capable of growing at light intensities below 5 μmol photons m^{-2} s^{-1}, presumably due to an overreduced plastoquinone pool in the thylakoid membrane. Upon selection for growth at light intensities below 5 μmol photons m^{-2} s^{-1}, a secondary mutant was generated that retained the CtaI deletion and had fully assembled photosystem II complexes; in this secondary mutant (pseudorevertant), oxygen evolution and respiratory activities were similar to those in the wild type. Functional complementation of the original CtaI-less strain to low-light tolerance by transformation with restriction fragments of genomic DNA of the pseudorevertant and subsequent mapping of the pseudoreversion site showed that the point mutation led to a Ser186Cys substitution in Sll1717, a protein of as-yet-unknown function and with a predicted ATP/GTP-binding domain. This mutation caused a decrease in the plastoquinone pool reduction level of thylakoids compared to that observed for the wild type. Based on a variety of experimental evidence, the most plausible mechanism to cause this effect is an activation of plastoquinol oxidation in thylakoids by the quinol oxidase CydAB that occurs without upregulation of the corresponding gene and that may be caused by an increased CydAB activity in thylakoids, conceivably due to altered CydAB sorting between cytoplasmic and thylakoid membranes. Sll1717 appears to be unique to *Synechocystis* sp. strain PCC 6803 and has a close homologue encoded in the genome of this organism. The transcript level of sll1717 is low, which suggests that the corresponding protein is regulatory rather than structural.

Cyanobacteria are a group of organisms in which photosynthetic and respiratory electron transport systems are found in the same internal membrane system, the thylakoids. The plastoquinone (PQ) pool, the cytochrome b_6f complex and soluble redox-active proteins are shared between the two electron transport systems (3, 4, 31). In addition, the respiratory electron transport chain is also found in the cytoplasmic membrane of these organisms. How targeting of these complexes occurs and how their synthesis is regulated are as yet unknown.

The PQ pool in thylakoids is essential to both photosynthetic and respiratory electron transport. It obtains electrons from photosystem II (PSII) complexes, respiratory dehydrogenases, and the acceptor side of photosystem I (PSI) via cyclic electron flow via NADP, ferredoxin-quinone reductase, or ferredoxin-NADP oxidoreductase interacting with the PQ pool (15). The PQ pool is oxidized by PSI and by the cytochrome *c* terminal oxidase via the cytochrome b_6f complex or directly by quinol oxidases such as a cytochrome *bd*-type quinol oxidase (13) or an alternative oxidase, the activity of which is controversial (7, 9, 17, 28, 29). In a mutant lacking PSI, electrons can flow from PSII to the respiratory chain in tens of milliseconds (35). If the capacity of transfer to the respiratory chain is exceeded and the PQ pool is completely reduced, charge recombination between Q_A^- (the reduced form of the first quinone-type electron acceptor in PSII) and the donor side of PSII complexes can occur in 0.5 to 1 s (5) if the water-splitting complex is in state S_2 or S_3. Among respiratory dehydrogenases found in *Synechocystis* sp. PCC 6803, only succinate dehydrogenase (SDH) and the type 1 NADPH dehydrogenase contribute to PQ pool reduction (6, 11), whereas the type 2 NADH dehydrogenase most likely plays only a regulatory role in the cell (16). The main terminal respiratory oxidase that contributes to oxidation of the PQ pool in thylakoids is CtaI (cytochrome *c* oxidase) (24); the quinol oxidase Cyd is active in the cell but is interpreted to be localized primarily in the cytoplasmic membrane (17), whereas its activity can be demonstrated in thylakoids under at least some conditions (7, 28). The alternative oxidase CtaII is much less characterized and may not be active as respiratory activity in the absence of CtaI and Cyd is very low (9, 28, 29).

The redox state of the PQ pool in thylakoids is perceived as a regulatory signal in the cell, both in chloroplasts (25) and in cyanobacteria (14, 22, 33). The thylakoid PQ pool redox state reflects the state of both photosynthetic and respiratory electron flow and influences transcript levels of many genes, especially those that encode photosynthetic proteins (1, 14, 22, 26, 34). Factors influencing the redox state of the PQ pool may therefore influence intracellular processes. To aid in studying these processes, a series of mutants with lesions in photosynthetic and respiratory proteins have been developed (7, 11, 17, 28, 29, 32, 33).

One of these mutants lacks CtaI (32). This mutant is unable to grow at low light intensity, even in the presence of glucose, presumably because of overreduction of the PQ pool in thylakoids. At low light intensity, CtaI, rather than the light-driven PSI, is the major terminal acceptor for electrons from the PQ pool. Using the low-light-sensitive CtaI-less mutant, we have generated second-site mutants (pseudorevertants) that have

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TABLE 1. Primers and a probe used for RT-PCR analysis employed in this study

Primer[a]	Sequence	Position
5′ slr2082	5′ GCGAATTTCTTTGGCTTTTGG 3′	1541120–1541140
3′ slr2082	5′ AACCCCCCCCATAATGGC 3′	1541181–1541163
slr2082 probe	5′ TGGCCACTTCCGGCATTTC 3′	1541143–1541161
5′ slr1379	5′ TGGCCTTTCAATTCGGTCTAA 3′	674118–674138
3′ slr1379	5′ CCCCTACGGATTCCGAAAAA 3′	674168–674149
5′ slr1380	5′ TGTCCTGTTGGATGGGTTTGA 3′	675421–675441
3′ slr1380	5′ GCCGTAAGGGACAAAATCCC 3′	675471–675452
5′ sll1717 (1)	5′ ATTTTGCACAAAAAAACGACGTGG 3′	966536–966513
3′ sll1717 (1)	5′ GCCGCATCTCGTTTTTGTCAAAT 3′	966465–966487
sll1717 probe	5′ TGCGGTGGAGGATTTAGATG 3′	966511–966492
5′ sll1717 (2)	5′ GTTACAGTCCCTCAACCAATTGC 3′	967107–967085
3′ sll1717 (2)	5′ CCACGTCGTTTTTTTGTGCAAAAT 3′	966513–966536
5′ sll0849	5′ CTTCGGCCATTCCCTCCTGTTCCT 3′	1349647–1349624
3′ sll0849	5′ GTGTTTTCCACCGTGGCACCGTGG 3′	1349237–1349260

[a] (1), primer used for RT-PCR of sll1717; (2), primer used for semiquantitative analysis of the sll1717 transcript level by reverse transcription and PCR.

regained the ability to grow at low light intensity in the presence of glucose. We show that a mutation in Sll1717, a protein with previously unassigned function, alters the redox state of the thylakoid PQ pool in this strain.

MATERIALS AND METHODS

Strains employed in this study and their growth conditions. *Synechocystis* sp. strain PCC 6803 was grown on BG 11 medium (30) supplemented with 5 mM glucose and buffered with 0.01 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) –NaOH (pH 8.2). Cells were grown at a light intensity of 40 μmol photons m^{-2} s^{-1} and at a temperature of 30°C. Solid growth medium for strain maintenance was supplemented with 1.5% (wt/vol) agar and 0.3% (wt/vol) sodium thiosulfate.

A cytochrome *c* oxidase deletion mutant (Δslr1137, the CtaI-less strain) was obtained from Schmetterer et al. (32) and contains a kanamycin resistance cassette in lieu of a complete sll1137. This strain was maintained similarly to the wild type, except for the presence of 50 μg ml^{-1} kanamycin on maintenance plates.

To generate a pseudorevertant strain of the cytochrome *c* oxidase deletion mutant, the CtaI-less strain was plated on solid BG 11 medium supplemented with 5 mM glucose and 50 μg ml^{-1} kanamycin and kept at 2 μmol photons m^{-2} s^{-1} and 30°C. The CtaI-less strain could not grow at this low light intensity. After 2 to 3 weeks, rarely occurring (frequency, $\sim 10^{-8}$) colonies of cells that could grow under these conditions were isolated and analyzed. Pseudorevertant strains were maintained at conditions similar to those of the CtaI-less strain, but at decreased light intensity (2 μmol photons m^{-2} s^{-1}).

Deletion of sll1717 was performed in both wild-type and CtaI-less strains by standard methods of cloning and antibiotic cassette replacement (23) using a chloramphenicol resistance cassette (10). A blunted NcoI/HincII fragment (restriction sites at nucleotides 967601 and 965952 of the *Synechocystis* chromosome, respectively; numbering according to CyanoBase) of a PCR product containing sll1717 was cloned into the HincII restriction site of pUC19. A 1.3-kbp SmaI/HindIII fragment carrying a chloramphenicol resistance gene was blunted and inserted into the sll1717 clone that was restricted by EcoRV, leading to deletion of an internal portion (nucleotides 166 to 825, counting from the translation start site) of sll1717. Complete segregation of the deletion strains was confirmed by PCR. These strains were maintained similarly to the CtaI-less strain, except for the addition of 50 μg ml^{-1} chloramphenicol to the growth medium.

The PSI-less strain containing a Ser186Cys substitution in Sll1717 was obtained from the CtaI-less strain that contained this substitution by the following procedure. (i) slr1137 was reintroduced by transformation with a PCR product of a wild-type gene copy and selection for growth at 2 μmol photons m^{-2} s^{-1}; the transformants with restored CtaI were kanamycin sensitive and grew about 25% faster than the pseudorevertant strain. (ii) Deletion of *psaAB* was carried out with a plasmid construct to replace *psaAB* by a chloramphenicol resistance cassette (8).

Procedure of DNA isolation and secondary mutation mapping. Chromosomal DNA was isolated by a previously published method (18) from liquid cultures of the CtaI-less low-light-tolerant pseudorevertant strain harvested at an optical density at 730 nm (OD_{730}) of ~0.5 to 0.6.

Secondary mutation mapping was based on functional complementation of the Δslr1137 strain to growth at low light intensity by transformation with restriction fragments of genomic DNA of the CtaI-less pseudorevertant strain. The general principle and procedure for mapping by functional complementation using genomic restriction fragment fractions have been previously described (36). The following enzymes were used for the restriction: BamHI, BglII, KpnI, NheI, PstI, and SmaI. Size fractionation and isolation of the restriction fragments were performed as previously described (20). The transformation procedure was optimized (19) to maximize the number of transformants tolerant of low light intensity. Transformant colonies appeared after 16 to 21 days upon growth at 2 μmol photons m^{-2} s^{-1} and at a temperature of 30°C. Out of 14 groups of restriction fragments that were size fractionated from the restriction mixture after digestion of genomic DNA with each of the restriction enzymes mentioned above, only one fraction (containing the secondary mutation) for each restriction enzyme functionally complemented the CtaI-less strain to growth at low light intensity. The region of the *Synechocystis* sp. strain PCC 6803 chromosome carrying the secondary mutation was deduced, based on the size range of restriction fragments that were effective in functional complementation.

Fluorescence induction and emission measurements. For fluorescence induction measurements, liquid cultures were harvested at the mid-exponential phase of growth (OD_{730}, ~0.5 to 0.6) by centrifugation (5,000 × *g*), concentrated to a chlorophyll concentration of 10 μg ml^{-1}, and incubated in darkness for 1 min. Fluorescence induction of this cell suspension was recorded using a Walz PAM fluorometer. Fluorescence measurements were also performed on suspensions to which 10 μM 3-(3′, 4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and/or 60 μM 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB) was added before dark incubation. In the latter case, 5 mM sodium ascorbate was added to keep DBMIB in a reduced state, which does not quench chlorophyll fluorescence. The areas above the chlorophyll fluorescence induction curves measured in the presence of DBMIB versus DCMU were calculated and used to evaluate the electron accepting capacity of the PQ pool according to Berry et al. (7).

To monitor the kinetics of PQ pool reduction by SDH, the chlorophyll fluorescence yield was measured over time in the presence of 1 mM KCN and 60 μM DBMIB with flashes of weak measuring light that did not have an actinic effect. The duration of each flash and the interval between the flashes was 8 s. The fluorescence amplitude was recorded for each flash; an increased fluorescence yield is indicative of reduced Q_A that was formed by reverse electron transfer from the highly reduced PQ pool (12).

77K fluorescence emission spectra were recorded upon excitation of cell sus-

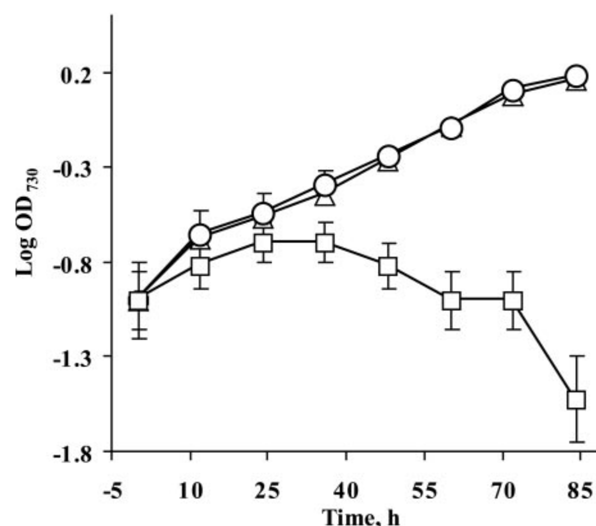

FIG. 1. Growth curve of the *Synechocystis* sp. strain PCC 6803 wild type (triangles), the CtaI-less strain (squares), and a pseudorevertant of the CtaI-less strain that is tolerant to low light intensity (circles). Cells were grown photomixotrophically at a light intensity of 2 μmol photons m^{-2} s^{-1}. The growth curves of the wild type and pseudorevertant partially overlap. Values were averaged from 10 independent experiments.

TABLE 2. Functional characteristics of the photosynthetic and respiratory electron transport chains of the strains employed in this study[a]

Strain	Oxygen evolution rate (% of wild-type value)	Oxygen uptake rate (% of wild-type value)	Ratio of areas over the fluorescence induction curve (+DBMIB/+DCMU)
Wild type	100	100	4.8 ± 0.3
CtaI-less	93 ± 16	53 ± 15	2.6 ± 0.4
Pseudorevertant (CtaI-less/Sll1717 S186C)	97 ± 14	92 ± 17	4.6 ± 0.5
Δsll1717	97 ± 13	96 ± 16	4.9 ± 0.4
CtaI-less/Δsll1717	95 ± 15	55 ± 14	2.5 ± 0.5

[a] Values represent the mean and standard deviation from five independent experiments. The wild type had an oxygen-evolving activity of 251 ± 26 μmol of O_2 (mg Chl)$^{-1}$ h^{-1} and an oxygen uptake rate of 41 ± 8 μmol of O_2 (mg Chl)$^{-1}$ h^{-1}. The ratio of the area above the fluorescence induction curve measured in the presence of 60 μM DBMIB (+5 mM ascorbate) versus 10 μM DCMU was used to determine the number of electrons that could be accommodated by the PQ pool.

pensions (chlorophyll concentration, 1 μg ml^{-1}) by 435-nm light with a FluoroMax spectrofluorometer.

P700 measurements. Cell cultures were harvested by centrifugation (1,000 × *g*) at the mid-exponential phase of growth (OD_{730}, ~0.5 to 0.6), concentrated to a chlorophyll concentration of 10 μg ml^{-1}, and incubated in darkness for 10 min. After the dark incubation, changes in light reflectance at 820 nm were measured as a function of actinic illumination with a Walz PAM fluorometer according to Howitt et al. (15). To block the oxidation of the PQ pool by respiratory oxidases (in case of the CtaI-less strain and the pseudorevertant, primarily quinol oxidase), KCN was added to the cell suspension to the final concentration of 1 mM.

Oxygen evolution and oxygen uptake assays. The steady-state rate of oxygen evolution in intact cells was determined as previously described (20). Oxygen uptake of intact cells in darkness was measured using a Clark-type electrode at a chlorophyll concentration of 50 μg ml^{-1}. The level of cyanide-resistant oxygen consumption was measured in the presence of 5 mM KCN. Respiratory electron flow via the cytochrome b_6f complex versus via a quinol oxidase was estimated by comparing oxygen uptake of the cells with and without 1 mM pentachlorophenol (PCP), a quinol oxidase inhibitor (29). Measuring respiration in cells in the presence of 20 μM DBMIB monitors respiration through quinol oxidase (7).

RNA isolation and RT-PCR. RNA isolation from liquid cell cultures harvested at mid-exponential growth stage was performed essentially as previously described (21). Total RNA samples were treated with RNase-free DNase and used for cDNA synthesis. Reverse transcription was performed using the iScriptcDNA synthesis kit (Bio-Rad) according to the protocol suggested by the manufacturer.

For semiquantitative analysis of sll1717 transcript levels, cDNA was used in a PCR with the primers 5′ sll1717 (2), and 3′ sll1717 (2), the sequences of which are presented in Table 1, using the following temperature profile: 94°C for 3 min; and 35 cycles, each consisting of 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s. Equal-volume aliquots were withdrawn from the reaction mixture after 10, 20, 25, and 35 cycles of amplification; loaded on a 1.2% agarose gel; separated by electrophoresis; and stained by ethidium bromide.

For real-time PCR (RT-PCR), amplification of cDNA corresponding to slr1379 (CydA) and slr1380 (CydB), a SYBR Green system was used, whereas detection of slr2082 (CtaII) and sll1717 cDNA required use of the TaqMan system. In both cases, relevant reaction kits (i*Taq* SYBR Green Supermix with 6-carboxy-X-rhodamine [ROX] for SYBR Green reactions and i*Taq* Supermix with ROX for TaqMan reactions) were supplied by Bio-Rad. Sequences of primers and the probes used in TaqMan reactions are shown in Table 1. The thermal profile of the RT-PCRs was as follows: stage 1, 95°C for 10 min; stage 2, 95°C for 15 s and 60°C for 15 s, repeated 40 times. Primer and probe design and the RT-PCR protocol were optimized using Primer Express software by Applied Biosystems. RT-PCRs were run using an ABI Prism 7900 HT Sequence Detection system and analyzed by Sequence Detection software, version 2.1 (both by Applied Biosystems).

RESULTS

Characterization of the CtaI-less strain. Photomixotrophic growth of the CtaI-less strain was inhibited at low light intensity (Fig. 1), whereas the wild-type strain demonstrated only a slight decrease in growth rates relative to rates at a higher light intensity. The oxygen consumption rate of the photomixotrophically grown CtaI-less strain measured without additional exogenous glucose provided shortly before the respiration measurement was about half that of the wild type (Table 2), which may be related to the lack of one of the terminal oxidases. It should be noted that under conditions of glucose-supported respiration (glucose added shortly before the measurement), the oxygen uptake rate of the CtaI-less strain was comparable to that of the wild-type strain (42 ± 6 μmol O_2 (mg chlorophyll [Chl])$^{-1}$ h^{-1}), in line with earlier measurements under these conditions (17, 32). The CtaI-less strain had a normal oxygen evolution rate (Table 2). Moreover, according to 77K fluorescence emission spectra, the 685- and 695-nm fluorescence intensities (originating from PSII) versus that at 725 nm (originating from PSI) were similar to that of the wild type (Fig. 2). These features are indicative of normally assembled and active PSII centers (Fig. 2; Table 2); therefore, electron flow from PSII to the PQ pool in the CtaI-less strain is expected to be unaltered. However, on the basis of the ratio of the area over

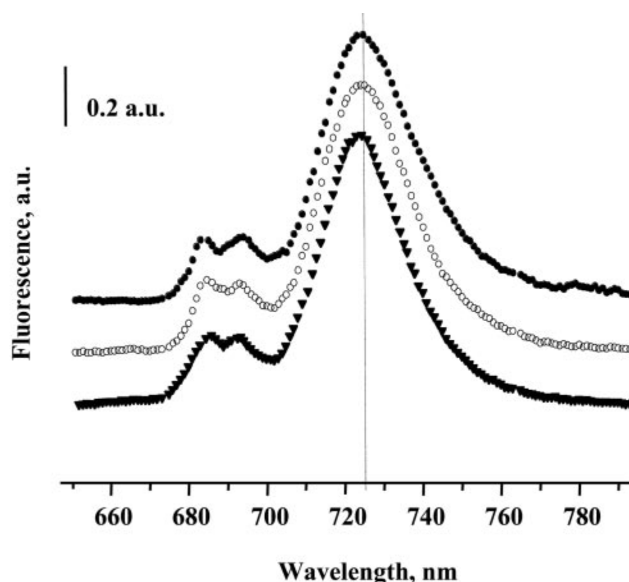

FIG. 2. 77K fluorescence emission spectra of intact cells of the *Synechocystis* sp. strain PCC 6803 wild type (top), the CtaI-less strain (middle), and the CtaI-less pseudorevertant (bottom). Excitation was at 435 nm. Cells were grown at a light intensity of 40 μmol photons m^{-2} s^{-1}. Fluorescence intensities were normalized to the fluorescence intensity at 725 nm, and curves were offset relative to each other. a.u., arbitrary units.

TABLE 3. Comparison between the experimental restriction fragment sizes of pseudorevertant DNA that complemented the CtaI-less mutant to growth at low light intensity and the theoretical restriction pattern of the sole region of the *Synechocystis* sp. strain PCC 6803 genome that fits these criteria

Enzyme	Size of DNA fragments in fraction that complements the Δslr1137 strain to dark tolerance (kbp)	Actual size of the fragment (bp)	Position of the DNA fragment that includes secondary mutation site (CyanoBase no.)
BamHI	32.5–35.0	33,386	944891–978276
BglII	15.0–17.5	15,852	954302–970153
KpnI	7.5–10.0	8,105	960016–968120
NheI	12.5–15.0	13,155	959238–972392
PstI	22.5–25.0	23,890	947668–971557
SmaI	7.5–10.0	8,648	961631–970278

the fluorescence induction curve in the presence of DBMIB versus that of DCMU, the PQ pool in thylakoids of this mutant was more reduced after a 1-min dark adaptation than that of the wild type (Table 2). Whereas the F_0 and F_{max} levels of fluorescence were rather comparable in the two strains, the fluorescence induction in the presence of DBMIB was faster in the CtaI-less strain than in the wild type, indicating that the PQ pool can accommodate fewer electrons and Q_A^- builds up more rapidly under these conditions. This is consistent with the observations by Howitt et al. (15), based on monitoring of the relative redox poise of the PQ pool using a Q electrode. This suggests that CtaI activity contributes significantly to electron transport out of the PQ pool in thylakoids.

Selection of the CtaI-less pseudorevertant and mapping of the secondary mutation. Upon selection of the CtaI-less strain for growth at nonpermissive, low light intensity, occasionally colonies were found that grew under these conditions and that therefore perhaps were able to cope with a reduced PQ pool in thylakoids. Eleven such colonies were obtained (the frequency of their occurrence was about 10^{-8}) and propagated. PCR amplification and sequencing of the slr1137 region from these strains indicated that they retained the deletion of the *ctaI* gene and that wild-type copies were absent. Therefore, tolerance to low light intensity was not due to reacquisition of slr1137 and most likely was the result of mutations elsewhere in the genome.

Functional complementation of the CtaI-less strain with restriction fragments of genomic DNA from one of the pseudorevertants allowed mapping of the mutation to the region between nucleotides 961631 (a SmaI site) and 968120 (a KpnI site), according to the numbering in CyanoBase (Table 3). Overlapping parts of this region were amplified and were used for functional complementation of the CtaI-less strain to growth at low light intensity. Those fragments that functionally complemented the CtaI deletion were sequenced. Sequencing showed a C-to-G substitution in position 966917 (numbering according to CyanoBase) that caused a Ser186Cys (TCT-to-TGT) mutation in Sll1717. This mutation was found to be present in

FIG. 3. Primary structure of Sll1717 and its homologue Sll1273 in *Synechocystis* sp. strain PCC 6803. The predicted ATP/GTP-binding motif of Sll1717 and Sll1273 is in boldface type. The arrow indicates Ser186, which is mutated in the CtaI-less pseudorevertant.

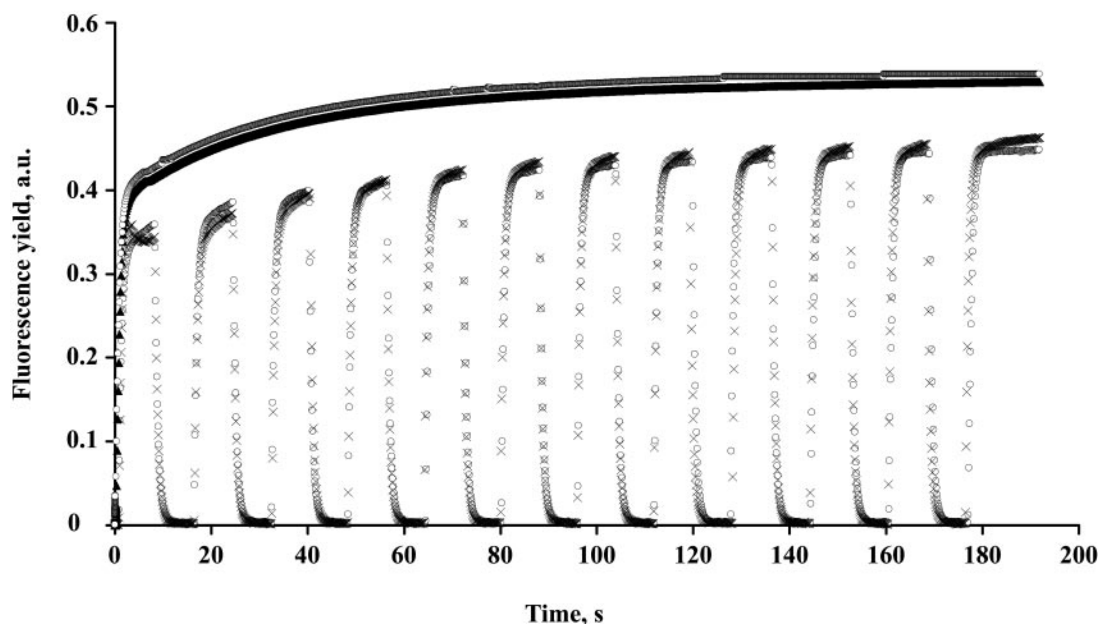

FIG. 4. Fluorescence yield of the PSI-less strain (○) and the PSI-less/Sll1717S186C strain (x) measured over time in the presence of 1 mM KCN and 60 μM DBMIB with flashes of weak measuring light that did not have an actinic effect. To prevent fluorescence quenching by oxidized DBMIB, 5 mM sodium ascorbate was added to the sample. The duration of each flash was 8 s, and the interval between the flashes was of similar length. The rise and decay kinetics of the signal upon turning on or off the light are artifacts, due to the time constant of the instrument that was set to a high value to be able to monitor fluorescence at very low, non-actinic intensities of measuring light. The upper curve reflects the maximum fluorescence yield recorded in actinic light in the presence of 10 μM DCMU in both the PSI-less strain (▲) and the PSI-less/Sll1717S186C strain (○) and reflects accumulation of reduced Q_A when electron transfer to Q_B is blocked. a.u., arbitrary units.

all 11 originally selected pseudorevertant colonies capable of growing at 2 μmol photons m^{-2} s^{-1}.

Properties of Sll1717. Sll1717 consists of 511 amino acids and has a predicted ATP/GTP-binding motif (Fig. 3). The closest homologue of sll1717 is sll1273 in the genome of *Synechocystis* sp. strain PCC 6803. The two corresponding proteins share similarity along the entire lengths of the molecules (Fig. 3). However, the sll1717 and sll1273 genes are unique for this cyanobacterial strain, and close homologues were not found in other sequenced organisms, not even in other sequenced cyanobacteria. According to a BLAST search, the closest Sll1717 homologue outside of *Synechocystis* is CAG36742, a hypothetical protein from the sulfate-reducing delta-proteobacterium *Desulfotalea psychrophila* LSv54, with 22% identity over 468 residues at the protein level.

Redox state of the thylakoid PQ pool in the pseudorevertant. The *Synechocystis* sp. strain PCC 6803 strain that lacks cytochrome *c* oxidase and contains a Ser186Cys substitution in Sll1717 was capable of growing at light intensities as low as 2 μmol photons m^{-2} s^{-1} (Fig. 1). Whereas after photomixotrophic growth and without addition of extra glucose before the respiration measurement the original CtaI-less strain had a reduced respiratory rate, under the same conditions the pseudorevertant displayed respiratory activity similar to that of the wild type (Table 2). To monitor the redox state of the PQ pool in thylakoid membranes in darkness, fluorescence induction in the presence of DBMIB (inhibiting at cytochrome b_6f) or DCMU (inhibiting oxidation of the first quinone-type electron acceptor, Q_A) was measured. The fluorescence induction curves of the wild type and the CtaI-less pseudorevertant were very similar. The area over the fluorescence induction curve in the presence of DBMIB versus DCMU that represents the electron-accepting capacity of the PQ pool in thylakoids was calculated (7). In the CtaI-less pseudorevertant, the thylakoid PQ pool redox state in darkness was found to be comparable with that in the wild type but not with that in the CtaI-less strain (Table 2). Therefore, either downregulation of respiratory plastoquinone reduction or upregulation of respiratory plastoquinol oxidation appears to have occurred in this second-site mutant of the CtaI-less strain. Changes in PSII abundance or efficiency do not appear to occur, according to low-temperature fluorescence emission spectroscopy (Fig. 2) and oxygen evolution assays (Table 2). As electron flow from PSII to the PQ pool has not been affected by the Ser186Cys mutation in Sll1717, effects of the secondary mutation in Sll1717 on respiratory processes or on cyclic electron transfer around PSI are best compatible with our experimental observations. However, as no changes in the $P700^+$ rereduction rate after illumination were observed (data not shown) and as photosynthetic electron flow is minimal at the low light intensity used (2 μmol photons m^{-2} s^{-1}; PSI activity is insufficient to keep up with respiratory electron transfer into the PQ pool to allow growth of the original CtaI-less strain at this light intensity), the attention will focus on effects of the Sll1717 mutation on respiratory activities.

Effect of the secondary mutation in Sll1717 on PQ pool reduction by respiratory dehydrogenases. The Sll1717 mutation might either block respiratory electron flow into the PQ pool or enhance flow out of the pool. There are very few methods to monitor respiratory electron flow into the PQ pool. Probably the most sensitive way to do so is to measure the PQ

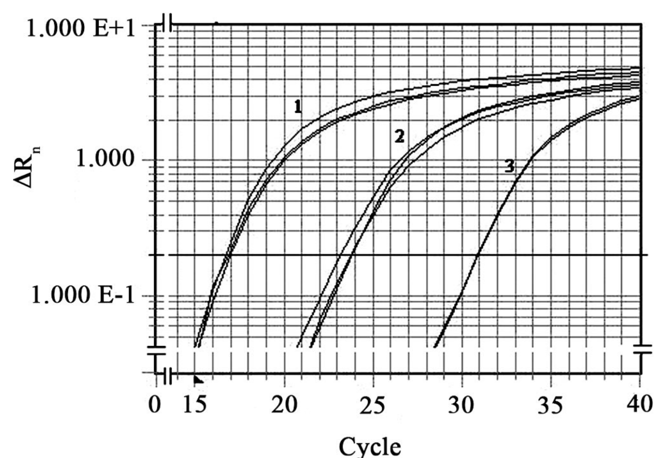

FIG. 5. Accumulation of RT-PCR products generated from slr1379 (*cydA*) transcripts using total RNA isolated from the wild-type strain, the CtaI-less strain, or the pseudorevertant of the CtaI-less strain (Δslr1137/Sll1717 S186C) as a template. The following dilutions of the total RNA preparation were used as templates: 1:20 (1), 1:2,000 (2), and 1:200,000 (3). ΔRn is baseline-subtracted fluorescence from the PCR product normalized to an internal dye (ROX). The C_T value is the number of PCR cycle at which the increase of fluorescence (and therefore the cDNA amplification) is maximally logarithmic. The C_T values for the slr1379 amplification in all three strains were very close to each other at each dilution of the RNA preparation and were 16.80 ± 0.14 (1), 23.58 ± 0.34 (2), and 31.00 ± 0.18 (3).

redox state through variable chlorophyll fluorescence: a reduced PQ pool will lead to reduction of Q_A, which in turn leads to increased chlorophyll fluorescence (16). However, in cyanobacteria retaining PSI, the variable fluorescence yield is much lower than in those without PSI (35). For this reason, PSI (*psaAB*) was deleted from the pseudorevertant. To determine whether the flow of electrons from respiratory dehydrogenases to the PQ pool was affected by the mutation in Sll1717, changes in the chlorophyll fluorescence yield in the PSI-less pseudorevertant in essential darkness were monitored in the presence of 1 mM KCN (inhibiting both quinol oxidase and cytochrome oxidase) and 60 μM DBMIB (inhibiting the cytochrome b_6f complex) using weak measuring light flashes that did not significantly excite PSII (Fig. 4). As before, 5 mM ascorbate was added to minimize fluorescence quenching by oxidized DBMIB. In the presence of both KCN and DBMIB, electron flow from the PQ pool to terminal oxidases in the respiratory chain is blocked, and PQ pool reduction in darkness reflects electron transfer from the respiratory dehydrogenases (SDH and type 1 NADPH dehydrogenase). The rise in chlorophyll fluorescence reflects accumulation of reduced Q_A due to reverse electron flow from the overreduced PQ pool (16). Our experiments indicate that in the absence of actinic illumination (with only very weak, non-actinic measuring light) there was no difference in the kinetics in the change of chlorophyll fluorescence yield between the PSI-less strain and the PSI-less strain carrying a Ser186Cys substitution in Sll1717 (Fig. 4). Therefore, the secondary mutation in Sll1717 did not affect the rate of respiratory PQ reduction.

Effect of the secondary mutation in Sll1717 on PQ pool oxidation by quinol oxidase. Another functional explanation that is compatible with the data presented thus far is that the Sll1717 mutation activates a quinol oxidase in thylakoids either by upregulation of the corresponding genes or by posttranscriptional processes. Our RT-PCR analysis indicated that the transcript levels for both subunits of quinol oxidase, CydA (Fig. 5) and CydB (data not shown), are very similar in the wild type, the CtaI-less strain, and the CtaI-less pseudorevertant of *Synechocystis* sp. strain PCC 6803. Therefore, upregulation of *cydAB* was not observed in the CtaI-less pseudorevertant.

To determine whether increased quinol oxidase activity was responsible for the increased oxygen consumption of the CtaI-less pseudorevertant, the oxygen consumption rate was measured in the presence of different electron transport inhibitors: 5 mM KCN (inhibits electron transport to molecular oxygen in terminal oxidases), 20 μM DBMIB (inhibits the cytochrome b_6f complex), or 1 mM PCP (inhibits quinol oxidase). Our results indicate that the increased oxygen consumption of the pseudorevertant strain was inhibited by both KCN and PCP but was not sensitive to DBMIB (Table 4). This indicates that the increased respiration rate is due to increased quinol oxidase activity that can consume electrons from the thylakoid PQ pool.

Role of an alternative oxidase in PQ pool oxidation. Another possible contributor to thylakoid PQ pool oxidation is an alternative oxidase, CtaII, for which genes are present but whose activity in *Synechocystis* is controversial. We have not been able to detect a *ctaII* transcript by RT-PCR in any of the strains employed in this study. Moreover, in our laboratory we have not been able to detect significant activity of CtaII in strains lacking *ctaI* and *cydAB* (17). Therefore, a contribution of this alternative oxidase to increased respiration in the pseudorevertant is unlikely.

Effect of the secondary mutation on sll1717 expression. To determine whether functional complementation by the Ser186Cys substitution in Sll1717 was caused by inactivation of the corresponding gene, sll1717 deletion mutants were generated in both the CtaI-less and wild-type backgrounds. The Δsll1717/CtaI-less strain was found to be unable to grow at 2 μmol photons m^{-2} s^{-1}, like the CtaI-less strain (data not shown), indicating that low-light tolerance in the CtaI-less pseudorevertant was conveyed by the Ser186Cys mutation in Sll1717 and that this mutation did not inactivate or destabilize the protein. However, upon deletion of sll1717, the strains became more sensitive to elevated temperatures (Fig. 6). No other functional effects of the sll1717 deletion were observed (data not shown).

TABLE 4. Oxygen consumption of *Synechocystis* sp. strain PCC 6803 wild-type and mutant strains in the presence of inhibitors[a]

Strain	Measured oxygen uptake, μmol O_2 (mg Chl)$^{-1}$ h^{-1}			
	No inhibitors added	+20 μM DBMIB	+5 mM KCN	+1 mM PCP
Wild type	41 ± 8	8 ± 1	1 ± 0.2	38 ± 4
CtaI less	22 ± 3	21 ± 2	0	3 ± 1
Pseudorevertant (CtaI-less/Sll1717 S186C)	38 ± 4	39 ± 5	1 ± 0.2	8 ± 1

[a] Values represent the mean and standard deviation from five independent experiments.

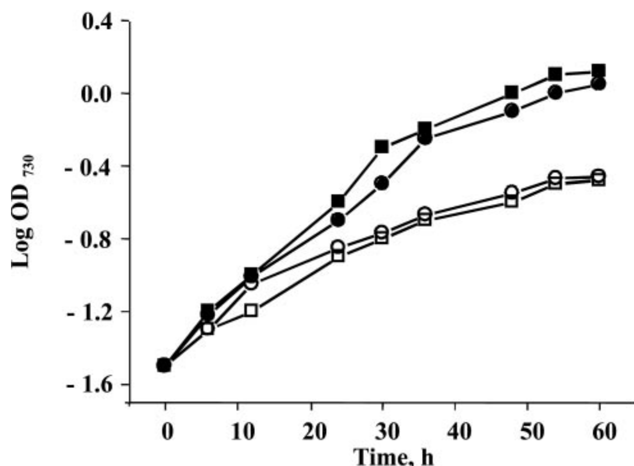

FIG. 6. Effect of the sll1717 deletion on photomixotrophic growth of *Synechocystis* sp. strain PCC 6803 at elevated temperatures. Cultures of the wild type (closed squares) and the CtaI-less mutant (closed circles) were grown at 40 μmol photons m^{-2} s^{-1} and at a temperature of 39°C. Open symbols represent growth curves of the corresponding strains with a sll1717 deletion.

Transcript levels of sll1717. To determine transcript levels of sll1717, RT-PCR was carried out. As indicated in Fig. 7A, the sll1717 transcript levels were similar in the wild type, the CtaI-less strain, and the pseudorevertant. The sll1717 transcript level was much lower than that of the *psbDI* gene (Fig. 7B). A low abundance of the sll1717 transcript would be consistent with a regulatory, rather than a structural, role of Sll1717 in the cell.

DISCUSSION

Thylakoid PQ pool redox homeostasis in the CtaI-less pseudorevertant and the physiological role of Sll1717. Overreduction of the PQ pool in cyanobacterial thylakoids appears to be lethal (8, 32, 33). At higher light intensity, the PQ pool is oxidized by PSI, whereas at low light intensity (<5 μmol photons m^{-2} s^{-1}) PSI activity is insufficient for PQ oxidation and respiration becomes key to retain PQ redox homeostasis in thylakoids. According to our results (Table 2) and Q-electrode measurements (15), the CtaI-less strain has an overly reduced PQ pool. This is why the CtaI-less strain cannot grow in darkness or at light intensities below 5 μmol photons m^{-2} s^{-1}, even if glucose is present. The pseudorevertant described in this study obviously has regained a mechanism to maintain PQ redox homeostasis in thylakoids at low light intensity in the absence of CtaI.

The pseudorevertant that survives at low light intensity and without CtaI activity could do so either by downregulating the number of electrons that flow into the PQ pool from respiratory (mainly SDH) (11) and photosynthetic complexes (PSII or PSI via cyclic electron flow) or by upregulating electron flow out of the PQ pool in thylakoids through either the quinol oxidase or an alternative oxidase. A third possibility is that cells no longer respond to a highly reduced thylakoid PQ pool in a way that leads to lethal effects. This third possibility can be excluded, as the redox state of the thylakoid PQ pool in the CtaI-less pseudorevertant that grows at low light intensity is comparable to that in the wild type after dark adaptation (Table 2). Of the two remaining explanations, slow reduction of the thylakoid PQ pool is inconsistent with our experimental results: the rate of thylakoid PQ pool reduction by respiratory

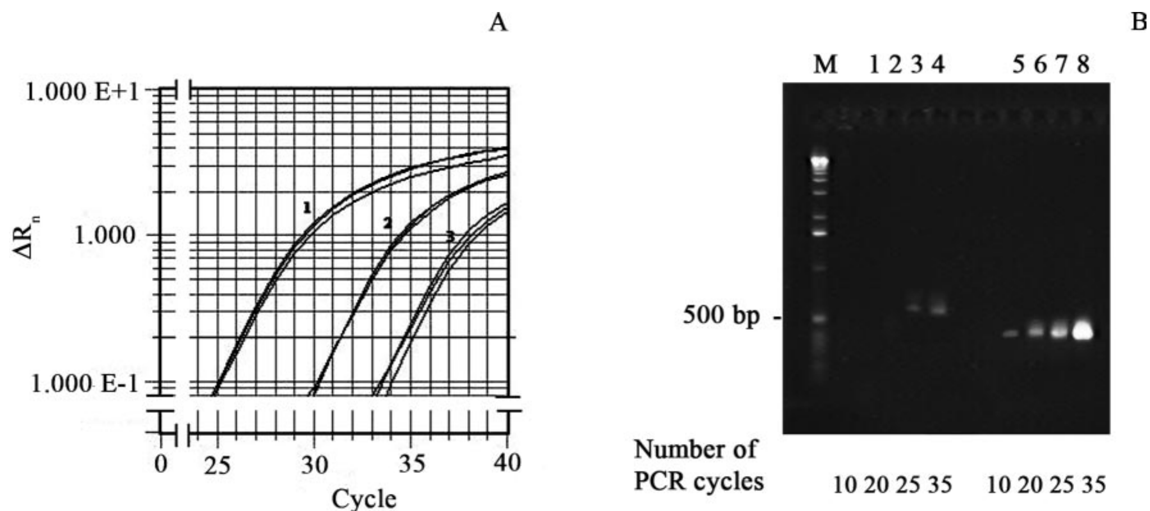

FIG. 7. (A) Accumulation of sll1717 RT-PCR products using total RNA isolated from the wild type strain, the CtaI-less strain, and the low-light-tolerant pseudorevertant of the CtaI-less strain as a template. Primers 5′ sll1717 (1) and 3′ sll1717 (1) and the sll1717 probe (Table 1) were used for the PCR. The following dilutions of the total RNA preparation were used as a template: 1:100 (1), 1:2,000 (2), and 1:40,000 (3). ΔRn is baseline-subtracted fluorescence from the PCR product normalized to an internal dye (ROX). The C_T value is the number of PCR cycles at which the increase of fluorescence (and therefore the cDNA amplification) is maximally logarithmic. The C_T values for sll1717 amplification in all three strains were very close to each other at each dilution of the RNA preparation and were 26.24 ± 0.09 (1), 31.37 ± 0.02 (2), and 34.89 ± 0.14 (3). (B) Accumulation of RT-PCR products corresponding to sll1717 (lanes 1 to 4) and *psbDI* (lanes 5 to 8) transcripts using total RNA isolated from wild-type *Synechocystis* sp. strain PCC 6803 as a template. Primers 5′ sll1717 (2) and 3′ sll1717 (2) were used to amplify sll1717 (Table 1). The number of PCR cycles to which the samples have been exposed has been indicated. Molecular weight markers (M) are in the left lane.

processes in the presence of KCN was similar in the pseudorevertant and the wild type, the PSII/PSI ratio was normal, and cyclic electron flow around PSI (monitored by $P700^+$ rereduction after illumination) was similar in the pseudorevertant and the CtaI-less strain.

Indeed, the second possibility (the pseudorevertant has induced a respiratory oxidase in thylakoids) is in line with our experimental results: the rate of respiratory oxygen consumption, measured without addition of exogenous glucose just before the measurement, increased in the pseudorevertant to levels comparable to those in the wild type (Table 2), and the redox state of the thylakoid PQ pool in darkness was similar to that in the wild type. In principle, the induced respiratory oxidase in thylakoids might be either the quinol oxidase Cyd or the alternative oxidase CtaII. In view of the results of inhibition experiments using PCP and the absence of measurable *ctaII* transcript levels in our RT-PCR experiments, our interpretation of the data is that quinol oxidase activity in thylakoids increased in the pseudorevertant (Table 2). This interpretation of increased activity of quinol oxidase in thylakoids in the pseudorevertant further supports the idea that a substantial portion of Cyd in the cell can be localized in thylakoids and that the distribution of quinol oxidase activity between thylakoid and cytoplasmic membranes depends on growth conditions (7), as well as on proteins such as Sll1717.

As argued above, our interpretation is that the Ser186Cys substitution in Sll1717 led to an upregulation of electron flow through quinol oxidase in thylakoids to an extent that was sufficient to compensate for the lack of cytochrome *c* oxidase activity. At this time, it is not clear how exactly this mutation functionally complemented the lack of CtaI. However, the S186C mutation in sll1717, observed in all 11 obtained pseudorevertants, is the only one so far to produce functional complementation of the CtaI-less strain to enable growth at 2 μmol photons m^{-2} s^{-1}. As may be expected from this observation, the mutation did not inactivate the corresponding gene, as the sll1717 deletion did not confer low-light tolerance in the CtaI-less strain.

Increased quinol oxidase activity in thylakoids of the CtaI-less pseudorevertant with a Ser186Cys substitution in Sll1717 was not a result of transcriptional upregulation of sll1717 and *cydAB*, as transcript levels of these genes were the same as in the wild type and the CtaI-less strain (Fig. 5 and 7). Mutated Sll1717 may play a role in increasing the stability and/or activity of quinol oxidase in thylakoids in some way. However, another straightforward interpretation of the data obtained on the pseudorevertant is that the Sll1717 mutation has changed the distribution of quinol oxidase between cytoplasmic and thylakoid membranes in favor of the latter. Indeed, altered distribution of quinol oxidase between cytoplasmic and thylakoid membranes as a function of growth conditions has been previously demonstrated (7).

Phylogenetic distribution of Sll1717. Sll1717 does not have highly conserved homologues in other species. However, the existence of a gene encoding a protein similar to Sll1717 in the same genome suggests a gene duplication event in the evolution of sll1717 that was followed by significant divergence. As quinol oxidase (Cyd) appears to be widely distributed among cyanobacteria according to available genome sequences, the absence of Sll1717 homologues in other cyanobacteria suggests that a distribution of this terminal oxidase between different membranes is not an issue in other cyanobacteria or that proteins with functions similar to those of Sll1717 but with very different primary structures may exist in the other organisms. There are other examples of proteins that are not ubiquitous, although they regulate processes that occur in a wide range of species. For instance, a two-component system that is involved in transduction of PQ redox signals in cyanobacteria (RppA and RppB) (22) is not found in chloroplasts, although PQ redox signals may be just as important in chloroplasts as in cyanobacteria (2, 25, 26, 27).

In summary, our experimental results suggest that CtaI in thylakoids can be functionally substituted by quinol oxidase upon a Ser186Cys mutation in Sll1717. Therefore, Sll1717 appears to be a protein with a regulatory effect on and presumably the localization of the quinol oxidase Cyd in *Synechocystis* sp. strain PCC 6803. This is one of the first pieces of the puzzle to help understand the role and localization of cyanobacterial quinol oxidase, and Sll1717 conceivably could be a new type of protein that modulates sorting of proteins that may be directed to different membranes, depending on environmental conditions.

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