Inactivation of the Open Reading Frame slr0399 in 
Synechocystis sp. PCC 6803 Functionally Complements
Mutations near the QA Niche of Photosystem II

A POSSIBLE ROLE OF Slr0399 AS A CHAPERONE FOR QUINONE BINDING*

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The Synechocystis sp. PCC 6803 triple mutant D2R8 with V247M/A249T/M329I mutations in the D2 subunit of the photosystem II is impaired in QA function, has an apparently mobile QA, and is unable to grow photoautotrophically. Several photoautotrophic pseudorevertants of this mutant have been isolated, each of which retained the original psbDI mutations of D2R8. Using a newly developed mapping technique, the site of the secondary mutations has been located in the open reading frame slr0399. Two different nucleotide substitutions and a deletion of about 60% of slr0399 were each shown to restore photoautotrophy in different pseudorevertants of the mutant D2R8, suggesting that inactivation of Slr0399 leads to photoautotrophic growth in D2R8. Indeed, a targeted deletion of slr0399 restores photoautotrophy in D2R8 and in other psbDI mutants impaired in QA function. Slr0399 is similar to the hypothetical protein Ycf39, which is encoded in the cyanelle genome of Cyanophora paradoxa; in the chloroplast genomes of diatoms, dinoflagellates, and red algae; and in the nuclear genome of Arabidopsis thaliana. Slr0399 and Ycf39 have a NAD(P)H binding motif near their N terminus and have some similarity to isoflavone reductase-like proteins and to a subunit of the eukaryotic NADH dehydrogenase complex I. Deletion of slr0399 in wild type Synechocystis sp. PCC 6803 has no significant phenotypic effects other than a decrease in thermotolerance under both photoautotrophic and photomixotrophic conditions. We suggest that Slr0399 is a chaperone-like protein that aids in, but is not essential for, quinone insertion and protein folding around QA in photosystem II. Moreover, as the effects of Slr0399 are not limited to photosystem II, this protein may also be involved in assembly of quinones in other photosynthetic and respiratory complexes.

The cyanobacterium Synechocystis sp. PCC 6803 is a useful molecular genetic system to study oxygenic photosynthesis and cell physiology of photosynthetic microorganisms. The organism is unique in that it combines several desirable properties: (i) Synechocystis sp. PCC 6803 is spontaneously transformable and incorporates exogenous DNA into its genome via double-homologous recombination (1, 2); (ii) the strain can grow photoheterotropically, thus enabling the creation of Synechocystis sp. PCC 6803 strains impaired in photosystem I (PS I) and/or photosystem II (PS II) function (for a recent review, see Ref. 3); and (iii) the entire genome sequence of Synechocystis sp. PCC 6803 is known (4). Because of these properties, a variety of molecular genetic approaches have been applied to study the role of particular proteins in this organism.

One of these approaches is the mapping and characterization of pseudorevertants, which carry second-site mutations restoring viability under conditions that are lethal for the original mutants. In the case of mutants that are impaired in photosynthesis, pseudorevertants with improved photosynthetic function and with secondary mutations in genes coding for proteins with known function have been isolated and analyzed (for example, see Refs. 5–11). These genes may be identical to the ones carrying the original mutations or may be at a different locus.

In eukaryotic genetic systems such as Chlamydomonas reinhardii or Arabidopsis thaliana mapping of a site of a suppressor mutation in an unrelated gene is a time-consuming and complicated project. However, with the advent of a known genomic sequence, in Synechocystis sp. PCC 6803 the process of mapping of genes that contain second-site mutations has been simplified and accelerated by development of a novel technique of functional complementation with size-separated restriction fragment pools (3). In this way, a functionally complementing gene can be identified using the pseudorevertant DNA without the need for library construction.

In the present study we have applied this technique to characterize frequently occurring photoautotrophic pseudorevertants of the obligate phototrophotropic D2R8 mutant (12), which has primary mutations in the QA-binding niche of the D2 protein that is part of the PS II reaction center complex. Surprisingly, the location of these pseudoreversions was found to map to slr0399, an open reading frame coding for a protein of an unknown function. As will be presented in this paper, we suggest that Slr0399 may function as a chaperone helping to insert QA into its site. Even though chaperone function has been well established for folding of soluble proteins (reviewed

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1 The abbreviations used are: PS I, photosystem I; PS II, photosystem II; PCR, polymerase chain reaction; QA, the primary electron-accepting plastoquinone in PS II; Qb, the second electron-accepting plastoquinone in PS II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DQ, tetramethyl-p-hexoquinone (duruquinone); PQ, plastoquinone; vitamin K, phylloquinone; kb, kilobase pair(s); HPLC, high performance liquid chromatography; IRL, isoflavone reductase-like protein; TES, N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid.
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recently in Refs. 13 and 14), much less is known about the possible involvement of chaperones in folding of membrane protein complexes and in insertion of cofactors.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions—Synechocystis sp.** PCC 6803 cells were grown at 30°C in BG-11 medium (15) supplemented with 5 mM glucose at the light intensity of 50 µmol photons m⁻² s⁻¹. Liquid medium was perfused with sterile air. Solid medium was supplemented with 1.5% agar, 0.3% sodium thiosulfate, and 10 mM MES/NaOH buffer, pH 5.2. The PS II inhibitor atrazine (20 µM) was added post-illumination for maintenance of obligate photoheterotrophic mutants with defects in PS II in order to avoid inadvertent selection for photoautotrophic pseudorevertants.

**Chromosomal DNA Isolation and Fractionation**—For the isolation of genomic DNA, Synechocystis sp. PCC 6803 cells were pelleted and incubated at 37°C for 20 min with 2 ml of saturated NaI solution (g/wt) of cells. After dilution of NaI with 5-10 volumes of water, cells were pelleted by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.6, and 2 mM EDTA buffer. After digestion of the chromosomal DNA with restriction endonucleases and size fractionation on a 0.4% agarose gel in TAE buffer (40 mM Tris-acetic acid, pH 8.0, and 1 mM EDTA), the gel was soaked in distilled water for 30 min and then in a 0.5 M NaCl/hexane mixture, at a flow rate of 1.5 ml/min (20). The absorbance of the eluate was monitored at 263 nm on a Beckman model 166 absorbance detector. The data were processed with the System Gold software (Beckman). Vitamin K₃ (Aldrich) was used as a standard for quantification of the amount of quinone detected. Quinone peaks were eluted and spectrally analyzed in oxidized form and after reduction with NaBH₄. Peak assignments were made on the basis of comparison with published absorption spectra (21, 22) of the oxidized and reduced quinone compounds.

**RESULTS**

**Isolation and Characterization of Pseudorevertants of the psbDI Triple Mutant D2R8**—The Synechocystis sp. PCC 6803 mutant D2R8 does not grow photoautotrophically due to two mutations in the Qₐ-binding de-loop of the D2 protein (V247M and A249T) that greatly alter the properties of QA and cause QA to be apparently mobile and replaceable by other quinones (12). This mutant lacks psbDII (the second gene coding for the D2 protein), and has three mutations in psbDI, leading to a V247M/A249T/M329I mutation combination. When the obligate photoheterotrophic D2R8 mutant was plated in the absence of glucose, photoautotrophic colonies were found to appear frequently. The estimated frequency was 10⁻⁵ to 10⁻⁶, which was about 2 or 3 orders of magnitude higher than the frequency of true reversion that we observe for obligate photoheterotrophic single-base change mutants.

In order to determine the genetic cause of the photoautotrophic nature of the D2R8 derivatives, the psbDI gene from three independent photoautotrophic D2R8 derivatives (D2R8R1, D2R8R2, and D2R8R3) was amplified by PCR and sequenced. Interestingly, in all three strains, the mutations V247M, A249T, and M329I remained present in the D2R8 pseudorevertant strains. Moreover, no additional mutations were found in psbDI that was isolated from the revertants. Corroborating these findings, PCR-amplified psbDII from the pseudorevertants failed to transform the initial D2R8 mutant to photoautotrophy, indicating that the site of pseudoreversion in the three pseudorevertant strains was located outside of psbDII. Total genomic DNA isolated from the same pseudorevertants transformed the D2R8 mutant to photoautotrophy with high frequency, establishing that the pseudoreversion of D2R8 to photoautotrophic growth is due to a single genetic event. We find cotransformation of different loci to be uncommon in *Synechocystis* sp. PCC 6803.

**Mapping of the Site of Pseudoreversion—Several genes or gene clusters coding for structural PS II proteins that might have domains close to the QA site (psbA2, psbA3, psbC, psbH, and psbEPLJ) were amplified by PCR from genomic DNA of the three pseudorevertants. These PCR products were examined for their ability to transform the original mutant to photoautotrophy. All of these genes tested failed to complement D2R8, indicating that they did not contain the site of the secondary mutation.**

In order to identify the locus or loci responsible for the pseudoreversion in the three strains, a novel mapping technique of functional complementation with size-separated restriction fragment pools was applied (3), making use of the genomic restriction map of *Synechocystis* sp. PCC 6803 that has been constructed for 16 enzymes based on the genomic
sequence. The genomic DNA from pseudorevertant D2R8R1 was isolated and purified gently to avoid fragmentation. About 25 μg of genomic DNA was completely digested with one of the following 10 enzymes (BamHI, BglII, EcoRI, EcoRV, KpnI, NheI, PstI, ScaI, SmaI, and XbaI) and size-separated on a 0.4% agarose gel. Each of the 10 lanes of the gel was cut into 20–25 fractions, containing DNA fragments of size categories between 1 and 35 kb. Every fraction was collected in a separate micro-centrifuge tube, and DNA was extracted and used to transform the original D2R8 mutant as described under “Experimental Procedures.” The capability to perform photoautotrophic growth was used as the selection criterion. Only one DNA fraction in each of seven restriction digestions complemented the D2R8 mutant, indicating that these fractions contained the restriction fragment carrying the secondary mutation (Table I). However, no complementation was observed after transformation with DNA fractions generated by restriction with BamHI, KpnI, and NheI (Table I). This may indicate that either (i) a restriction site of these enzymes is too close to the locus of the secondary mutation, not leaving a large enough flanking region to facilitate efficient homologous recombination in Synechocystis sp. PCC 6803; or (ii) the restriction fragment containing the secondary mutation generated by these enzymes was less than 1 kb or longer than 35 kb and was not represented in the complementation test.

The size ranges of the seven restriction fragments that led to functional complementation of the original mutant were compared with the size-sorted restriction map of the entire Synechocystis sp. PCC 6803 chromosome (3) in order to determine a single region in the genome that fitted this unique restriction pattern. Only one genomic location was found to yield restriction fragments compatible with the sizes observed for each of the seven complementing restriction fragment collections. This location was 2,147,054–2,149,574 base pairs (numbering according to CyanoBase Kazusa DNA Research Institute, Japan), corresponding to a 2,528-base pair EcoRI/ScaI fragment. This region of the genome should contain the site of the pseudoreversion.

To verify this finding, this EcoRI/ScaI fragment was amplified by PCR from pseudorevertants D2R8R1, D2R8R2, and D2R8R3, and from the wild type, cloned into the pACYC184 vector (yielding plasmids pD2R8R1, pD2R8R2, pD2R8R3, and pWT, respectively), and used to transform the D2R8 mutant. Indeed, PCR products cloned from the pseudorevertants, but not the wild type, could transform the original mutant to photoautotrophy.

The Genome Region Containing the Site of Pseudoreversion—As shown in Fig. 1A, the complementing 2,528-base pair EcoRI/ScaI fragment contained two complete open reading frames (slr0398 and slr0399) and two partial ones (slr0397 and slr0400). The sizes and genome locations of the corresponding restriction fragments harboring the secondary mutation were determined after the locus of pseudoreversion had been mapped based on complementation test results for the other restriction enzymes.

| Restriction enzyme | As determined from complementation test | As determined from comparison with the Synechocystis sp. PCC 6803 restriction map |
|--------------------|----------------------------------------|-----------------------------------------------|
|                    | Size range of complementing DNA fraction | Location of the complementing fragment within the genome | Size of the complementing fragment |
| BglII              | 23–35        | 2,131,568–2,160,099 | 28.53 |
| EcoRI              | 4–15         | 2,147,054–2,151,425 | 4.37 |
| EcoRV              | 16–18        | 2,158,546–2,176,552 | 17.51 |
| PstI               | 9–11         | 2,140,213–2,150,458 | 10.25 |
| ScaI               | 19–23        | 2,127,089–2,149,574 | 22.49 |
| SmaI               | 17–19        | 2,144,053–2,163,009 | 18.96 |
| XbaI               | 11–14        | 2,140,080–2,151,209 | 11.13 |
| BamHI             | None         | 2,146,114–2,149,444 | 3.93 |
| KpnI*             | None         | 2,146,190–2,189,236 | 43.05 |
| NheI*             | None         | 2,146,264–2,152,677 | 6.05 |

* None of the DNA fractions of D2R8R1 generated by these three enzymes could complement D2R8 to photoautotrophic growth. The sizes and genome locations of the corresponding restriction fragments harboring the secondary mutation generated by these enzymes was less than 1 kb or longer than 35 kb and was not represented in the complementation test.
interact with the QA site as a whole rather than with individual residues.

Inactivation of slr0399—To verify the notion that deletion of a large part of Slr0399 leads to photoautotrophic growth in previously obligate photoheterotrophic PS II mutants with changes at the QA site, a plasmid was constructed (p\textsubscript{D}slr0399) in which a 620-base pair Bsa\textsubscript{BI}/Spe\textsubscript{I} fragment near the 3\textsuperscript{9} end of slr0399 was replaced by the kanamycin (Km) resistance cassette from pUC4K. To rule out possible polar effects of this deletion and/or Km insertion on the transcription of sequences located downstream of slr0399, another DNA construct was designed that contained intact slr0399, but where the Km resistance cassette was inserted at the Spe\textsubscript{I} restriction site adjacent to the stop codon of slr0399. Both constructs were used to transform the Synechocystis sp. PCC 6803 wild type, the D2R8 strain, and the D2 mutant S254F (see Table II), selecting for resistance to kanamycin. The complete segregation of the introduced deletions was demonstrated by PCR (Fig. 2); as Synechocystis sp. PCC 6803 contains multiple genome copies per cell, demonstration of segregation prior to functional analysis is essential.

As expected, targeted inactivation of slr0399 restored photoautotrophic growth in the photoheterotrophic p\textsubscript{D}R8 and S254F (Table III). However, slr0399 inactivation had essentially no effect on the properties of the wild type (Table III). The photoautotrophic growth rate, the PS II content (as determined from the ratio of chlorophyll and the number of DCMU binding sites), the affinity of PS II for radiolabeled DCMU, the chlorophyll a content per cell, and the relative amount of variable fluorescence remained unchanged in wild type upon slr0399 inactivation. Moreover, the 77K chlorophyll fluorescence emission characteristics as well as the rate of photoinactivation of PS II electron transport upon illumination with saturating light remained unchanged (data not shown).

However, PS II properties of the D2R8 and S254F mutants were altered significantly upon introduction of the slr0399 inactivation construct, becoming more like those of the wild type. The photoautotrophic doubling time of the deletion mutants was 16–21 h, somewhat slower than that of the wild type, and the amount of PS II was increased 3-fold in both D2 mutants to about half of that in the wild type. Upon introduction of the slr0399 inactivation construct the dissociation constant of DCMU decreased from 66 and 31 nM in the initial mutants D2R8 and S254F, respectively, to values comparable to those in the wild type (Table III).

Insertion of a Km resistance cassette immediately downstream of slr0399 had no measurable phenotypic effect in D2R8, S254F, or the wild type (data not shown). This confirms that the phenotypic effects observed in the D2R8 pseudorevertants and in the D2 mutant strains with inactivated slr0399 are in fact due to slr0399 inactivation rather than to effects on expression levels of genes that happen to be cotranscribed with slr0399.

Slr0399 Effects on the D2 Mutants D2R8 and S254F—The
TABLE II

| Mutant               | Location of mutation(s) | Oxygen evolution in Hill reaction | Complementation to photoautotrophic growth by slr0399 from: |
|----------------------|-------------------------|----------------------------------|----------------------------------------------------------|
| psbD1 mutants (D2 protein) |                         |                                  |                                                          |
| S79F                 | AB-loop, in lumen        | +                                |                                                          |
| V247M/A249T          | Q_{A} niche              | +                                |                                                          |
| S254F                | Q_{A} niche              | +                                |                                                          |
| G255D                | Q_{A} niche              | +                                |                                                          |
| A260V                | Q_{A} niche              | +                                |                                                          |
| G279D                | E-helix                 | +                                |                                                          |
| G285S/G288D          | E-helix                 | +                                |                                                          |
| R294W                | C terminus, in lumen     | +                                |                                                          |
| psbA2 mutant (D1 protein) |                         |                                  |                                                          |
| \(\Delta\)N266–N267  | Q_{B} niche              |                                  |                                                          |
| psbB mutants (CP47 protein) |                       |                                  |                                                          |
| \(\Delta\)T271–K277  | E-loop, in lumen         |                                  |                                                          |
| \(\Delta\)K277–E283  | E-loop, in lumen         |                                  |                                                          |
| \(\Delta\)T304–L309  | E-loop, in lumen         |                                  |                                                          |
| \(\Delta\)P422–E428  | E-loop, in lumen         |                                  |                                                          |
| \(\Delta\)T304–L309  | E-loop, in lumen         |                                  |                                                          |
| \(\Delta\)P422–E428  | E-loop, in lumen         |                                  |                                                          |

a Described in Ref. 23.
b These are the mutations present in the D2R8 strain (12).c S. Ermakova-Gerdes and W. Vermaas, unpublished data.
d Described in Ref. 24.e Described in Ref. 25.

Fig. 2. PCR amplification of the slr0399 gene using as templates total chromosomal DNA from the wild type (lane 2), slr0399\(^{-}\) (lane 3), D2R8 (lane 4), D2R8/slr0399\(^{-}\) (lane 5), S254F (lane 6), and S254F/slr0399\(^{-}\) (lane 7) strains. One primer located upstream and the other downstream of slr0399 have been used for the PCR amplification. Estimated sizes of the PCR products in kb are indicated on the right. Lane 1, 1-kb ladder.

D2R8 mutant has an apparently mobile \(Q_{A}\) resulting in inhibition of PS II electron transport by artificial quinones, most prominently duroquinone (DQ) (the \(I_{50}\) for inhibition of oxygen evolution in this mutant is 2 \(\mu\)M). Moreover, in the absence of artificial quinones, induction of variable fluorescence in D2R8 is very slow and the \(Q_{A}/\text{donor side charge recombination kinetics have become slower}\) (12). For this reason, the \(Q_{A}\) properties of the D2R8/slr0399\(^{-}\) strain were characterized. As indicated in Fig. 3, in the D2R8/slr0399\(^{-}\) strain the \(I_{50}\) value for the inhibition of oxygen evolution by DQ was about 25 \(\mu\)M, which is an order of magnitude higher than that for the D2R8 mutant. However, DQ is still a more potent inhibitor in the D2R8/slr0399\(^{-}\) strain than in wild type. Other quinones (2,5-dichloro-p-benoquinone, 2,5-dimethyl-p-benoquinone) that inhibited electron transfer in the D2R8 mutant (12) had little effect on electron transfer in the D2R8/slr0399\(^{-}\) strain (data not shown). Moreover, the D2R8/slr0399\(^{-}\) strain displayed reasonably normal fluorescence induction kinetics (data not shown) and the yield of variable fluorescence (Table III) had increased significantly compared with D2R8. In addition, the rates of the \(Q_{A}\) oxidation by \(Q_{B}\) (in the absence of DCMU) and by charge recombination with the PS II donor side (in the presence of DCMU) in the D2R8/slr0399\(^{-}\) strain were similar to those in the wild type (Table IV).

This reversal toward wild type properties upon inactivation of slr0399 was not specific for D2R8. In the S254F mutant fluorescence and other PS II properties were altered as well, although not as drastically as in D2R8, and were restored to essentially wild type characteristics upon inactivation of slr0399 (Tables III and IV).

Prenylquinone Analysis—The results presented above are indicative of an alteration of \(Q_{A}\) function in D2 mutants, but not in wild type, upon inactivation of slr0399. One possibility to lead to this phenotype is that the slr0399 gene product influences the quinone composition and/or the amount of quinone in the membranes of the organism. To determine whether this may be the case, all prenyllipids, including prenylquinones, were isolated from the D2R8, D2R8/slr0399\(^{-}\), wild type, and slr0399\(^{-}\) strains and separated by reverse-phase HPLC. HPLC separation of prenyllipids from the wild type and the slr0399\(^{-}\) strain is presented in Fig. 4, indicating no major differences between these two strains, although the level of phyloquinone had been somewhat decreased in the mutant. The prenylquinone composition of the other two strains was also similar (data not shown). Only PQ and phyloquinone (\(\text{vitamin K}_{1}\)) were found as prenylquinones in \(\text{Synechocystis}\) sp. PCC 6803, similar to what has been reported before in other cyanobacterial species (26, 27). No new prenylquinone species were detected upon deletion of slr0399, indicating that the mechanism of the restoration of PS II function in pseudorevertants is not due to accommodation of a quinone different than PQ at the altered \(Q_{A}\) site.

Possible Functions of Slr0399—The results above indicate that Slr0399 is not involved with quinone synthesis, but a role of this protein as a chaperone in plastquinone insertion into nascent photosystem II seems certainly a viable hypothesis.
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**Table III**

| Strain        | Photoautotrophic doubling time | Oxygen evolution | Chl/DCMU binding site ratio | Chl/DCMU binding site ratio | K<sub>DCMU</sub> | (F<sub>m</sub> - F<sub>i</sub>)<sup>2</sup>F<sub>i</sub> | K<sub>B</sub> |
|---------------|-------------------------------|-----------------|-----------------------------|-----------------------------|----------------|-------------------------------|----------|
| D2R8          | ∞                             | 100             | 3700 ± 600                  | 15                          | 66 ± 11        | 0.024 ± 0.007                 | 0.36 ± 0.01 |
| D2R8/Slr0399<sup>-</sup> | 16                            | 190             | 1060 ± 120                  | 52                          | 22 ± 2         | 0.37 ± 0.02                   | 0.08 ± 0.008 |
| S254F         | ∞                             | 120             | 3090 ± 110                  | 18                          | 31 ± 6         | 0.22 ± 0.03                   | 0.49 ± 0.01 |
| S254F/Slr0399<sup>-</sup> | 21                            | 250             | 1110 ± 110                  | 50                          | 19 ± 0.5        | 0.49 ± 0.02                   | 0.49 ± 0.02 |
| Wild type     | 12                            | 320             | 550 ± 30                    | 100                         | 17 ± 1         |                                |          |
| slr0399<sup>-</sup> | 13                            | 315             | 540 ± 120                   | 102                         | 19 ± 1         |                                |          |

**Fig. 3. Inhibition of steady-state oxygen evolution in continuous light by DQ in the D2R8 (●), D2R8/Slr0399<sup>-</sup> (▲), and wild type (○) strains.** Oxygen evolution was measured at saturating light intensity and in the presence of 0.5 mM K<sub>3</sub>[Fe(CN)]<sub>6</sub> (which does not penetrate cells) to keep DQ oxidized.

Slr0399 consists of 326 amino acids and is similar to the putative protein of unknown function Ycf39 that is encoded in the cyanelle genome of *Cyanothecaceae paradoxa* (<sup>2</sup> 53% identity, 73% similarity) and in the chloroplast genomes of non-green algae including *Ochrosphaera neapolitana* (<sup>2</sup> 65% similarity, 43% identity), *Oxotricha sinensis* (<sup>4</sup> 69% similarity, 45% identity) (<sup>38</sup>), *Porphyra purpurea* (<sup>5</sup> 72% similarity, 50% identity) (<sup>29</sup>), and *Cyanidium caldarium* (<sup>5</sup> 55% similarity, 35% identity). It is also similar to the predicted translation product of an *A. thaliana* nuclear gene (70% similarity and 58% identity) located on chromosome 4, BAC clone F23E12.7

An alignment of Slr0399 and its homologues from non-green algae and *Arabidopsis* is provided in Fig. 5. Regions of high similarity between Slr0399 and Ycf39 proteins are scattered throughout the protein. The only clearly identifiable functional domain in Slr0399 is a conserved NAD(P)/H-binding motif near the N terminus of the protein (Fig. 5). This putative nucleotide-binding domain contains a β-α-β-α-β motif, which is present in all classical dinucleotide binding proteins (for review, see Ref.

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<sup>2</sup> V. L. Stirewalt, C. B. Michalowski, W. Löffelhardt, H. J. Bohnert, and D. A. Bryant, GenBank<sup>®</sup> accession no. U30821.

<sup>3</sup> V. A. R. Huss, A. C. Tietze, and C. Julius, C., GenBank<sup>®</sup> accession no. X90677.

<sup>4</sup> GenBank<sup>®</sup> accession no. Z67753.

<sup>5</sup> GenBank<sup>®</sup> accession no. U58804.

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<sup>7</sup> M. Bevan, H. Hilbert, M. Braun, H. Holzer, A. Brandt, A. Duesterhoeft, J. Hoheisel, T. Jesse, L. Heinjen, P. Vos, H. W. Mewes, K. F. K. Mayer, and C. Schueler, GenBank<sup>®</sup> accession no. AL022604.

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30), and which interacts with the adenosine pyrophosphoryl moiety of the cofactor (NAD, NADP, or possibly FAD). Residues 3–32 of Slr0399 constitute the fingerprint region (β-α-β), derived from known structures of dinucleotide-binding enzymes (<sup>31</sup>, <sup>32</sup>). This region contains a glycine-rich phosphate-binding consensus sequence GXGXGXXG and six conserved hydrophobic residues at characteristic locations (marked with asterisks in Fig. 5). The absence of a conserved Asp or Glu at the carboxyl end of the β-α-β motif and the presence a conserved Arg residue instead (Fig. 5) suggest that Slr0399 binds NADPH rather than NADH (<sup>33</sup>, <sup>34</sup>). Moreover, a fingerprint motif present in FAD-binding enzymes (<sup>35</sup>) is not obvious in Slr0399, arguing against the possibility that FAD would serve as a cofactor in this protein.

The position of this highly conserved region with the NAD(P)/H-binding motif so close to the N-terminal end of the protein sequence suggests that Slr0399/Ycf39 are not processed in the cytoplasm or in eukaryotes when this protein is chloroplast-encoded. Therefore, Slr0399 is expected to remain in the cytoplasm in *Synechocystis* sp. PCC 6803 (<sup>36</sup>) and Ycf39 is expected to be located in the chloroplast stroma and to not be translocated into the lumen. The presence of a putative chloroplast targeting leader sequence in the *A. thaliana* nuclear-encoded Ycf39 protein (PSORT software; Refs. 36 and 37) is in agreement with targeting into the chloroplast stroma (data not shown).

Slr0399 and its Ycf39 homologues from eukaryotes are mostly hydrophilic. Slr0399 has a single hydrophobic domain that is long enough to span the thylakoid membrane (Tyr-139 to Leu-160), but because in Ycf39 homologues this region sometimes carries charges it is unlikely that this domain is an actual membrane-spanning region. Also, this region is unlikely to form an α-helix, and therefore, this protein is likely to not span the membrane.

**Slr0399 Effect on Thermotolerance**—As indicated earlier, a possible explanation of the data presented in this paper is that Slr0399 is a chaperone-like protein that aids in, but is not essential for, quinine insertion and protein folding around QA in photosystem II. Lack of chaperone-like proteins sometimes leads to a thermosensitive phenotype (<sup>41</sup>). To determine the temperature sensitivity in relation to the presence of Slr0399, the wild type and slr0399<sup>-</sup> *Synechocystis* sp. PCC 6803 strains were first grown at 30 °C at 50 µmol photons m<sup>-2</sup> s<sup>-1</sup> light intensity and were then diluted and transferred to 39 °C at the same light intensity. This temperature is close to the temperature maximum (40–44 °C) for photoautotrophic growth of *Synechocystis* sp. PCC 6803 wild type (<sup>42</sup>). The photoautotrophic growth rate of both strains immediately after transfer to 39 °C was essentially zero. This is consistent with all the photosynthetic parameters being much lower than those of the wild type (Table III).

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<sup>8</sup> Software used for the protein secondary structure analysis was as follows: hydropathy analysis using algorithm of Kyte and Doolittle (<sup>38</sup>), DAS (<sup>39</sup>), TMpred, and SOSUI (<sup>40</sup>).
Inactivation of slr0399 in Synechocystis

Chlorophyll a fluorescence decay traces were deconvoluted assuming the presence of two exponential components; if a one-component deconvolution provided an equally good fit, the second component was omitted. $A_1$ and $A_2$ represent the amplitudes of the two phases (normalized to give a sum of 100); ($t_{1/2}^1$), and ($t_{1/2}^2$) are the corresponding decay half-times. Data were reproducible within about 15%. Note that the variable fluorescence intensity of D2R8 is very small, and in this strain the error therefore is larger (up to about 25%).

| Strain                  | $A_1$ | $t_{1/2}^1$ | $A_2$ | $t_{1/2}^2$ | $A_1$ | $t_{1/2}^1$ | $A_2$ | $t_{1/2}^2$ |
|-------------------------|------|------------|------|------------|------|------------|------|------------|
| D2R8                    | 100  | 830        |      |            |  0.19|  60        |  0.92|            |
| D2R8/slr0399$^-$        |  78  |  510       |  22  |  3.2       |  40  |  0.18      |  60  |  0.92      |
| S254F                   |  83  |  430       |  17  |  4.2       |  54  |  0.35      |  46  |  2.2       |
| S254F/slr0399$^-$      |  53  |  230       |  47  |  1.4       |  56  |  0.21      |  44  |  0.99      |
| Wild type               |  65  |  340       |  35  |  2.0       |  48  |  0.24      |  52  |  0.98      |
| slr0399$^-$             |  64  |  300       |  36  |  1.9       |  48  |  0.21      |  52  |  0.74      |

39 °C was very similar and almost twice as fast as observed at 30 °C. However, after about 5 cell divisions, the slr0399$^-$ culture abruptly stopped dividing and eventually died, whereas the control strain continued growing at a rapid rate (Fig. 6A). The cessation of growth of the slr0399$^-$ strain was related to the number of cell divisions rather than to the length of 39 °C exposure because at limiting light intensity (9 μmol photons m$^{-2}$ s$^{-1}$) where the growth rate of the wild type and slr0399$^-$ strains had decreased by a factor of 3, the cell division in the slr0399$^-$ strain stopped after a 3-fold longer heat exposure (corresponding again to 5 cell divisions) (data not shown). The inability of the slr0399$^-$ strain to sustain photoautotrophic growth at 39 °C was not due solely to inactivation of the PS II complex, since addition of glucose did not alleviate the cessation of growth (Fig. 6B). Furthermore, in the presence of glucose slr0399$^-$ cells stopped dividing even earlier, after about 3 to 4 cell divisions at 39 °C (Fig. 6B). This indicates that Slr0399 appears to serve a chaperone function involving complexes other than PS II as well. Indeed, quinone-binding complexes are common in both photosynthetic and respiratory electron transfer, and therefore a role of Slr0399 that goes beyond PS II is not unexpected.

**FIG. 4.** HPLC analysis of prenyllipids isolated from membranes of the wild type (A) and slr0399$^-$ (B) strains of Synechocystis sp. PCC 6803. The peaks identified are chlorophyll a (Chl), pheophytin (Pheo), phytoquinone (Vit K$_1$), and plastoquinone (PQ). Only oxidized forms of prenylquinones are present since membranes were treated with 0.5 mM K$_3$[Fe(CN)$_6$] prior to extraction.

**DISCUSSION**

A novel technique of functional complementation with size-separated restriction fragment pools (3) used in this work for the localization of a pseudoreversion has become feasible with the availability of the entire Synechocystis sp. PCC 6803 genome sequence (4) and, hence, its genomic restriction map. This method exploits the ability of this naturally transformable cyanobacterium to integrate exogenous DNA into its genome by homologous recombination (1, 2). Application of this procedure in Synechocystis sp. PCC 6803 is a powerful and elegant approach toward mapping the sites of secondary mutations in pseudorevertants, as well as of spontaneous mutations and mutations introduced via random mutagenesis. A main factor determining the suitability of this approach is whether a strong selection for screening of complemented transformants is available. In the case of the work described here, this strong selection was provided by restoration of photoautotrophic growth.

In all D2R8 pseudorevertants that have been mapped, photoautotrophic growth was restored due to secondary mutations in slr0399, an open reading frame on the Synechocystis sp. PCC 6803 chromosome. Targeted deletion of the entire 3'-terminal half of slr0399 also restored photoautotrophic growth in D2R8. The D2R8 pseudorevertants and the D2R8/slr0399$^-$ mutant showed increased PS II levels as compared with D2R8 (Table III), and the functional characteristics of QA were restored to close to wild type values (Table IV and Fig. 3). Interestingly, inactivation of slr0399 also restored photoautotrophic growth and PS II properties in some other obligate phototrophic psbD1 mutants that had alterations around the QA site and that retained some oxygen evolution. However, other PS II mutants could not be restored by inactivation of slr0399 (Table II), suggesting a specific interaction between Slr0399 and the QA$_b$ site of PS II.

The Synechocystis sp. PCC 6803 open reading frame slr0399 is expected to encode a 36-kDa protein, which shows high similarity with hypothetical protein Ycf39 (Fig. 5). It is noteworthy that the ycf39 gene, present in chloroplast genomes in non-green algae, was transferred to the nuclear genome later in evolution of plants. This protein contains a putative...
NAD(P)H-binding motif and has an overall similarity with two groups of proteins. One group is the family of isoflavone reductase-like (IRL) proteins that are present in different plants species (43–48). Typically, Slr0399 is 25% identical and 40% similar to IRL proteins from plants (Fig. 5); the level of similarity to other groups of reductases is much lower (data not shown). Slr0399 also shares similarity (about 20% identity and 40% similarity) with NueM9 from the hyperthermophilic bacterium *Aquifex aeolicus* that has been identified as a subunit of a NADH:ubiquinone oxidoreductase on the basis of its sequence similarity with the 39-kDa subunit of the bovine mitochondrial complex I (49).

The IRL proteins have been grouped in a family solely on the basis of their high sequence similarity with isoflavone reductases, which catalyze the reduction of α,β-unaturated ketones and which are involved in biosynthesis of isoflavonoid phytoalexins in legumes in response to fungal infection (50–52). However, IRL proteins have been identified in plants that do not synthesize isoflavonoid phytoalexins in response to pathogen attack (53), and therefore IRLs are likely to have broader functions. IRLs have been implied to function in response to oxidative stress in *Arabidopsis* (44), prolonged sulfur starvation in maize (45), and to UV radiation in harvested grapefruit (48). It has been suggested that all isoflavone reductase-like proteins are oxidoreductases utilizing NAD(P)H as a cofactor, which have various substrates that may or may not be related structurally to flavonoids (43, 44).

The similarity of Slr0399 with NueM9 from *Aquifex aeolicus*
and with 39/40-kDa subunit of the NADH:ubiquinone oxidoreductase complex I of fungi and mammals (54, 55) may be important, as these proteins may interact with quinones. The peripheral 39/40-kDa subunit of complex I in eukaryotes has no homologue in the “minimal” 14-subunit bacterial respiratory NADH dehydrogenase. The precise function of this subunit remains unknown.

The fact that inactivation of slr0399 can complement different mutations in the QA- binding region of the D2 protein and does not complement mutations in several other PS II subunits suggested to us that Slr0399 may be involved with PQ metabolism or biosynthesis, PQ incorporation into PS II centers, QA suggested to us that Slr0399 may be involved with PQ metabolism (for example, leading to accumulation of quinones that can bind tightly to the altered QA site in the D2R8 mutant and that are functional at this site) is countered by our observation that the slr0399 inactivation mutant has a temperature-sensitive phenotype (Fig. 6). The fact that this phenotype persists even in the presence of glucose (when no PS II activity is needed for growth) suggests that the function of Slr0399 is not limited to assembly of PS II, but may involve other quinone-binding complexes such as PS I and the NADH:ubiquinone oxidoreductase complex I.

In the Synechocystis sp. PCC 6803 genome, there are two other open reading frames encoding putative proteins that are similar to the N-terminal 150–200 amino acids of Slr0399: slr1218 (31% identity and 52% similarity of the corresponding polypeptide with Slr0399) and slr0317 (23% identity and 42% similarity). The calculated molecular masses of Slr1218 and Slr0317 are 24 and 32 kDa, respectively. Both proteins contain a NAD(P)H-binding domain near the N terminus, suggesting that these two hypothetical proteins are dehydrogenases or reductases, but they may differ from Slr0399 in their specific function.

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