2-1-2004

Four Novel Genes Required for Optimal Photoautotrophic Growth of the Cyanobacterium Synechocystis sp. Strain PCC 6803 Identified by In Vitro Transposon Mutagenesis

Shulu Zhang
Louisiana State University

Susan M. Laborde
Louisiana State University

Laurie K. Frankel
Louisiana State University

Terry M. Bricker
Louisiana State University

Recommended Citation

Zhang, S., Laborde, S., Frankel, L., & Bricker, T. (2004). Four Novel Genes Required for Optimal Photoautotrophic Growth of the Cyanobacterium Synechocystis sp. Strain PCC 6803 Identified by In Vitro Transposon Mutagenesis. *Journal of Bacteriology, 186* (3), 875-879. https://doi.org/10.1128/JB.186.3.875-879.2004

JOURNAL OF BACTERIOLOGY, Feb. 2004, p. 875–879 Vol. 186, No. 3
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.3.875–879.2004

Four Novel Genes Required for Optimal Photoautotrophic Growth of the Cyanobacterium *Synechocystis* sp. Strain PCC 6803 Identified by In Vitro Transposon Mutagenesis

Shulu Zhang, Susan M. Laborde, Laurie K. Frankel, and Terry M. Bricker*

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

Received 28 August 2003/Accepted 29 October 2003

Four novel *Synechocystis* sp. strain PCC 6803 genes (*sll1495*, *sll0804*, *slr1306*, and *slr1125*) which encode hypothetical proteins were determined by transposon mutagenesis to be required for optimal photoautotrophic growth. Mutations were also recovered in *ccmK4*, a carboxysome coat protein homologue, and *me*, the decarboxylating NADP$^+$-dependent malic enzyme. This is the first report that these known genes are required for optimal photoautotrophy.

Photosynthesis is one of the most important biological processes and occurs in a very diverse set of organisms ranging from prokaryotes to eukaryotes. Recently, much effort has been directed towards understanding the structure and function of proteins involved in photosynthesis (photosystem I, photosystem II, cytochrome b_6/f complex, Calvin-Benson cycle enzymes, etc.). While much progress has been made in the understanding of the functional organization of these proteins, relatively little is known concerning the organization of other protein components which must be involved in the regulation, assembly, and turnover of the proteins involved in photosynthesis. Cyanobacteria are photoautotrophic gram-negative eubacteria capable of performing oxygenic photosynthesis in a manner quite similar to that in eukaryotic algae and higher plants. *Synechocystis* sp. strain PCC 6803 is a naturally competent unicellular cyanobacterium and has proved to be one of the best model organisms for studying the mechanism and regulation of oxygenic photosynthesis (15). We are interested in identifying the genes required for oxygenic photosynthesis. In this study, we used a hyperactive Tn*5*-based in vitro transposition system to introduce random insertional mutations into *Synechocystis* and have identified a number of mutants which are incapable of undergoing optimal photoautotrophic growth. Here we describe the production, identification, and characterization of a number of these mutants. The structure and possible function of the affected genes in these mutants will also be discussed.

A glucose-tolerant strain of *Synechocystis* sp., PCC 6803 (15), was used as a parental control and as the DNA recipient strain in the present study. Cells of both the control strain and the derivative photosynthetic mutants were maintained under photoheterotrophic growth conditions at 30°C with a light intensity of 20 μmol of photons m^{-2} s^{-1} (fluorescent light) in liquid BG-11 growth medium (ATCC medium 616) supplemented with 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-KOH (pH 8.2), 5 mM glucose, and 10 μM DCMU [*N*-(3,4-dichlorophenyl)-*N*′-dimethylurea]. Liquid cultures were bubbled continuously with air. For autotrophic cell culture, the glucose and DCMU were omitted. For cultures grown on plates, the BG-11 medium was supplemented with 1.5% agar and 0.3% sodium thiosulfate. When appropriate, kanamycin was included in the media at a final concentration of 10 μg/ml.

A *Synechocystis* genomic library which had been subjected to in vitro transposon mutagenesis and then amplified in *Escherichia coli* (1, 2) was kindly provided by D. Bhaya at the Carnegie Institute. This DNA was used to transform parental *Synechocystis* cells which are naturally competent and which exhibit high rates of homologous recombination (15). After selection on kanamycin-containing photoheterotrophic growth medium, followed by multiple transfers to allow sorting out, ca. 10,000 individual kanamycin-resistant cell lines were screened for the ability to grow photoautotrophically on agar plates. Those cell lines that repeatedly failed to grow photoautotrophically but which did grow photoheterotrophically (17 cell lines) were identified as putative photosynthetic mutants and selected for further analysis.

Southern blot analysis indicated that all 17 cell lines exhibited single transposon insertions (data not shown). The location of the transposon insertion was identified either by direct genomic sequencing (1) or by inverse PCR (8). Since the *Synechocystis* genome has been sequenced (11), the identification of the genes disrupted by the transposon mutagenesis is facile. Growth rates in liquid culture under both photoheterotrophic and photoautotrophic conditions at 30°C with a light intensity of 20 μmol of photons m^{-2} s^{-1} were monitored for 10 days. The cell sizes of the control strain and the mutants were similar (±10%) as determined by differential interference contrast microscopy. O_2 evolution activity during mid-log-phase growth was assayed by oxygen polarography with 1 mM bicarbonate as an electron acceptor.

We have determined the transposon insertion sites for 14 out of 17 mutants which we have isolated (Table 1). For three of the mutants, both direct genomic sequencing and inverse PCR failed repeatedly. Among the 14 identified mutants, 13 mutants each had a single insertion within the protein-encoding region, resulting in a premature truncation of the encoded protein. The 4YE2 mutant, unlike the other 13 mutants, had

* Corresponding author. Mailing address: Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803. Phone: (225) 578-1555. Fax: (225) 578-7258. E-mail: BTBRIC@LSU.EDU.

TABLE 1. Photosynthetic mutants identified in this study

Mutant	Gene designation[a]	Name	Annotation	Function
4YE2	*slr0906*	*psbB*	PSII[b] light-harvesting core protein CP47	PSII structural protein
RB12	*sll0851*	*psbC*	PSII light-harvesting core protein CP43	PSII structural protein
3DH6	*sll1127*	*menB*	Naphthoate synthase[c]	Menaquinone synthesis
5AH9	*sll1127*	*menB*	Naphthoate synthase[c]	Menaquinone synthesis
3DC8	*sll1127*	*menB*	Naphthoate synthase[c]	Menaquinone synthesis
AH4	*slr0492*	*menE*	*O*-Succinylbenzoic acid-CoA ligase[c]	Menaquinone synthesis
DDE5	*slr0492*	*menE*	*O*-Succinylbenzoic acid-CoA ligase[c]	Menaquinone synthesis
AAE6	*slr1839*	*ccmK4*	CO_2-concentrating mechanism protein K homologue[c]	CO_2 fixation, required for optimal photoautotrophy[d]
5ND9	*slr1839*	*ccmK4*	CO_2-concentrating mechanism protein K homologue[c]	CO_2 fixation, required for optimal photoautotrophy[d]
3WEZ	*slr0721*	*me*	Malic enzyme	Pyruvate metabolism, required for optimal photoautotrophy[d]
4YD9	*slr1125*	NA[e]	Probable glucosyltransferase[f]	Required for optimal photoautotrophy[d]
3ZA12	*slr1306*	NA	Hypothetical protein[f]	Required for optimal photoautotrophy[d]
4BA2	*sll0804*	NA	Hypothetical protein[f]	Required for optimal photoautotrophy[d]
CG4	*sll1495*	NA	Hypothetical protein[f]	Required for optimal photoautotrophy[d]

[a] *Synechocystis* sp. strain PCC 6803 open reading frame designation.
[b] PSII, photosystem II.
[c] Please note that the different mutations within the same gene are independent and bear transposon insertions at different locations.
[d] This study.
[e] NA, not applicable with no gene name or function yet determined.
[f] Cyanobase annotation (http://www.kazusa.or.jp).

the single insertion in the 3′ noncoding region of the *psbB* gene. It should also be noted that for three genes, *menB*, *menE*, and *ccmK4*, multiple independent transposon insertions were observed (Table 1).

A total of 10 genes were affected by the insertion of the transposon in the 14 identified mutants (Table 1). These 10 genes include six which had been previously identified and four hypothetical genes. The majority of the known genes which have been disrupted encode components known to be involved in photosynthesis. These include *psbB* (*slr0906*), which encodes the photosystem II core protein CP47, and *psbC* (*sll0851*), which encodes the photosystem II protein CP43. These are known essential structural components of photosystem II (3, 4). The *ccmK4* gene (*slr1839*) encodes the carbon dioxide-concentrating mechanism protein homologue 4. This is one of the four carboxysome coat protein homologues present in *Synechocystis*. The carboxysome has been hypothesized to be involved in concentrating carbon dioxide for photosynthetic carbon fixation (5, 14). This is the first report indicating that this particular *ccmK* homologue is required for optimal photoautotrophy. The *menB* gene (*sll1127*) encodes the enzyme 1,4-dihydroxy-2-naphthoate synthase, and *menE* (*slr0492*) encodes the enzyme *O*-succinylbenzoic acid coenzyme A (CoA) ligase. These enzymes function in the menaquinone biosynthetic pathway leading to the biosynthesis of phylloquinone, the secondary electron acceptor of photosystem I (9, 10). The recovery of mutations in these known genes indicated that the screen employed in this study allows the identification of genes required for photoautotrophy.

In addition to these components, insertional inactivation of the *me* gene (*slr0721*), which encodes the malic enzyme (decarboxylating malate oxidoreductase, EC 1.1.1.39), leads to a loss of optimal photoautotrophy. This enzyme catalyzes the oxidative decarboxylation of malate into pyruvate. It is unclear, at this time, why inactivation of this gene would cause the loss of optimal photoautotrophic growth. At least two hypotheses could explain this result. First, the malic enzyme could participate in the carbon-concentrating mechanism of *Synechocystis*. Recently Yang et al. (16) have hypothesized that a C_4-like pathway may operate in *Synechocystis* involving phosphoenolpyruvate carboxylase and the malic enzyme. Their hypothesis was based on results obtained from metabolic flux analysis of the metabolism of ^{13}C-labeled glucose under photoheterotrophic and mixotrophic conditions. Second, it is possible that the malic enzyme is involved in photorespiratory glycolate metabolism (6). Further studies testing these (and other) possibilities are ongoing.

The growth characteristics of the mutants 4YD9, 3ZA12, 4BA2, and CG4, which bear transposon insertions in the hypothetical genes *slr1125*, *slr1306*, *sll0804*, and *sll1495*, respectively, were further characterized. Figure 1 shows the growth curves which were obtained for these mutants in liquid culture under either photoheterotrophic (Fig. 1A) or photoautotrophic (Fig. 1B) conditions. Under photoheterotrophic conditions all of the mutants grew at rates very similar to that observed for the control strain. Under photoautotrophic conditions, however, all of the mutants exhibited significantly retarded growth rates compared to the control. The control strain grew about 4 times faster than the mutants 4BA2 and 3ZA12 and 12 times faster than the mutant 4YD9.

The mutant CG4 exhibited an unusual biphasic growth curve which was fully reproducible in each of three independent experiments that we performed. We have observed that this mutant exhibits a high rate of spontaneous reversion, recovering its ability to grow photoautotrophically. Sequencing of several of these revertants, however, indicated that the transposon

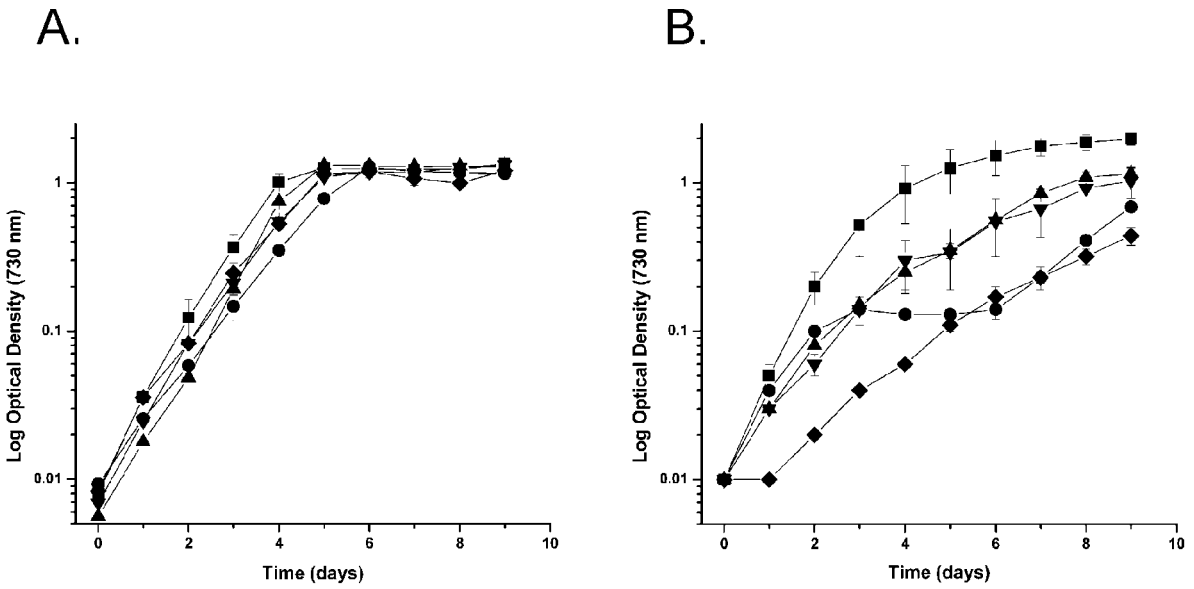

FIG. 1. Growth of the mutants CG4, 4YD9, 4BA2, and 3ZA12 in comparison to the parental control *Synechocystis* strain. (A) Photoheterotrophic growth. (B) Photoautotrophic growth. Symbols: ■, control strain; ●, CG4; ♦, 4YD9; ▲, 4BA2; ▼, 3ZA12. These data are the averages of three experiments; standard deviations are represented by vertical bars. Please note that in some instances the error is smaller than the symbols used.

insertion in *sll1495* had been maintained (data not shown). Apparently, secondary mutations at distant unidentified sites complement the loss of *sll1495* function. The accumulation of such second-site suppressor mutations which lead to growth at control rates during the photoautotrophic growth experiment may account for the biphasic growth curve observed.

We have also measured the whole-chain electron transport rates (H_2O to CO_2) in these mutants and the control strain. For most of the mutants, only modest decreases in electron transport were observed (70 to 80% of wild-type rates). In all cases, the observed rates were somewhat lower for the photoautotrophically grown cells than for those grown photoheterotrophically. Apparently, the lesions which engender the loss of optimal photoautotrophy in these mutants do not dramatically affect the ability to carry out whole-chain electron transport. Other systems such as cofactor assembly-stability, membrane assembly, photosynthate utilization, etc., must be affected in these mutants, leading to the loss of optimal photoautotrophic growth. The 4YD9 mutant, however, did exhibit significant loss of whole-chain electron transport activity, evolving oxygen at about 35% of the control rate when grown under photoautotrophic conditions. Additionally, this mutant exhibited an olive-green color which was significantly different from the brilliant blue-green coloration of the control strain and other mutant strains. Whole-cell absorption spectra for this mutant and the control strain at constant cell numbers are shown in Fig. 2. The mutant cells contain less than one-half of the chlorophyll *a* and no detectable phycobiloproteins. We hypothesize that this mutant may possess a defect in thylakoid membrane assembly-stability. A more complete description of the defects present in this mutant will appear elsewhere.

What are the characteristics of these hypothetical genes which are required for optimal photoautotrophy? The 4YD9 mutant exhibits a transposon insertion in the *slr1125* gene (Fig. 3A), which encodes a hypothetical protein of 402 amino acids and is predicted (PSORT-B, http://www.psort.org/ [7]) to be cytoplasmically localized. The protein is similar (up to 35% identity) to a few other known proteins including zeaxanthin glucosyltransferase (*crtX*) from *Erwina uredovora*, glycosyltransferase from *Streptomyces coelicolor*, and UDP glucuronosyltransferase from *Mus musculus*. Although the gene is annotated as encoding zeaxanthin glucosyltransferase in GenBank, we feel that this assignment is premature since the strongest region of similarity is in the C terminus of the protein, which is predicted to encode the glucosyltransferase domain. Similarity to other regions of the *Erwinia* zeaxanthin glucosyltransferase is at a significantly lower level.

The mutant 3ZA12 contains a transposon insertion in the

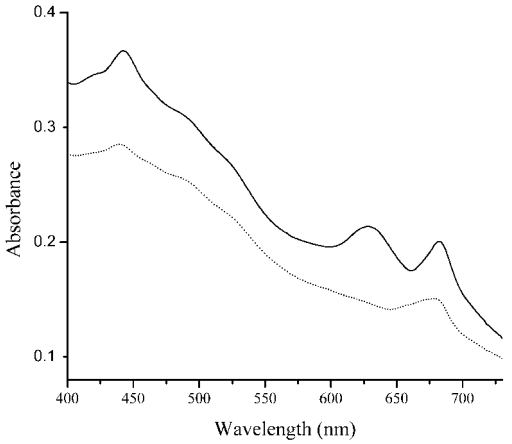

FIG. 2. Absorption spectrum of the control *Synechocystis* strain (solid line) and the mutant 4YD9 (dashed line). The control strain has been offset for clarity.

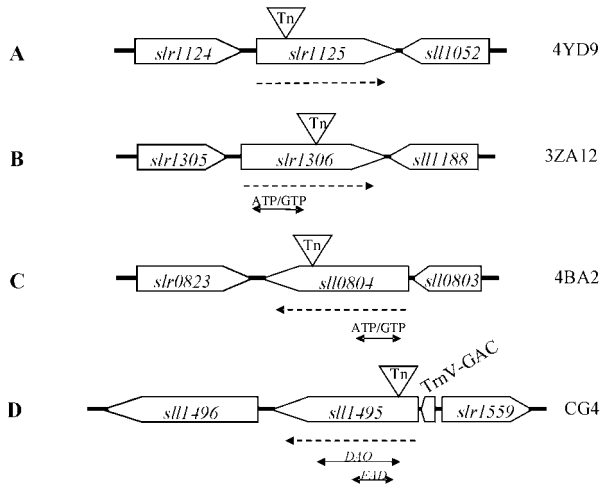

FIG. 3. Structural features of the four hypothetical genes *slr1125* (A), *slr1306* (B), *slr0804* (C), and *slr1495* (D) disrupted in the mutants 4YD9, 3ZA12, 4BA2, and CG4, respectively. Open reading frames in the vicinity of the targeted gene are shown, and the broken line below a gene indicates the direction of transcription. Putative functional domains are indicated by solid arrows. Tn, transposon; *DAO*, D-amino acid oxidase domain; *FAD*, flavin adenine dinucleotide binding domain; ATP/GTP, nucleotide-binding domain. Domains and/or motif assignments were obtained at http://www.pedant.gsf.de or, after BLAST searches, at http://www.ncbi.nlm.nih.gov.

slr1306 gene (Fig. 3B), which encodes a hypothetical protein of 485 amino acids. The protein contains an ATP-GTP-binding-site motif A (P loop) and has some similarities (up to 30% amino acid identities) to a number of other cyanobacterial hypothetical proteins including Slr1462 from *Synechocystis* and Alr4172 from *Nostoc* sp. Recently, this gene has been identified as one of the 181 cyanobacterial signature genes (13). These are genes which are present in the eight available cyanobacterial genomes but which are not present in other eubacteria.

The mutant 4BA2 contains a transposon insertion in the *sll0804* gene (Fig. 3C), which encodes a hypothetical protein of 453 amino acids. The protein contains an ATP-GTP-binding-site motif A (P loop) and has some similarities (up to 49% identical amino acids) to several other hypothetical cyanobacterial proteins including Alr0904 from *Nostoc* and Sll0503 from *Synechocystis*. This protein is predicted to be localized to the inner membrane by PSORT-B and is predicted to contain two transmembrane α-helices in the C-terminal third of the protein. Additionally, it contains two strongly predicted coiled-coil motifs (http://www.ch.embnet.org [12]).

The mutant CG4 bears a transposon insertion in the *sll1495* gene (Fig. 3D), which encodes a hypothetical protein of 397 amino acids. The Sll1495 protein appears to be a dehydrogenase and contains both a D-amino acid oxidase (*DAO*)-binding motif and a flavin adenine dinucleotide-binding domain. The protein has a high level of similarity (70% identical amino acids) to Alr2826 from *Nostoc* sp. Additionally, it shares strong similarity (50% identity) to a number of proteins which have been identified as possible transcriptional regulators, including a putative γ-aminobutyrate DTP gene cluster repressor from *Salmonella* sp. and the putative transcriptional regulator VCA0147 from *Vibrio cholerae*. This raises the interesting possibility that the Sll1495 protein may be a redox-active transcriptional regulator. No direct evidence supporting this hypothesis, however, is available at this time.

The *sll1495* gene is flanked by *sll1496* (mannose-1-phosphate guanyltransferase) and a valyl tRNA. These three genes could be organized in a possible operon. The insertion of the transposon in the *sll1495* gene could give rise to polar effects with respect to the *sll1496* gene, yielding the observed phenotype. To test this possibility, insertional mutagenesis was used to introduce a kanamycin resistance cassette in the noncoding DNA located between the *sll1495* and *sll1496* genes. The phenotype of this insertional mutant was identical to that of the control strain (data not shown). This indicated that the transposon insertion into the *sll1495* gene was responsible for the observed phenotype of the CG4 mutant and that polar effects with respect to *sll1496* were not occurring.

Transposon mutagenesis has proved to be a useful tool in the identification of genes required for photoautotrophy. In most genomes which have been sequenced, 30 to 40% of the identified genes have no known function. The identification of a substantive phenotype for such hypothetical genes is a required first step toward the elucidation of their role in cellular metabolism. While the specific functions of the hypothetical genes which we have identified in this study have not, at this time, been determined, it is clear that they are required for optimal photoautotrophic, but not photoheterotrophic, growth. We hypothesize that these genes may function in the regulation, assembly, and/or turnover of the proteins involved in the photosynthetic process.

Support for this project was provided by grants from the Department of Energy and the National Science Foundation to L.K.F. and T.M.B.

REFERENCES

1. **Bhaya, D., A. Takahashi, and A. R. Grossman.** 2001. Light regulation of type IV pilus-dependent motility by chemosensor-like elements in *Synechocystis* PCC6803. Proc. Natl. Acad. Sci. USA **98:**7540–7545.
2. **Bhaya, D., A. Takahashi, P. Shahi, and A. R. Grossman.** 2001. Novel motility mutants of *Synechocystis* strain PCC 6803 generated by in vitro transposon mutagenesis. J. Bacteriol. **183:**6140–6143.
3. **Bricker, T. M.** 1990. The structure and function of CPa-1 and CPa-2 in photosystem II. Photosynth. Res. **24:**1–13.
4. **Bricker, T. M., and L. K. Frankel.** 2002. The structure and function of CP47 and CP43 in photosystem II. Photosynth. Res. **72:**131–146.
5. **Cannon, G. C., S. Heinhorst, C. E. Bradburne, and J. M. Shively.** 2002. Carboxysome genomics: a status report. Funct. Plant Biol. **29:**175–182.
6. **Colman, B.** 1992. Glycolate metabolism in cyanobacteria, p. 155–168. *In* H. Stabenau (ed.), Phylogenetic changes in peroxisomes of algae. University of Oldenburg, Oldenburg, Germany.
7. **Gardy, J. L., C. Spencer, K. Wang, M. Ester, G. E. Tusnády, I. Simon, S. B. Hua, K. de Fays, C. Lambert, K. Nakai, and F. S. L. Brinkman.** 2003. PSORT-B: improving protein subcellular localization prediction for Gram-negative bacteria. Nucleic Acids Res. **31:**3613–3617.
8. **Huang, G., L. Zhang, and R. G. Birch.** 2000. Rapid amplification and cloning of Tn5 flanking fragments by inverse PCR. Lett. Appl. Microbiol. **31:**149–153.
9. **Johnson, T. W., G. Shen, B. Zybailov, D. Kolling, R. Reategui, S. Beauparlant, I. R. Vassiliev, D. A. Bryant, A. D. Jones, J. H. Golbeck, and P. R. Chitnis.** 2000. Recruitment of a foreign quinone into the A1 site of photosystem I. I. Genetic and physiological characterization of phylloquinone biosynthetic pathway mutants in *Synechocystis* sp. PCC 6803. J. Biol. Chem. **275:**8523–8530.
10. **Johnson, T. W., B. Zybailov, A. D. Jones, R. Bittl, S. Zech, D. Stehlik, J. H. Golbeck, and P. R. Chitnis.** 2001. Recruitment of a foreign quinone into the A1 site of photosystem I. In vivo replacement of plastoquinone-9 by media-

supplemented naphthoquinones in phylloquinone biosynthetic pathway mutants of *Synechocystis* sp. PCC 6803. J. Biol. Chem. **276:**39512–39521.
11. **Kaneko, T., and S. Tabata.** 1997. Complete genome structure of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Plant Cell Physiol. **38:** 1171–1176.
12. **Lupas, A., M. Van Dyke, and J. Stock.** 1991. Predicting coiled coils from protein sequences. Science **252:**1162–1164.
13. **Martin, K. A., J. L. Siefert, S. Yerrapragada, Y. Lu, T. Z. McNeill, P. A. Moreno, G. M. Weinstock, W. R. Widger, and G. E. Fox.** 2003. Cyanobacterial signature genes. Photosynth. Res. **75:**211–221.
14. **Price, G. D., S. M. Howitt, K. Harrison, and M. R. Badger.** 1993. Analysis of a genomic DNA region from the cyanobacterium *Synechococcus* sp. strain PCC7942 involved in carboxysome assembly and function. J. Bacteriol. **175:** 2871–2879.
15. **Williams, J. G. K.** 1988. Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. Methods Enzymol. **167:**766–778.
16. **Yang, C., Q. Hua, and K. Shimizu.** 2002. Metabolic flux analysis in *Synechocystis* using isotope distribution from ^{13}C-labeled glucose. Metab. Eng. **4:**202–216.