Journal of Photoscience, Vol. 3, No. 1, pp. 23–28, 1996
Printed in the Republic of Korea.

ISSN 1225-8555/96

A NOVEL PHOTOHETEROTROPHIC MUTANT FOR *psaB* GENE OF *Synechocystis* sp. PCC 6803 GENERATED FROM TARGETED MUTAGENESIS

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(*Received* 5 *March* 1996; *accepted* 3 *April* 1996)

Abstract — To investigate the structure and function of photosystem I, cartridge mutagenesis technique was used to inactivate the *psaB* gene of photosystem I. From the screen, many strains which have potential defects in photosystem I were generated. Biochemical analysis revealed that B2, one of the mutant, had a reduced amount of chlorophyll. Electron transfer activity from photosystem II to photosystem I as oxygen uptake was the rate of 64 % of wild type. Also B2 showed a decreased photosystem I activity when measured by 77 K fluorescence emission spectrum. Particularly, immunodetection analysis showed that the B2 had reduced amount of PsaA/PsaB, but a normal range of PsaC and PsaD. Here we present a photoheterotrophic mutant for *psaB* gene as a unique model strain for future study of structural/functional relationship and biogenesis of photosystem I.

INTRODUCTION

Oxygenic photosynthesis occurs in a wide variety of organisms from cyanobacteria to higher plants. The structure of photosystem complex has been highly conserved during evolution[1]. Since the cyanobacterial genome is small, it is easy to manipulate genetically[2]. *Synechocystis* sp. PCC 6803 takes up and incorporates exogenous DNA into the genome by homologous recombination or other mechanism[3,4], thus allowing generation of specific mutants by targeted gene interruption or deletion[5,6]. Also the cyanobacterium *Synechocystis* 6803 is capable of growing on glucose medium without light allowing isolation of mutants defective in photosynthesis. So, the cyanobacterium *Synechocystis* sp. PCC 6803 provides an attractive model system to study the organization and function of photosystem.

Electron transfer components of photosystem I in plants, algae, and cyanobacteria appear to be bound by at least three polypeptides, PsaA, PsaB, and PsaC[7]. The PsaC protein has a molecular weight of approximately 9 kDa and is much like a ferrodoxin with two [4Fe-4S] centers[8]. Among other small polypeptides, PsaD is thought to be ferrodoxin binding site on the reducing side of photosystem I[9-11]. Targeted mutagenesis technique has been used to find out the function of certain protein in photosystem. Several photosystem I or II mutants were made by using the technique[5,6,10,12]. In this paper, we used targeted mutagenesis to investigate structural and functional relationship in core polypeptides of photosystem I. We report the generation and characterization of a mutant of *Synechocystis* 6803 which has a mutation at the *psaB* gene in photosystem I.

MATERIALS AND METHODS

Cyanobacterial strains and culture. The glucose-tolerant strain of the cyanobacterium *Synechocystis* sp. PCC 6803 was used as wild type. Wild type and mutant cells of *Synechocystis* 6803 were grown in Medium C or BG 11 with 1.5 % glucose or 1.5 % glucose and selective antibiotics (5 μg/ml chloramphenicol) for culturing mutant cells at 28°C under a light intensity of 21 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Cells at the early exponential phase were harvested for characterizations. Growth of cells was monitored by measuring A_{730} of cultures.

Targeted mutagenesis of PSI. The scheme for inactivation of the *psaB* gene is given in Figure 1. A *Bgl*II restriction endonuclease site in *psaB* gene was used to

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† *Abbreviations* : A_{730}, absorbance at 730 nm; HEPES, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidenedi fluoride; CAT, chloramphenicol acetyltransferase; Cm^R, chloramphenicol resistance

clone a DNA fragment containing a gene encoding chloramphenicol acetyltransferase that confers chloramphenicol resistance. Wild type *Synechocystis* sp. cells were transformed with the resultant plasmid, pOV300. Transformation was carried out according to the method previously described[6]. The transformants were selected and segregated for a few generations by single colony selection on BG 11 plates containing 1.5 % glucose and 5 μg/mL of chloramphenicol.

Spectroscopic methods. Absorbency spectra were recorded on a Beckman DU 7500 spectrophotometer (Beckman, Fullerton, CA., U.S.A). Pigments were extracted from cells using 90 % methanol. The amount of chlorophyll in whole cell was determined according to Williams[6].

Measurements of photosynthetic electron transport. Rates of oxygen evolution or uptake were determined according to the method of Xu et al.[10] by using a Clark type oxygen electrode unit (Yellow Springston Inc., USA) at 25 °C at a light intensity of 1,600 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Fluorescence spectra measurements. Fluorescence emission spectra were determined using a SLM-AMINCO (SLM instruments, Urbana, IL., USA) 8100 instrument. For 77 K emission spectra, intact cells were adjusted to a concentration of 50 μg chlorophyll/mL in 25 m*M* HEPES/NaOH, pH = 7.0 for spectra measured at liquid nitrogen temperature. The excitation wavelength was 590 nm, and the excitation and emission bandwidths were 8 and 2 nm, respectively.

Analytical gel electrophoresis and immunoblotting. Preparations of thylakoid membranes from *Synechocystis* were performed by following the method of Rögner et al[13]. with a minor modification. Thylakoid membranes were solublized in the presence of 2 % SDS and 1 % 2-mercaptoethanol. Electrophoresis of proteins was carried out using a slab gel prepared with 14 % acrylamide and 6 *M* urea. After tricine-SDS-PAGE, proteins were electrotransferred to PVDF membrane using a Bio-Rad Transblot system. Immunodetection was performed by using a Flash western kit (Stratagene cloning system) according to manufacturer's instruction. Antibodies against PsaA/PsaB, PsaC or PsaD subunits were kindly provided by Dr. Anastasios Melis, Dr. Hirozo Oh-oka or Dr. Nathan Nelson, respectively.

RESULTS AND DISCUSSION

To inactivate the *psaB* gene of *Synechocystis* 6803, a plasmid designated as pOV300 was constructed. pOV300 carries a fragment encoding chloramphenicol acetyl transferase (CAT) from the plasmid pHP45Ω-Cm[14] at *Bgl*II site of *psaB* gene of *Synechococcus* 7002 in plasmid pAQPR80[15]. Partial restriction maps of the insert region of pOV300 and of the *Synechocystis* 6803 *psaA-psaB* operon are shown in Figure 1. *Synechocystis* 6803 maintained under photoheterotrophic conditions was transformed with the plasmid pOV300. Selection for chloramphenicol resistance

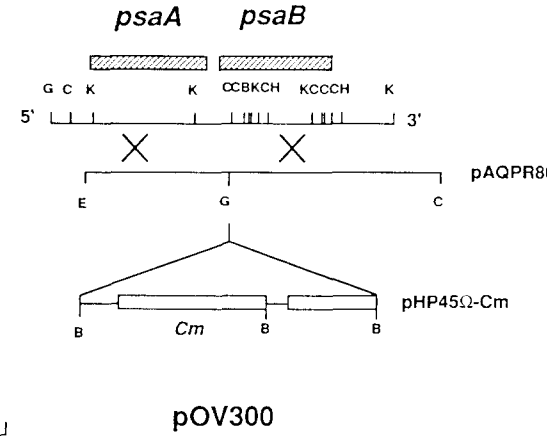

Figure 1. Restriction map of the insert region from plasmid pOV300 and of the *psaA-psaB* operon of *Synechocystis* sp. PCC 6803. Protein coding regions for *psaA* and *psaB* are shown as hatched boxes. Cloned *Synechocyoccus* 7002 DNA in pOV300 is indicated as a line (derived from pAQPR80[14]), while the Cm^R gene of the plasmid pHP45Ω-Cm[15] is indicated as a dotted box. Potential cross-over integration into *Synechocystis* sp. PCC 6803 chromosome are shown as crosses. Restriction sites are; G, *Bgl*II; C, *Hinc*II; K, *Kpn*I; B, *Bam*HI; H, *Hind*III; E, *Eco*RI.

(Cm^R) colonies was performed under heterotrophic culture condition (continuous light, 1.5 % glucose and 17μg/mL chloramphenicol). Several Cm^R colonies were selected and streaked at least five times to obtain full segregation of strains carrying mutation. Since *Synechocystis* 6803 maintains multiple copies of its genome per cell[12], complete segregation of a mutation may only be accomplished when the presence of wild type copies provides no selective advantage for the cells. Plasmid pOV300 is not able to replicate for itself in *Synechocystis* 6803 and this bacterium has an active homologous recombination system. So Cm^R colonies would most likely have a gene replacement event, as depicted in Figure 1. Unexpectedly, many mutants isolated display various colors (blue, green, olive, orange, and yellow) which is different from blue-green of wild type[16]. Among them, one Cm^R transformant, B2 has the most different color, brown, therefore was chosen and analyzed further.

Growth of the mutant and wild type strains was monitored by measuring A_{730} of cultures (Fig. 2). In the presence of 1.5 % glucose in the Medium C, B2 grew at a slightly different rate compared to wild type strain (photoheterotrophy). As shown in Table 1, wild type can grow photoautotrophically although doubling time at photoautotrophy was approximately 1.5-fold longer than that at photoheterotrophy. In contrast, B2 was unable to grow in the absence of glucose displaying a property common to all known photosystem-deficient mutants of *Synechocystis*

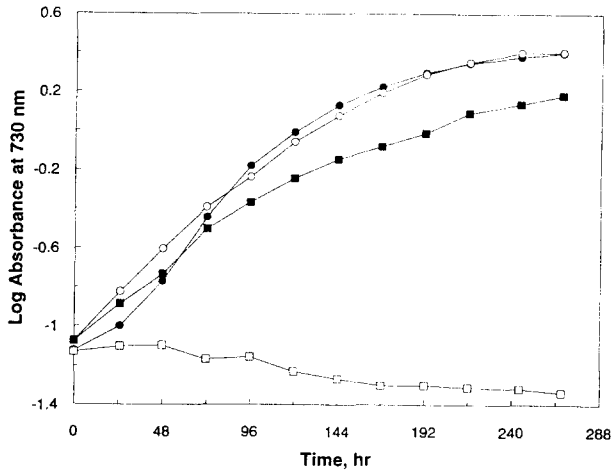

Figure 2. Photoautotrophic and photoheterotrophic growth of wild type and B2 strain of *Synechocystis* sp. PCC 6803 in Medium C. Actively growing cells of wild type (*filled symbol*) and B2(*open symbol*) were pelleted, then resuspended in the Medium C (*squares*) or Medium C containing 1.5% glucose (*circles*). The growth of these cultures was monitored by measuring their absorbance at 730 nm. Each treatment was replicated three times to determine averages.

6803[17].

To determine the effect of photosystem I mutation in B2 on pigment composition, thylakoid membranes were extracted by using 80 % acetone. Absorption spectra of pigment extracts from wild type and B2 are shown in Figure 3. B2 showed a decrease around 665 nm which was absorption region of chlorophyll, and an increase between 440 and 550 nm, due to carotenoids. The amount of chlorophyll from whole cells or from isolated membranes of wild type and B2 is presented in Table 1. About 60 % reduction of chlorophyll per cell was observed in B2 mutant compared to wild type. Also the amount of chlorophyll in membranes from B2 was reduced when normalized to equal amount of protein. Since photosystem I binds over 100 chlorophyll molecules per reaction center, P700[18], and more than 90 % of total chlorophyll in *Synechocystis* 6803 for light-grown cells is present in photosystem I[13], the reduction of over 60 % in chlorophyll content in B2 cells seems to be primarily due to a reduction of photosystem I core polypeptides. Also, it seems that the reduced amount of chlorophyll present in B2 results in allowing brown color in a culture state.

The light-dependent oxygen evolution or uptake measurements have been used as indication of the rates of photosynthetic electron transfer. For B2 cells, no oxygen evolution could be measured in overall and photosystem II chain, which is probably due to the second mutation in oxygen evolving complex of

Table 1. Some characteristics of wild type and photosystem mutant of *Synechocystis* sp. PCC 6803. Doubling times were determined by monitoring growth in Medium C. Chlorophyll (chl) contents were estimated for cultures at the same stage of growth in Medium C with or without 1.5% glucose. The datas were averages of at least four samples for each cell type.

	Wild type	B2
Doubling time (h)	42-52(59-73)[a]	40-54(NG)[b]
Chlorophyll contents		
Whole cells ($\mu g/A_{730}$)	4.94	1.99
Membranes (μg chl/mg protein)	211.3	59.1
Oxygen evolution (μmol O_2/mg chl/h)		
Whole chain (H_2O to CO_2)	149.1	<10
PSII (H_2O to DCBQ)[c]	446.2	<10
PSI(Asc/DAD to MV)[c]	-354.9[d]	-225.9

[a] The value in parenthesis is doubling time measured under condition of photoautotrophic growth.[b] NG, no growth. [c] DCBQ, 2,6-dichloro-p-benzoquinone; Asc, sodium ascorbate; DAD, 3,6-diaminodurene; and MV, methyl viologen.[d] Negative numbers indicate O_2 uptake via Mehler reaction.

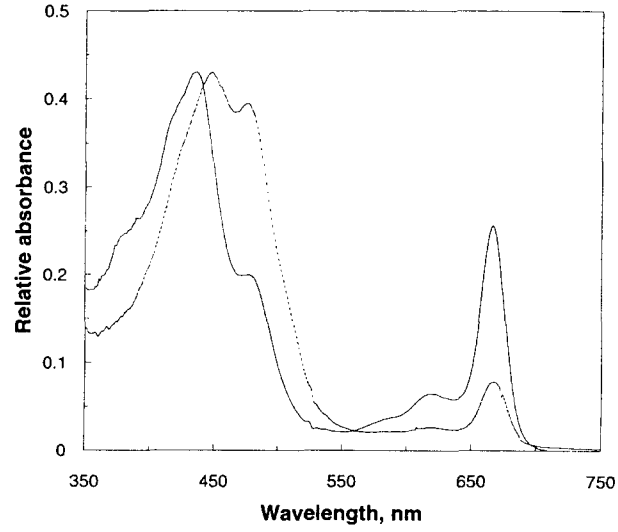

Figure 3. Absorption spectra of pigment extract for wild type and B2 of *Synechocystis* sp. PCC 6803. Thylakoids from wild type (solid line), and mutant cell B2 (dashed line) were extracted with 80 % acetone, and the absorption spectrum for the extracts was measured. Chlorophyll *a* absorbs around 435 and 665 nm; carotenoids absorb 450-525 nm region. Spectra were normalized at maximum absorbance value of 0.43 to facilitate spectral comparison.

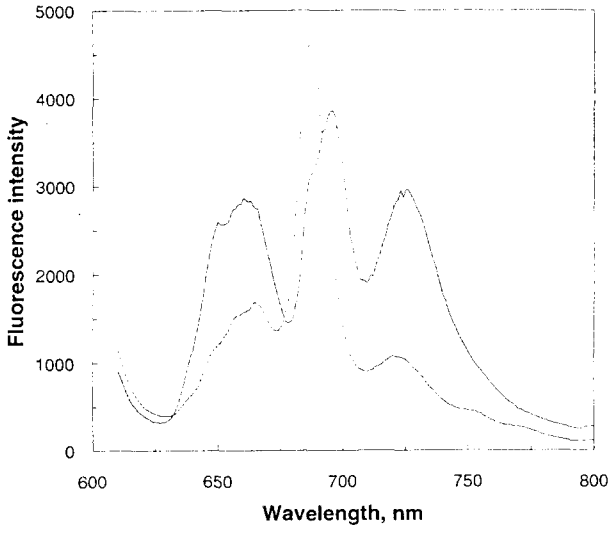

Figure 4. Fluorescence emission spectra of intact cells of wild type (solid line) and B2 (dashed line) monitored at 77 K. Excitation was at 590 nm (phycobilin excitation). The chlorophyll concentration was 50 μg/ml. The bandwidths of the excitation and emission monochromators were 8 and 2 nm, respectively.

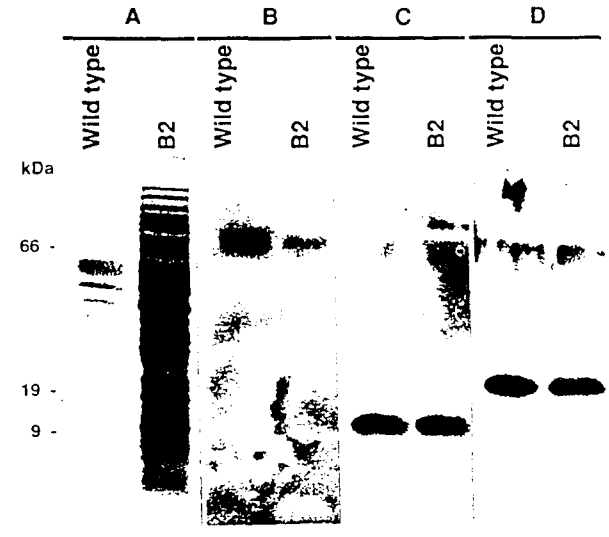

Figure 5. Immunodetection of photosystem I subunits in thylakoid membranes of wild type and mutant cells. (A) Thylakoid membranes containing 0.5 μg of chlorophyll for each lane were separated by tricine-SDS-urea PAGE containing 14 % acrylamide and 6 *M* urea. Separated proteins were transferred to PVDF membranes and probed with antibodies for (B) PsaA/PsaB, (C) PsaC, and (D) PsaD proteins. Immunoreaction was enhanced by chemilumin ascence, which was visualized by using goat anti-rabbit alkaline phosphatase and a chemiluminascent substrate. Molecular mass markers are indicated on left.

photosystem II during segregation (Table 1). To measure photosystem I activity as the rate of oxygen uptake in intact cells, photosystem II activity was inhibited with dichloromethylurea, then ascorbic acid and 3,6-diaminodurene were used as electron donors to photosystem I. The rates of oxygen uptake via a Mehler reaction exerted by photosystem I activity of B2 was approximately 64 % of wild type.

Fluorescence emission spectra at 77 K have been used to assess the efficiency of excitation energy transfer from phycobilisomes to photosystem I[19]. Figure 4 shows 77 K fluorescence emission spectra of wild type and B2 cells. Both spectra showed 665 (phycobilin), near 695 (photosystem II) and 725 nm (photosystem I) peak with 590 nm excitation. The emission band at 665 nm of B2 was lower, suggesting some abnormality in phycobilins or binding between phycobilin and chlorophyll in photosystem II in the mutant. There was an increase of fluorescence near 695 nm, and a decrease of fluorescence at 725 nm. It was considered because energy of photosystem II received from phycobilisomes did not transfer to photosystem I efficiently caused by conformational change of photosystem I in the mutant. These suggested B2 had a photosystem I complex different from the one of wild type, which could not receive energy from photosystem II normally.

Since electron transport assay and 77 K fluorescence emission spectra demonstrated reduced photosystem I activity in the mutant, immunoblot analysis was performed. Thylakoid proteins from wild type and B2 were separated by tricine-SDS-urea PAGE (Fig. 5A), transferred to membrane, and probed with antibodies for photosystem I proteins (Fig. 5). Polyclonal antibodies for PsaA and PsaB detected to signals in both wild type and B2, but the intensity of the signal for B2 was greatly reduced compared to wild type (Fig. 5B). This result supports possibility that mutation is occurred in the *psaA* and/or *psaB* gene. The anomalous behavior of the P700-apoproteins, that is migration at a lower molecular weight than the predicted 83 kDa, has been observed previously in SDS-PAGE, and is probably due to the highly hydrophobic nature of these proteins[20]. Antibodies for PsaC and PsaD, peripheral proteins of photosystem I reaction center, cross-reacted to protein of ~9 kDa (Fig. 5C) and 19 kDa (Fig. 5D) in wild type and B2, respectively. In contrast to PsaA/PsaB proteins, signals for PsaC and PsaD proteins are similar in wild type and B2, suggesting that expression of *psaC* and *psaD* genes are not affected.

To determine whether the change of pigment composition in B2 mutant owing to *psaB* gene inactivation or to other unexpected genetic alterations of the mutant B2, Southern blots analyses of genomic DNAs from wild type and mutant B2 were performed. When the CAT gene of pHP45Ω-Cm was

used as hybridization probe, no hybridization signal was detected in B2 mutant as well as in wild type (data not shown). When the *psaA* and *psaB* genes from pAQPR80 was used as probe, no alteration for *psaA* and *psaB* genes was detected in mutant (data not shown). Since mutants were selected on BG11 agar plates containing chloramphenicol and the plasmid pOV300 does not have a replication origin for *Synechocystis* 6803 strain, it is likely that during the targeted mutagenesis the CAT gene of pOV300 was integrated into the host *Synechocystis* 6803 chromosomal DNA at other than the targeted locus when homologous recombination between the *psaA* and *psaB* genes of pOV300 and the psa*A* and psa*B* genes of *Synechocystis* 6803 chromosomal DNA occurred (Fig. 1). It is also possible that B2 mutant carries second site mutation(s), for example, at the chloramphenicol binding site of ribosome, during selecting and segregating Cm^R mutants. Taken together, a favorable explanation based on Southern and western analyses are that a small deletion or a point mutation was introduced into *psaB* gene in B2. If this is the case, the minor mutation may affect in reducing the amount of PsaB protein in B2 and thus also reducing the activity of electron transport of photosystem I complex. We, however, do not know yet how photosystem I complex can be constructed with small amount of PsaA/PsaB, and normal range amount of PsaC and PsaD.

To study reaction center of photosystem I, we generated several mutants potentially having defects in photosystem I, and analyzed one of the mutants, B2 in detail. In previous works performed in other laboratories, the mutation in *psaA* and/or *psaB* genes blocks stable assembly of photosystem I, and a similar phenotype was observed in an insertional inactivation mutant for *psaB* in *Synechocystis* 6803[5], and in chemically generated mutants for *psaB* in *Chlamydomonas reinhardtii*[21]. The B2 photoheterotrophic mutant, although generated in an attempt similar to those in previous works, seems to have a subtle mutation at the *psaB* locus, thus provides a unique model system for future study of photosystem I.

Acknowledgement — This work was in part supported by a grant from Korea Basic Science Institute (PE00079 and PN00017). The authors wish to thank Dr. Pyung-Lim Han (Hanhyo Science Institute, Korea) for proofreading of manuscript.

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