The Establishment of Conditions to Efficiently Screen Photosynthesis-Deficient Mutants of Synechocystis sp. PCC 6803 by Nitrofurantoin Treatment

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A method based on the susceptibility of photosynthetic organisms to nitrofurantoin under illumination was established to screen mutants of Synechocystis sp. PCC 6803 deficient in the function of photosystem II, which were created by random PCR mutagenesis targeted to the psbAII gene coding for the D1 protein of the photosystem II reaction center. In this method, cyanobacterial colonies on a nitrocellulose membrane on a BG11 agar plate were treated with nitrofurantoin at 1.0 mM under white light at 40 μE m–2 s–1 for 2 h, and then kept under normal conditions without nitrofurantoin so that surviving cells could grow. This method was also shown to be useful for screening mutants deficient in the function of photosystem I.

Key words: Nitrofurantoin — Photosystem II — psbA gene — Random mutagenesis — Screening — Synechocystis sp. PCC 6803.

Abbreviations: EPR, Electron Paramagnetic Resonance; FT-IR, Fourier Transform Infrared.

Introduction

PSII is unique in that it can generate a high redox potential to utilize water molecules as the electron donor. The function of the photosystem is supported by a pigment–protein complex called the PSII reaction center consisting of the D1 and D2 proteins (Nanba and Satoh 1987). These two proteins are homologous in the amino acid sequence to the L and M subunits, respectively, of the purple bacterial reaction center, whose structure has been elucidated by X-ray crystallographic analysis (Deisenhofer et al. 1984). The sequence homology, together with the biophysical and biochemical evidence for the reaction mechanism, suggests that both photosystems are evolutionally related and thus that the structure of the PSII complex can be predicted based on analogy to the purple bacterial counterpart. However, an important aspect of the complex that can not be predicted from this analogy is the structure supporting the unique functions of PSII, i.e. the generation of high redox potential and the oxidation of water molecules.

A number of studies have been directed towards elucidating the unique structure of PSII. These included EPR, FT-IR, and resonance raman spectroscopies, as well as X-ray crystallographic analyses (e.g. Zouni et al. 1998). However, despite these extensive studies, the structure of PSII has not yet been visualized in detail.

To identify the role of specific amino acid residues in the structure and function of the PSII reaction center, mutational analyses targeted to the D1 or D2 protein have been conducted with partial success (e.g. Debus et al. 1988). Site-directed mutagenesis has been the predominant strategy in these analyses. However, the basic limitation of this method, in general, is that only the amino acids that are predicted to be important based on other established evidence can be targeted to the mutation. In contrast, random mutagenesis has the advantage of including the challenge of discovering unexpected structure-function relationships across a wide region on a protein. This method has therefore been introduced to the study of PSII targeting of the D1 or D2 subunit (e.g. Narusaka et al. 1995, Narusaka et al. 1998, Ermakova-Gerdes et al. 1996). For this purpose, Synechocystis sp. PCC 6803 offers a convenient system because of its unique property of easy transformation and its potential capacity for heterotrophic growth (Williams 1988).

The success of random mutational analysis, however, is largely dependent on the availability of appropriate screening methods, as well as on the number of mutations in the targeted region. For screening PSII-deficient mutants, a simple method, although not direct, is to select organisms based on the impairment or loss of photoautotrophy in growth. A convenient selection method is to use chemicals that selectively kill organisms with normal photosynthetic activity (Schmidt et al. 1977, Astier et al. 1984). The use of nitrofurantoin treatment in liquid culture has therefore been introduced to isolate the spontaneous photosynthesis mutants of Synechocystis sp. PCC 6803, which has resulted in the identification of ctpA gene coding for the endo-protease involved in the carboxyl-terminal cleavage of the precursor D1 protein (Shestakov et al. 1994).

In the present study, we established a method using nitrofurantoin to select photosynthesis-deficient mutants of Synechocystis sp. PCC 6803 on nitrocellulose membranes, which can successfully be applied to the screening of random mutants created by PCR mutagenesis targeted to a wide region on the D1 protein.

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Materials and Methods

Experimental organisms and growth conditions

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was used for analysis in this study. The Cm4A-1 strain of the cyanobacterium, which was donated by Dr. Debus, carries inactivated *psbA* and *psbAIII* genes partially substituted by spectinomycin- and chloramphenicol-resistant cassettes, respectively (Debus et al. 1988). The KC and ΔC strains constructed from the Cm4A-1 strain were used as controls; the KC strain is a wild-type photosynthesis strain that has a kanamycin-resistant cassette insertion downstream from the *psbAIII* gene, and the ΔC strain has an inactivated *psbAII* gene created by the partial substitution of the *psbAII* gene with a kanamycin-resistant cassette (Yamasato et al. 1998). The PSI-less strain was provided by Dr. Vermaas; the *psaA-psbB* operon is inactivated in this strain by partial substitution with a chloramphenicol-resistant cassette (Shen et al. 1993).

The cyanobacterial cells were grown at 30°C either in an air-bubbling liquid BG11 medium (Rippka 1988) supplemented with 5 mM HEPES (pH 7.5) or on a BG11 agar plate (Williams 1988). Chloramphenicol (5 μg ml⁻¹), spectinomycin (5 μg ml⁻¹), kanamycin (5 or 20 μg ml⁻¹), and glucose (5 mM) were added to the medium when necessary. The light from white fluorescent bulbs was supplied to the culture at 28 μE m⁻² s⁻¹, except for the PSI-less strain in which the intensity was reduced to approximately 5 μE m⁻² s⁻¹ to avoid the photobleaching of pigments (Shen et al. 1993).

Construction of plasmids and transformation

The pBS-psbA2 plasmid, which contains the whole region of *psbAII* gene supplemented by upstream and downstream sequences (cloned by Dr. Ishiura), was used to construct pKC, pALIK, and pRK plasmids (Yamasato et al. 1998). The pKC and pALIK plasmids were used to create KC and ΔC strains, respectively. The pRK plasmid, which was used to create the random *psbAII* mutants to introduce random amino acid substitutions to the D1 protein, was constructed in the following manner: the random PCR method (Cadwell and Joyce 1992) was applied to the *psbAII* gene in the pBS-psbA2 plasmid, using two primers, i.e. the psbA2 C primer (5'-GGTACGGTGGAATTCTGGTG-GTGAGCTAC-3'); the underlined part indicates the *EcoRI*-site) and the psbA2 B primer (5'-CAATCAGGAATTCTACTGFTG-3'); the random PCR products and the pKC plasmids were ligated at the restriction sites, *EcoRI* and *HinII* (Yamasato et al. 1998), and the products were then introduced to *E. coli*-competent cells (JM109 strain). The pRK plasmids were then amplified and purified.

Transformation of the *Synechocystis* sp. PCC 6803 strain was carried out as described by Williams (1988). In typical experiments, approximately 10⁴ host cells (Cm4A-1 strain) in 100 μl of BG11 medium mixed with 1–10 μg of plasmid DNA was incubated for 4 h under the growth conditions and then spread onto a nitrocellulose membrane on the BG11 agar plate supplemented with glucose (5 mM). After 1 d of incubation, 100 μl of kanamycin solution (2 mg ml⁻¹) was added underneath the agar layer (40 ml), and on the next day, the membrane was transferred to the new BG11 plate containing glucose (5 mM), chloramphenicol (5 μg ml⁻¹), spectinomycin (5 μg ml⁻¹), and kanamycin (20 μg ml⁻¹).

Nitrofurantoin treatments and measurement of fluorescence kinetics

Nitrofurantoin (97%, code No. 86, 044-1, Aldrich) was dissolved in dimethylformamide to a final concentration of 200 mM. The harvested KC cells from liquid culture in the exponential growth phase were adjusted to a concentration of 10 μg Chl ml⁻¹ using fresh BG11 medium and, after the addition of nitrofurantoin, the cells continued to grow at 30°C either in the dark or in the light (28 μE m⁻² s⁻¹). After designated incubation periods, the cells were harvested by centrifugation, resuspended in BG11 medium, and then subjected to the measurement of Chl fluorescence using a PEA fluorescence detector (Hansatech Instruments) after 10 min of dark incubation. The samples were excited at 650 nm, and fluorescence emissions longer than 700 nm were detected.

In the plate culture, cyanobacterial colonies on nitrocellulose membrane on a BG11 agar plate, which were grown for 1–2 weeks to reach approximately 0.5 mm in diameter to enhance genome segregation, were subjected to screening by the following procedure: (1) the nitrocellulose membrane was transferred to the BG11 agar plate supplemented with nitrofurantoin (1.0 mM) and then treated with light at 40 μE m⁻² s⁻¹ for 2 h at 30°C; (2) after the treatment, the membrane with cyanobacterial colonies was transferred to a new BG11 agar plate supplemented with glucose (5 mM), chloramphenicol (5 μg ml⁻¹), spectinomycin (5 μg ml⁻¹), and kanamycin (20 μg ml⁻¹), and further incubated for another 12–24 h at 30°C under weak light illumination (approximately 5 μE m⁻² s⁻¹) to extract the nitrofurantoin remaining in the membrane; (3) the nitrocellulose membrane was again transferred to the other BG11 agar plate supplemented with glucose and three antibiotics and kept for 1–2 weeks to isolate colonies growing normally.

Results and Discussion

Effect of nitrofurantoin treatment on Chl fluorescence

In the experiment shown in Fig. 1a, the cell suspension of the KC strain was incubated at a concentration of 10 μg Chl ml⁻¹ in the BG11 medium containing 1.0 mM nitrofurantoin in the light (28 μE m⁻² s⁻¹). The Chl fluorescence kinetics was measured for the nitrofurantoin-treated cells harvested at different time intervals (0, 1, 2, 4, 8 h) after resuspending in the nitrofurantoin-free medium at 10 μg Chl ml⁻¹. The nitrofurantoin treatment induced a marked time-dependent decrease in the Fv/Fm value of the Chl fluorescence caused by the increase in the Fv/Fm level (Fig. 1a). The decrease in the Fv/Fm value in the present case represents an inactivation of the PSII, although the precise mechanism has not been analyzed in detail. A slight decline in the Fv/Fm value was also induced by the nitrofurantoin treatment in the dark (Fig. 1b). However, the effect was markedly accelerated by illumination during the treatment, resulting in a complete loss of the variable portion within 8 h at 1.0 mM nitrofurantoin. We speculate that this accelerated inactivation of PSII under light is responsible, at least in part, for the reported effects of this chemical compound on the viability of photosynthetic organisms in liquid culture (Shestakov et al. 1994). Therefore, we attempted to determine the optimal conditions for screening *Synechocystis* sp. PCC 6803 colonies deficient in the function of photosynthetic electron transport, utilizing the nitrofurantoin treatment. However, in the experiment, cyanobacterial cells were treated with nitrofurantoin on an agar plate, rather than in liquid culture, to avoid picking up cells of the same genotype propagated during incubation in liquid medium.

Effect of nitrofurantoin treatment on viability

In the experiment shown in Fig. 2, three photosynthesis mutants of *Synechocystis* sp. PCC 6803, i.e. KC, ΔC and PSI-less strains, were grown on nitrocellulose membranes for 7–
10 d, and the colonies on the membranes were then treated with nitrofurantoin at 1.0 mM for 2 h at 30°C, either in the dark or in the light (40 μE m⁻² s⁻¹ of white light). In the control experiment, practically no inhibition of growth could be detected in these three mutants under the dark condition; i.e. all of these strains grew normally during the additional 7–10 d incubation (Fig. 2, (D) for KC strain). The same treatment under the light condition (40 μE m⁻² s⁻¹), on the other hand, resulted in the extinction of almost all colonies in the case of the KC strain after an additional 7–10 d of incubation; in contrast, ΔC and PSI-less strains continued to grow normally after the treatment, and no difference could be detected in relation to the dark control (Fig. 2, (L)).

Even after this treatment, a small fraction of KC colonies survived for unknown reasons, i.e. the local inhomogeneity in the membrane, physiological cell condition, and appearance of

![Graph](image)

**Table 1** Nucleotide substitutions in random mutants †

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Growth</th>
<th>Substitutions</th>
<th>No. of substitutions</th>
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<tr>
<td>NfRK-A48</td>
<td>PS−</td>
<td>GAA→AAA</td>
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<td></td>
<td>E333K</td>
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<tr>
<td>NfRK-A49</td>
<td>PS−</td>
<td>GGT→GGA</td>
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<td>g201g</td>
<td></td>
</tr>
<tr>
<td>NfRK-A50</td>
<td>PS−</td>
<td>TGG→AGG</td>
<td>3</td>
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<td></td>
<td>W284R</td>
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</tr>
<tr>
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<td>PS−</td>
<td>ACC→GCC</td>
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<td></td>
<td></td>
<td>T228A</td>
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<tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>M183L</td>
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</tr>
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</table>

† Nitrofurantoin resistant mutants shown in Fig. 3.

‡ The nucleotide substitutions on psbAII gene and the amino acid substitutions on D1 protein are indicated by upper and lower lines, respectively; silent mutations are indicated by small letters.

PS−, loss of autotrophy.

* Stop codon.
Screening of photosynthesis-deficient mutants

The spontaneous mutants. Survival of the KC strain was markedly increased in response to treatment at lower concentrations of nitrofurantoin. In contrast, both a further increase in the concentrations of nitrofurantoin during treatment and the prolonged treatment time caused marked destruction of KC colonies, even growth inhibition in the case of the ΔC strain. Interestingly, the PSI-less strain was more tolerant than the ΔC strain under high intensity light (300 μE m⁻² s⁻¹) (data not shown). The same screening was achieved at 1.5 mM of nitrofurantoin with 1–2 h of treatment or 2.0 mM of nitrofurantoin with 1 h of treatment, under 40 μE m⁻² s⁻¹.

In addition to nitrofurantoin, some other chemical compounds, such as metronidazole, p-hydroxymercuribenzoate, and tetracycline, have been reported as being used in screening the photosynthesis-deficient mutants of the *Synechocystis* sp. PCC 6714 strain and *Chlamydomonas reinhardtii* (Schmidt et al. 1977, Astier et al. 1984, Joset 1988). These compounds are expected to be used in a manner similar to that reported in the present study. The mechanism of selective killing by metronidazole has been proposed by Schmidt et al. (1977), with the reaction pathway related to PSI being responsible for the effect. A similar mechanism can be assumed in the case of nitrofurantoin, as the PSI-less mutant was found to be markedly more tolerant than the PSII-less mutant, i.e. the ΔC strain, under our experimental conditions.
Selection of random PSII-deficient mutants

The method of screening by nitrofurantoin treatment described above was applied to the isolation of PSII-deficient mutants created by random mutagenesis of the psbAII gene coding for the D1 protein of the PSII reaction center. The pRK plasmids, in which the psbAII gene carries randomly substituted base pairs in the region of Ser148–Ala357 on the D1 protein, were used to transform the Cm4A-1 cells as described in Materials and Methods. The colonies of transformants on nitrocellulose membranes, which had been incubated for 1–2 weeks at 20 μg ml−1 of kanamycin to enhance genome segregation, were treated with 1.0 mM nitrofurantoin for 2 h at 30°C, under 40 μM m−2 s−1 of white light, as described in the preceding section. Fig. 3 shows an example of a plate that contained approximately 300 kanamycin-resistant colonies of transformants before treatment. In response to nitrofurantoin treatment, almost all of the colonies on the membrane disappeared during the period of a successive 13 d of incubation in the absence of nitrofurantoin on a BG11 agar plate. However, five colonies on the membrane continued to grow progressively during the incubation period of a successive 13 d of incubation in the absence of nitrofurantoin on a BG11 agar plate. However, five colonies on the membrane continued to grow progressively during the incubation time, as shown in Fig. 3. The nucleotide sequence analysis for these five colonies confirmed that they carried nucleotide substitutions in the targeted region on the psbAII gene and had lost photoautotrophy (Table 1). In this way, we succeeded in isolating a number of novel mutants deficient in PSII activity, a deficiency that was confirmed to be due to specific amino acid substitution(s) on the D1 protein. The results of this analysis will be reported separately.

In previous studies, screening techniques based on photo-tolerance (Narusaka et al. 1995, Narusaka et al. 1999) and herbicide-tolerance (Narusaka et al. 1998) have been applied to select mutants of Synechocystis sp. PCC 6803 created by targeted in vitro random mutagenesis. In the present study, the nitrofurantoin screening based on the impairment of photosynthetic electron transport was confirmed to be very useful in selecting mutants deficient in the function of PSI and PSII.

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