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Rapid Report

Directed mutagenesis of the gene *psaB* of Photosystem I of the cyanobacterium *Anabaena variabilis* ATCC 29413

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Two large, hydrophobic polypeptides termed PSI-A and PSI-B of Photosystem I are thought, as a heterodimer, to harbor all redox centers and pigments required for primary charge separation. The gene *psaB* coding for PSI-B was cloned from *Anabaena variabilis*, a segment replaced by a drug-resistance cassette, and returned to *Anabaena* on a suicide plasmid. Double recombinants (mutants) grew only chemoheterotrophically, had a reduced chlorophyll content, and lacked Photosystem I.

Cyanobacteria are well-suited for genetic analysis of oxygenic photosynthesis. Since most transformable species can only grow photoheterotrophically (which requires some activity of Photosystem I), but not chemoheterotrophically, directed mutagenesis was mainly used to probe various aspects of Photosystem II (examples in Ref. 1). Nelson's group [2,3] has inactivated, in *Synechocystis* sp. PCC 6803, the genes *psaD* and *psaE* that code for small, peripheral subunits of Photosystem I (PS I). The mutants did not lose the activity of PS I completely, and could grow photoheterotrophically. Recently, 'light-activated', chemoheterotrophic growth of *Synechocystis* has been reported [4].

The cyanobacterium *Anabaena variabilis* strain FD, a facultative chemoheterotroph, can receive plasmid DNA by conjugation and recombine it with resident DNA [5]. With the method of *sacB*-mediated, positive selection for double recombinants [6], the strain has already been used to replace a gene coding for a proteinase by a mutated form [7]. We have now applied these techniques to the mutagenesis of the gene *psaB* that codes for a subunit of PS I, PSI-B. PSI-B is a hydrophobic polypeptide of about 80 kDa that, probably in a heterodimer with the homologous polypeptide PSI-A, harbours four of the six known redox centers and all the pigments of PS I except those of the outer antenna (recently reviewed in Ref. 8). The initial steps of charge separation occur within this heterodimer.

The major part of the *psaB* gene of *A. variabilis* was located from a library of *Hin*dIII-digested, size-fractionated genomic DNA in plasmid pBS(+) (Stratagene, Heidelberg, Germany) with two degenerate oligonucleotides corresponding to conserved regions of the DNA sequence of the gene (bp 1696 to 1721 and bp 1957 to 1976, numbering according to the *psaB*-sequence of *Synechococcus* sp. PCC 7002 [9]) as described [10]. One strand of the 2.5-kb-long insert was sequenced [10]. The insert covered the C-terminal part of the *psaB* product corresponding to amino-acid residue 103 to residue 733 of the homologous *Synechococcus* sequence [9], and a further 551 bp downstream of the *psaB* gene.

Mutation of the psaB gene. The cloned insert was transferred as a 2.5 kb *Hin*dIII fragment to vector pIC 20R [11], bp 665 to 1610 deleted by double digestion with *Bcl*I and *Sty*I and filled in with the Klenow enzyme. The Km^r/Nm^r drug cassette C.K3 was inserted as a *Sma*I fragment from pRL 448 [12], and the resulting mutated, partial *psa*B gene was transferred as an *Eco*RV/*Sma*I fragment into the unique *Nru*I site of pRL 270. Plasmid pRL 270 is a mobilizable, positive selection vector containing gene *sacB* [7]. Essential features of the resulting plasmid pMM III are shown in Fig. 1A.

Abbreviations: ATCC, American Type Culture Collection; bp, base pair(s); Chl, chlorophyll *a*; kb, kilobase pair(s); PCC, Pasteur Culture Collection; PS I, Photosystem I; P700, primary electron donor of Photosystem I.

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Mutation of Anabaena. Plasmid pMM III was transferred to *A. variabilis* strain FD by conjugation on filters [5] and exconjugants obtained in AA-based media [13], as follows: the filters were first incubated for 1 day on AA-medium solidified with 1% agar at 30 °C under white light of 3000 lux, then under the same conditions for 1 week on plates containing, in addition, kanamycin at 25 μg/ml. Thereafter, double recombinants were isolated and identified essentially as described [7], except that plates and liquid cultures were kept at 30 °C in darkness and all media were supplemented with 5 mM fructose, 1 mM sodium thiosulfate, 10 μg/ml (liquid) and 25 μg/ml (plates) of kanamycin and 10 mM sodium arsenate. Arsenate, to which cyanobacteria are fairly resistant [14] was included to keep cultures axenic. Three clones were obtained which were resistant to sucrose and to kanamycin, and thus potential double recombinants [6]. As the three clones had indistinguishable phenotypes, only one of them (mutant MM I) is described in more detail.

Characteristics of the mutant. The mutant did not grow photoautotrophically or photoheterotrophically (i.e., in the light in the presence of 5 mM fructose), but did grow in the dark on fructose, on plates and in liquid. The lack of photoheterotrophic grows suggests that the mutant is sensitive to light. In the following, pertinent properties of the dark-grown mutant are compared to those of the wild-type grown identically except for the omission of kanamycin.

Fig. 1A shows essential features of the suicide (integration) plasmid pMM III used for mutagenesis. The inserted drug cassette C.K3 contains a single *Hin*dIII restriction site. When Southern blots of *Hin*dIII-digested DNA from the wild type and the mutant MM I were probed with the plasmid (see Ref. 7 for the methods), the 2.5 kb band (wild type) was replaced by two bands of 1.8 kb and 0.8 kb (mutant, Fig. 1B), as expected (0.945 kb of the gene *psaB* were replaced by 1.1 kb of cassette C.K3). Digestion with *Eco*RI, which does not cut in the insert of pMM III, resulted in a single band of about 8.5 kb both for the wild type and the mutant (not shown).

Mutant MM I had a lower Chl content than the wild type (Fig. 2), consistent with the absence of PS I (see below). The Chl content was reduced about 4-times (Table I). Light-dependent CO_2 fixation, measured with $[^{14}C]CO_2$ as in Ref. 15, was strongly reduced: When calculated on a Chl basis, the mutant fixed CO_2 at 1.6% of the rate of the wild type (100% = 100–120 μmol CO_2 (mg Chl)$^{-1}$ h^{-1}), when calculated on a packed cell basis, at 0.4% of the rate of the wild type.

Membranes were isolated from the cells in dilute buffer (20 mM Tris, 0.1 M NaCl (pH 8.1)) by French press treatment followed by differential centrifugation

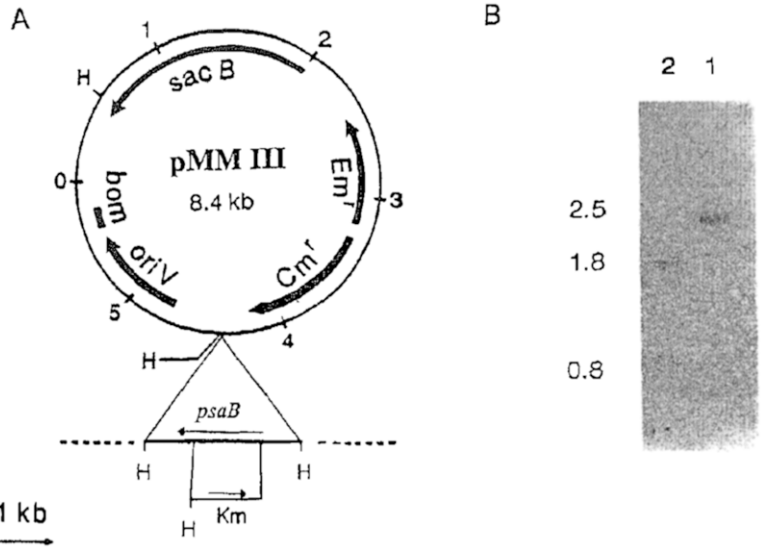

Fig. 1. (A) Essential features of plasmid pMM III which was used to mutagenize the psaB gene of *Anabaena variabilis*. The plasmid is based on the positive selection vector pRL 270 described in Ref. 7. Shown are the restriction sites for *Hin*dIII (H). Lengths are given in kb. Abbreviations: bom, basis of mobilization sequence (ori T region); Cm^r, chloramphenicol resistance gene; C.K3, kanamycin/neomycin resistance cassette; Em^r, erythromycin resistance gene; ori V, replication origin of pBR 322; *sacB*, gene from *Bacillus subtilis* coding for levansucrase. The double-headed arrow in the scale marker for the chromosomal portion. (B) Southern analysis of *A. variabilis* strain FD (lane 1) and its mutant MM I (lane 2). About 2 μg of *Hin*dIII-digested DNA was separated by agarose gel electrophoresis, blotted on nitrocellulose and probed with plasmid pMM III. The hybridization temperature was 68 °C. Cassette C.K3, which was inserted into the *psaB* gene of the mutant, contains a restriction site for *Hin*dIII. Sizes of the fragments, in kb, are indicated at the left. For further details, see text.

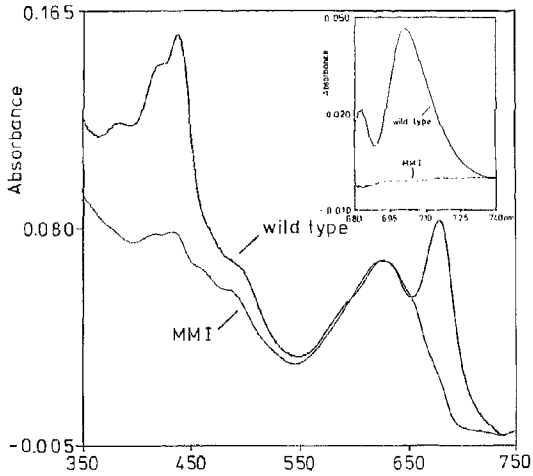

Fig. 2. In vivo absorption spectra of dark-grown *Anabaena variabilis* strain FD and its *psaB*-mutant MM I. The absorption maximum at 625 nm is principally that of phycocyanin, the maxima at 678 nm and 438 nm are those of chlorophyll *a*. The mutant had a strongly reduced chlorophyll content. Conditions: Dark grown cultures were suspended in glycerol/water (1:1) at a density of about 0.6 μl of packed cells/ml and absorption spectra were recorded at room temperature in a Kontron Uvikon 860 equipped with a scattered transmission accessory. The spectra were normalized at 750 nm. The *inset* shows ascorbate-reduced minus ferricyanide-oxidized difference spectra (see Ref. 17) of isolated thylakoids in the far-red region. The spectra were normalized at 740 nm. The maximum at 700 nm, present only in the wild type membranes, corresponds to P700, the primary electron donor of Photosystem I. Both samples contained membranes with 30 μg chlorophyll/ml.

TABLE I

Characteristics of chemoheterotrophically grown Anabaena variabilis FD and its psaB mutant MM I

	Wild Type	Mutant
Chlorophyll content (mg/ml packed cells) [a]	1.65	0.4
Polypeptides PSI-A and PSI-B [b]	present	not detected
Chlorophyll/P 700 (mol/mol) [c]	108	> 3000
Activity of Photosystem [d]	2190	< 50
Activity of Photosystem II [e]		
(a) per chlorophyll	179	529
(b) per packed cells	295	212

[a] Chlorophyll was assayed as in Ref. 16, the volume of the packed cells in graduated centrifuge tubes after centrifugation for 10 min at 3000 × *g*.

[b] Determined by SDS-PAGE of isolated membranes.

[c] Determined by redox difference spectroscopy as in Ref. 17.

[d] Activities are expressed as μmol O_2 consumed (mg chlorophyll *a*)$^{-1}$ h^{-1}. Assayed with isolated membranes as in Ref. 18. T = 25 °C, actinic red light (> 640 nm) of approx. 300 W m^2.

[e] Activities are expressed as μmol O_2 evolved (mg chlorophyll *a*)$^{-1}$ h^{-1} or μmol O_2 evolved (ml packed cells)$^{-2}$ h^{-1}. Assayed with intact cells as in Ref. 16, except that the electron acceptor system consisted of 0.4 mM *p*-benzoquinone, 10 mM $K_3Fe(CN)_6$; other conditions as above.

(10 min at 3000 × *g*, 1 h at 100000 × *g*), a treatment that removes the phycobiliproteins. In the red region of the spectrum, such membranes from the mutant exhibited a Chl maximum at 672 nm, compared to 677 nm in the wild-type membranes (not shown). The primary electron donor of PS I, P 700, was not detected in redox difference spectra of membranes from MM I (see insert of Fig. 2 and Table I). It also lacked PS I activity, measured as light-dependent electron transport from reduced cytochrome *c*-553 to methyl viologen, and lacked both polypeptides PSI-A and PSI-B (Table I).

However, the mutant did contain a normal amount of the principal antennae of Photosystem II, the phycobiliproteins (absorption maximum at 625 nm in Fig. 2), and showed Photosystem II activity, measured as light-dependent oxygen evolution with *p*-benzoquinone/$K_3Fe(CN)_6$ as electron acceptors (Table I). On a chlorophyll basis, this activity was about 3-fold higher in the mutant than in the wild-type, consistent with the reduced content of chlorophyll in MM I, but the activity was similar when related to the volume of packed cells (Table I).

Conclusions. To our knowledge, this is the first report on site-directed mutagenesis of one of the large subunits of PS I. The mutant did not contain any detectable PS I, an indication that the photosystem can not be built from homodimers of PSI-A. A similar conclusion has been reached from analysis of mutants of *Chlamydomonas* obtained by conventional mutagenesis [19]. Mutant MM I may facilitate a biochemical analysis of Photosystem II which was present in normal amounts. Only recently has the directed mutagenesis of a chloroplast gene, *psaC*, of the green alga *Chlamydomonas reinhardtii* with a drug-resistance cassette been achieved [20]. In combination with the available, rather advanced techniques for DNA transfer and for selection of double recombinants, the cyanobacterium *Anabaena variabilis* strain FD appears very well suited for a detailed analysis of PS I by site-directed mutagenesis.

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