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ORIGINAL ARTICLE

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A strain of *Synechocystis* sp. PCC 6803 without photosynthetic oxygen evolution and respiratory oxygen consumption: implications for the study of cyclic photosynthetic electron transport

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Abstract Cyclic electron transport around photosystem (PS) I is believed to play a role in generation of ATP required for adaptation to stress in cyanobacteria and plants. However, elucidation of the pathway(s) of cyclic electron flow is difficult because of low rates of this electron flow relative to those of linear photosynthetic and respiratory electron transport. We have constructed a strain of *Synechocystis* sp. PCC 6803 that lacks both PSII and respiratory oxidases and that, consequently, neither evolves nor consumes oxygen. However, this strain is still capable of cyclic electron flow around PSI. The photoheterotrophic growth rate of this strain increased with light intensity up to an intensity of about 25 μmol photons m^{-2} s^{-1}, supporting the notion that cyclic electron flow contributes to ATP generation in this strain. Indeed, the ATP-generating ability of PSI is demonstrated by the fact that the PSII-less oxidase-less strain is able to grow at much higher salt concentrations than a strain lacking PSI. A quinone electrode was used to measure the redox state of the plastoquinone pool in vivo in the various strains used in this study. In contrast to what is observed in chloroplasts, the plastoquinone pool was rather reduced in darkness and was oxidized in the light. This is in line with significant electron donation by respiratory pathways (NADPH dehydrogenase and particularly succinate dehydrogenase) in darkness. In the light, the pool becomes oxidized due to the presence of much more PSI than PSII. In the oxidase-less strains, the plastoquinone pool was very much reduced in darkness and was oxidized in the light by PSI. Photosystem II activity did not greatly alter the redox state of the plastoquinone pool. The results suggest that cyclic electron flow around PSI can contribute to generation of ATP, and a strain deficient in linear electron transport pathways provides an excellent model for further investigations of cyclic electron flow.

Keywords Cyclic electron flow · Photosystem · Plastoquinone pool · Quinone electrode · Respiratory electron transport · *Synechocystis* sp. PCC 6803

Abbreviations atrazine: 2-chloro-4-ethylamino-6-isopropylamin-*s*-triazine · DBMIB: 2,5-dibromo-3-methyl-6-isopro-pyl-*p*-benzoquinone · DCMU: 3-(3,4-dichlorophenyl)-1, 1-dimethylurea · NDH-1: Type-1 NADPH dehydrogenase · PCR: polymerase chain reaction · PQ: plastoquinone · PS: photosystem · Q-electrode: quinone electrode · UQ-1: ubiquinone-1

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Introduction

The major issue that will be addressed in this study is the design of a suitable system to study the possible physiological role of cyclic electron transport around photosystem I (PSI). In spite of extensive research involving biochemical methods and electron-transfer measurements (for a review, see Bendall and Manasse 1995) the cyclic electron transport pathways around PSI and their relative physiological importance are still unknown. In cyclic electron flow around PSI, electrons are returned from PSI to the linear electron transport chain [the plastoquinone (PQ) pool or the cytochrome b_6f complex] and can thus contribute to increased ATP synthesis without the generation of reducing power (NADPH). At least in cyanobacteria, the Type-1 NADPH dehydrogenase (NDH-1) is presumed to play a significant role in this process. A mutant in which *ndhB* (coding for one of the NDH-1 subunits) has been inactivated is unable to

concentrate inorganic carbon (Ogawa 1991) and has decreased levels of cyclic electron transport around PSI as compared to the wild type (Mi et al. 1992, 1995). When salt stress is applied to *Synechocystis* sp. PCC 6803, cyclic electron flow through NDH-1 increases (Tanaka et al. 1997; Jeanjean et al. 1998, 1999). In the absence of NDH-1, two additional pathways have become apparent (Jeanjean et al. 1998, 1999) but the molecular nature of these pathways remains to be elucidated.

Similar to the situation in cyanobacteria, in plants a cyclic electron-transfer pathway around PSI appears to involve NDH-1 and may function in adaptation to stress (Burrows et al. 1998; Kofer et al. 1998; Sazanov et al. 1998; Shikanai et al. 1998) such as salt stress. Salinity is a major factor limiting crop production in irrigated areas (Smirnoff 1998), and is known to retard growth and development of plants (Munns 1993). Stress adaptation requires energy that can be used for synthesis and accumulation of organic solutes that do not affect the physiology of the cell (glucosylglycerol in the case of *Synechocystis* sp. PCC 6803; Erdmann et al. 1992), extrusion of Na^+ from the cell (Erber et al. 1986; Fry et al. 1986; Molitor et al. 1986), or sequestering such ions into vacuoles (Munns 1993 and references therein). This energy appears to be provided by increased cyclic electron transport around PSI (Fork and Herbert 1993; Jeanjean et al. 1993; Hibino et al. 1996; Joset et al. 1996; Tanaka et al. 1997) and by increased respiratory electron transport (Fry et al. 1986; Molitor et al. 1986; Moser et al. 1991; Jeanjean et al. 1993).

The major reason why cyclic electron transfer around PSI is difficult to study is that competing electron-transfer pathways occur simultaneously at rates that generally are much higher than that of cyclic electron flow. To alleviate this problem, here we report the construction and characterization of a PSII-less, respiratory oxidase-less strain of *Synechocystis* sp. PCC 6803. This strain neither evolves nor consumes oxygen and appears to utilize cyclic electron transport around PSI for the generation of ATP. The identity of the components involved in such a cycle (which may turn out to be different than hitherto assumed) will be the subject of future studies.

Materials and methods

Biological material

Synechocystis sp. strain PCC 6803 was cultivated in air at 30 °C in modified BG-11 medium (Rippka et al. 1979) buffered with 10 mM Tes-NaOH (pH 8.0). The BG-11 modification consisted of partial substitution of $NaNO_3$ with an equal concentration of NH_4NO_3 (the final concentration of ammonia was 4.5 mM). For photomixotrophic or photoheterotrophic growth the medium was supplemented with 5 mM glucose. For growth on plates, 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate were added, and BG-11 was supplemented with antibiotics appropriate for the particular strain (25 μg ml^{-1} kanamycin, 25 μg ml^{-1} erythromycin, 40 μg ml^{-1} streptomycin and/or 25 μg ml^{-1} spectinomycin). Strains were grown at a light intensity of 50 μmol photons m^{-2} s^{-1} unless indicated otherwise. Growth was followed by measuring the optical density of the cells at 730 nm using a Shimadzu UV-160 spectrophotometer. For anaerobic or microaerobic growth the cultures were sparged with a 1% CO_2, 99% N_2 mixture.

Deletion construct for *psbB*

The deletion construct for *psbB* was created by amplifying the streptomycin resistance genes from RSF1010 by polymerase chain reaction (PCR), with the ATG start codon of the *strA* gene becoming part of an *Nco*I site, and with an *Sma*I site 50 bp downstream of the stop codon of *strB*. The amplified fragment was digested with *Nco*I and *Sma*I. This fragment, together with an *Sma*I/*Hin*dIII fragment from pKW1246 (Vermaas et al. 1987) that contained the 3′ and downstream regions of *psbB* (nucleotides 2,780,852–2,782,484; numbering according to CyanoBase (http://www.kazusa.or.jp/cyano/cyano.html), was cloned into the pF17 plasmid. pF17 contains the region immediately upstream of the *psbB* coding region and the first 5 bp of the *psbB* coding region (residues 2,779,411–2,779,513 according to CyanoBase), and carries a site-directed mutation introducing an *Nco*I site at the *psbB* start codon. The resulting plasmid carries a replacement of *psbB* by the streptomycin resistance cartridge, and has been named pΔpsbB (see Fig. 1A).

DNA preparation

DNA from *Synechocystis* sp. strain PCC 6803 was prepared essentially as described in Williams (1988).

Oxygen consumption and evolution

Measurements of oxygen consumption and evolution were carried out in a manner similar to that described previously for oxygen uptake (Vermaas et al. 1994) with the cells being dark-adapted for approximately 10 min prior to being assayed.

Chlorophyll *a*

Concentrations of chlorophyll *a* were determined according to Porra et al. (1989). Fluorescence emission spectra were determined using a Fluorolog 2 instrument (SPEX Industries Inc., Edison, N.J., USA). Cells were resuspended in 10 mM Hepes/NaOH (pH 7.4) in 50% (v/v) glycerol for measurements at liquid-nitrogen temperatures. The excitation and emission bandwidths were 3.5 and 0.5 nm, respectively.

Determination of salt tolerance

Initially, strains of *Synechocystis* sp. PCC 6803 were plated on BG-11 agar containing 0.5 M NaCl and glucose if required. If growth was observed at this NaCl concentration the strain was sequentially restreaked onto plates containing higher NaCl concentrations (0.1 M NaCl increments), until the maximum salt tolerance was determined. If growth was not observed the starting concentration of NaCl was lowered to 0.2 M and the above process was repeated. Once the maximal NaCl tolerance level had been determined on plates, the strain growing at this concentration was transferred to a liquid culture containing NaCl at a concentration 0.2 M below that of the plate, and the concentration subsequently increased in 0.1 M increments in successive sub-cultures to determine the maximal salt tolerance level of the strain in liquid culture.

P700 measurements

Cells were harvested when cultures were in the mid-exponential growth phase (OD_{730} = 0.4–0.7) and concentrated to an OD_{730} of approximately 40. Cells were dark-adapted for about 10 min prior

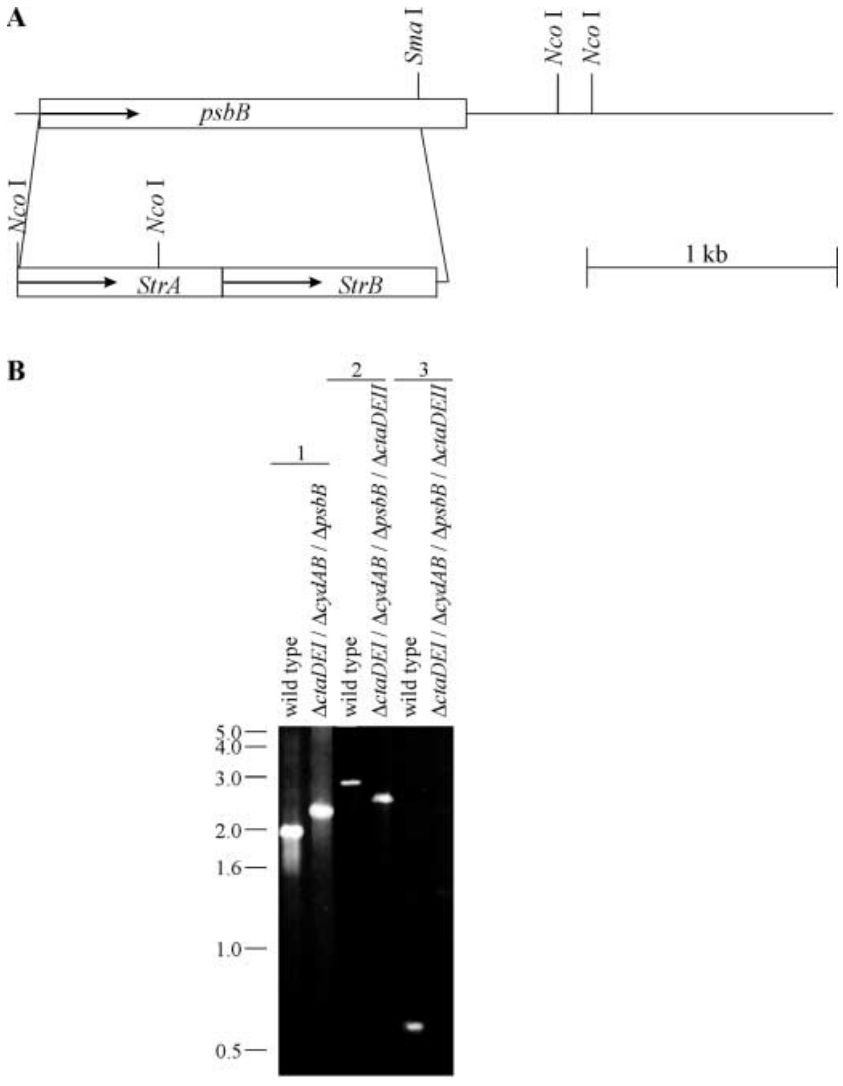

Fig. 1A, B Construction of the Δ*psbB* plasmid and PCR analysis of segregation of *psbB* and *ctaDIIEII*. **A** Schematic representation of *psbB* and the deletion construct. **B** PCR analysis of segregation of the *psbB* (*panel 1*) and *ctaDIIEII* (*panels 2–3*) deletions to homozygosity. The relative positions in the genome of *Synechocystis* sp. PCC 6803 of the primers used to test segregation are: *panel 1* 5′psbB 2,779,317–2,779,336 and 3′psbB 2,781,364–2,781,340; *panel 2* 5′ctaDEII 1,540,405–1,540,427 and 3′ctaDEII 1,543,358–1,543,335; *panel 3* 5′ ctaDEII, 1,540,405–1,540,427 and 3′ctaDII 1,541,070–1,541,050. Numbering is according to CyanoBase

to the measurement. After dark adaptation 400 µl of cells was placed in a cuvette and the reflectance at 820 nm was measured using a PAM fluorimeter (Walz, Effeltrich, Germany) fitted with an emitter detector unit ED 800T as described by Schreiber et al. (1988). Where indicated, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) were added to a final concentration of 5 µM. Actinic illumination at approximately 10,000 µmol photons m^{-2} s^{-1} was provided for 6 s using a Schott KL1500 light source (Walz).

Quinone electrode

The quinone electrode (Q-electrode) was set up essentially as described for plant mitochondria (Moore et al. 1988; Dry et al. 1989), membranes from *Rhodobacter capsulatus* (Zannoni and Moore 1990), and for pea thylakoids (Cleland 1998). Cells from cultures in mid-exponential growth phase were harvested and resuspended to $OD_{730} = 4$ in a medium containing 10 mM Hepes/NaOH (pH 7.4) and 50 mM KCl. A 10-ml aliquot of this cell suspension was placed in a stirred water-jacketed cuvette that was maintained at 28 °C. The cuvette contained a glassy carbon working electrode poised at a potential of +360 mV with respect to an Ag/AgCl reference electrode and was connected to a platinum auxiliary electrode via the medium. All electrodes were purchased from Bioanalytical Systems Inc., West Lafayette, Ind., USA. Exogenous ubiquinone-1 (UQ-1) was added to the reaction vessel to a final concentration of 0.2 µM to act as a redox mediator between the quinone pool in the cells and the electrodes. Current flow between the working and auxiliary electrodes was measured and digitized using a CV-50 W unit (Bioanalytical Systems Inc.) operating in time base mode with the sensitivity set at 100 nA V^{-1}. The data were captured on a computer using software provided with the CV-50 W. A halogen lamp provided illumination with the light being passed through a filter that cut out wavelengths below 430 nm. The light intensity was modulated with a combination of reflectance and wire mesh filters.

Results

Construction of the PSII-less, oxidase-less strain

A strain of *Synechocystis* sp. PCC 6803 that lacks cytochrome oxidase (CtaI) and a putative cytochrome *bd*-type quinol oxidase (CydAB) (Howitt and Vermaas 1998) was transformed with pΔpsbB, and transformants were selected for resistance to streptomycin. Transformants were cultured in the presence of glucose, atrazine (2-chloro-4-ethylamino-6-isopropylamin-*s*-triazine) and

streptomycin to allow segregation of the wild-type and mutant genome copies. Segregation to homozygosity was initially demonstrated by the absence of photoautotrophic growth and confirmed by PCR (Fig. 1B, Section 1). The resulting strain was transformed with a deletion construct for *ctaDEII* encoding a putative secondary quinol oxidase (CtaII) (Howitt and Vermaas 1998) and transformants were selected for resistance to spectinomycin. Transformants were then cultured in the presence of increasing concentrations of spectinomycin to allow segregation to homozygosity to occur. Segregation was confirmed by PCR (Fig. 1B, Section 2). As the deletion construct was shorter than the wild-type copy of *ctaDIIEII*, thus possibly discriminating against detection of the wild type, a second PCR was performed using a primer 5′ of the deletion and one internal to the deletion. A 650-bp fragment was seen in wild type but no PCR product was seen in the deletion strain (Fig. 1B, Section 3), confirming that the strain was homozygous for the deletion. This strain was designated as the PSII-less oxidase-less strain and has the genotype Δ*psbB* Δ*ctaDIEI* Δ*ctaDIIEII* Δ*cydAB*. The deletion of PSII and the respiratory oxidases was further confirmed by analysis of respiratory and photosynthetic oxygen consumption/evolution rates in the dark and light, respectively. In darkness, oxygen was consumed at rate of approximately 8 μmol O_2 (mg Chl)$^{-1}$ h^{-1} and was insensitive to 5 mM KCN; this rate is similar to that seen in the respiratory oxidase-less strain (Howitt and Vermaas 1998) and this oxygen uptake is unrelated to respiratory processes. Upon illumination of the cells no change in the oxygen consumption rate was seen and no oxygen was evolved (data not shown).

Growth analysis of the PSII-less oxidase-less strain

At low light intensity this strain grew slowly (Table 1). However, the growth rate increased with the light intensity up to approximately 25 μmol photons m^{-2} s^{-1}; above this light intensity the growth rate remained stable. As the lack of photosynthesis and respiration is likely to affect the ATP level and therefore potentially nutrient uptake, doubling times were also determined at a light intensity of 50 μmol photons m^{-2} s^{-1} under conditions of nutrient limitation (Table 2). Reduction of the N or S concentrations to 3% of normal (0.63 mM and 9 μM, respectively) slowed the growth of the wild type to a doubling time of about 20 h. A similar slowing down was seen for the PSII-less and oxidase-less strains. The PSII-less oxidase-less strain and the PSI-less strain, which already had a doubling time of about 20 h under the conditions used, were not affected by the reduced N concentration in the growth media. This suggests that nutrient uptake at decreased N concentration was not rate-limiting for growth in these strain. A similar pattern was seen for growth of the PSI-less strain at a reduced S concentration. However, the PSII-less/oxidase-less mutant grew at least twice slower or was unable to grow at all when the S concentration in the media was reduced to 3% of normal (Table 2), suggesting that insufficient energy reserves were available to cope with the reduced sulfate availability. Sulfate concentrations of at least 8% of normal (≈24 μM) in the media were required for reasonable growth of the PSII-less oxidase-less strain (data not shown).

As addition of NaCl to the growth medium has been shown to increase rates of cyclic electron transport around PSI in *Synechocystis* sp. PCC 6803 (Tanaka et al. 1997; Jeanjean et al. 1998, 1999) and is likely to increase the ATP demand of the cell, the NaCl tolerance levels of various strains were determined in order to see if deletion of PSII and the respiratory oxidases adversely affected salt tolerance. The results are shown in Table 3.

Table 1 Doubling times of the PSII-less, oxidase-less strain of *Synechocystis* sp. PCC 6803 at different light intensities. Values listed are means ± SD from at least three determinations. *LAHG*: Light Activated Heterotrophic Growth (growth in darkness, with 15 min of light every day that presumably serves to activate a blue-light receptor)

Light intensity (μmol photons m^{-2} s^{-1})	Doubling time (h)
100	21.2 ± 1.0
50	23.0 ± 1.2
25	19.7 ± 1.2
15	24.3 ± 2.2
7	28.8 ± 2.1
2	36.4 ± 1.1[a]
LAHG	No growth

[a]Six determinations were performed and cultures only grew in 50% of these; the number shown is the average from the three cultures that grew

Table 2 Doubling times of the wild-type and PSII-less, oxidase-less strains of *Synechocystis* sp. PCC 6803 grown under nutrient limitation at a light intensity of 50 μmol photons m^{-2} s^{-1}. All media contained normal BG-11 ingredients supplemented by 5 mM glucose, except that either the total N or S content was reduced to the level indicated. Values are means ± SD from at least three determinations

Medium composition	Doubling Time (h)				
	Wild type	PSII-less, oxidase-less	PSII-less	PSI-less	oxidase-less
BG-11	10 ± 2.1	19 ± 1.1	16 ± 4.3	20 ± 1.8	11 ± 2.5
10% N (2.1 mM)	18 ± 2.9	21 ± 1.3	21 ± 1.8	21 ± 3.1	21 ± 1.2
3% N (0.63 mM)	19 ± 1.8	23 ± 2.2	24 ± 0.9	20 ± 2.5	22 ± 2.4
10% S (30 μM)	13 ± 3.1	22 ± 3.6	20 ± 0.8	22 ± 3.3	19 ± 1.1
3% S (9 μM)	19 ± 2.8	40 ± 4.1[a]	26 ± 2.3	21 ± 2.6	24 ± 3.6

[a]Growth under these conditions was not always observed, possibly depending on the physiological state of the cells before sulfur starvation. The data presented here represent the experiments where growth was obtained under these conditions

Deletion of PSI had a much greater effect on the level of salt tolerance than deletion of PSII and/or the terminal oxidases, suggesting that PSI is more important than either PSII or respiration to produce the ATP required to adapt to and tolerate salt stress (Table 3).

Characterization of electron transport

The light-intensity dependence of the growth rate of the PSII-less oxidase-less strain together with the salt sensitivity of the PSI-less strain suggest that indeed cyclic electron transport around PSI contributes to generation of ATP. To determine electron flow through PSI we measured the reduction kinetics of $P700^+$ (the primary electron donor of PSI) after turning off actinic illumination. The half-time of this process gives an indication of the rate of electron donation to PSI. The results are shown in Table 4. No significant difference in $P700^+$ reduction rates was seen between wild type, the oxidase-less strain, or the PSII-less oxidase-less strain. Even addition of various inhibitors of electron transport had little effect on $P700^+$ reduction. However, the reduction kinetics in all strains were slow, indicating that the electron donors to PSI are predominantly oxidized after illumination. This supports the concept of an oxidized PQ pool in the light, and will be expanded upon in the *Discussion*.

Table 3 Salt (NaCl) tolerance of various strains of *Synechocystis* sp. PCC 6803. Salt tolerance levels were determined in the presence of 5 mM glucose at a light intensity of 50 μmol photons m^{-2} s^{-1} except for the PSI-less strain which was determined in the presence of 15 mM glucose at a light intensity of 5 μmol photons m^{-2} s^{-1}. The salt-tolerance level is the maximum NaCl concentration in which the strains can grow

Strain	NaCl tolerance (M)
Wild type	1.2
PSI-less	0.3
PSII-less	0.7
PSII-less oxidase-less	0.6
Oxidase-less	0.9

Table 4 Half time of $P700^+$ reduction after turning off the actinic light. Values given are means ± SD of at least three determinations. Both DBMIB and DCMU were added to a final concentration of 5 μM. All strains of *Synechocystis* sp. PCC 6803 were grown at a light intensity of 50 μmol photons m^{-2} s^{-1} in the presence of 5 mM glucose. Dense samples of intact cells were illuminated for 6 s at 10,000 μmol photons m^{-2} s^{-1} before the strong actinic light was turned off and the reduction of $P700^+$ was measured

Additions to reaction vessel	$t_{1/2}$ (ms)		
	Wild type	Oxidase-less	PSII-less, oxidase-less
None	311 ± 30	344 ± 34	338 ± 22
+ DBMIB	486 ± 39	614 ± 23	480 ± 49
+ DCMU	372 ± 58	339 ± 20	380 ± 91
+ DBMIB + DCMU	548 ± 60	618 ± 64	531 ± 36

The PSII-less oxidase-less strain of *Synechocystis* sp. PCC 6803 that was created has a simplified electron transport chain and shows a light dependence of photoheterotrophic growth that is consistent with a functional role of cyclic electron transport around PSI. However, the light dependence might also be interpreted by over-reduction of the PQ pool at low light intensity due to PSI activity that is significantly below that of electron input from stromal components. An over-reduction of the PQ pool may be detrimental to growth as radicals may be produced that can slow cell growth or cause cell death (Asada 1999). In order to determine the redox state of the PQ pool in vivo we constructed a Q-electrode, which has been used successfully to determine the redox state of the quinone pool in plant mitochondria (Moore et al. 1988; Dry et al. 1989), membranes from *Rhodobacter capsulatus* (Zannoni and Moore 1990), and pea thylakoids (Cleland 1998), and adapted it for use with whole cells of *Synechocystis* sp. PCC 6803. Ten milliliters of a cell suspension ($OD_{730} \approx 4$) and an exogenous UQ-1 concentration of 0.2 μM were empirically determined to be the optimal concentrations to give a good signal, without interfering with photosynthetic or respiratory electron transport processes in the cell. At this concentration UQ-1 served as redox mediator between the membrane and the soluble phase, but did not serve as a highly effective electron acceptor or donor in lieu of PQ. At higher UQ-1 concentrations a signal of greater amplitude was seen in the Q electrode, but these concentrations caused an increased respiratory oxygen consumption rate and decreased photosynthetic oxygen evolution rates at non-saturating light intensities in the wild type (data not shown). This suggests that at higher concentrations UQ-1 can act as a mobile electron carrier between PSII in thylakoids and the respiratory electron transport chain on the cytoplasmic membrane.

A sample Q-electrode trace for the PSII-less oxidase-less strain is shown in Fig. 2. The cells were allowed to equilibrate in darkness until a steady current was reached. Then UQ-1 was added, and the measurement was continued until a steady current was reached. As this strain contains no respiratory oxidases, the steady-state current in darkness in the presence of UQ-1 was taken to represent the fully reduced PQ pool (Q_{red}): under these conditions electrons are provided to the pool by NDH-1 (Mi et al. 1995) and succinate dehydrogenase (Cooley et al. 2000), but no reducing equivalents in the PQ pool can be consumed. This is similar to the situation in oxidase-containing strains in the presence of 1–5 mM KCN, where in darkness full reduction of the PQ pool has been observed (Vermaas et al. 1994). The light was then turned on and the redox poise of the PQ pool was measured at different light intensities. As this strain lacked PSII the steady-state current at the maximum light intensity was taken to represent a fully oxidized PQ pool (Q_{ox}). In strains that contained PSII the level corresponding to full oxidation

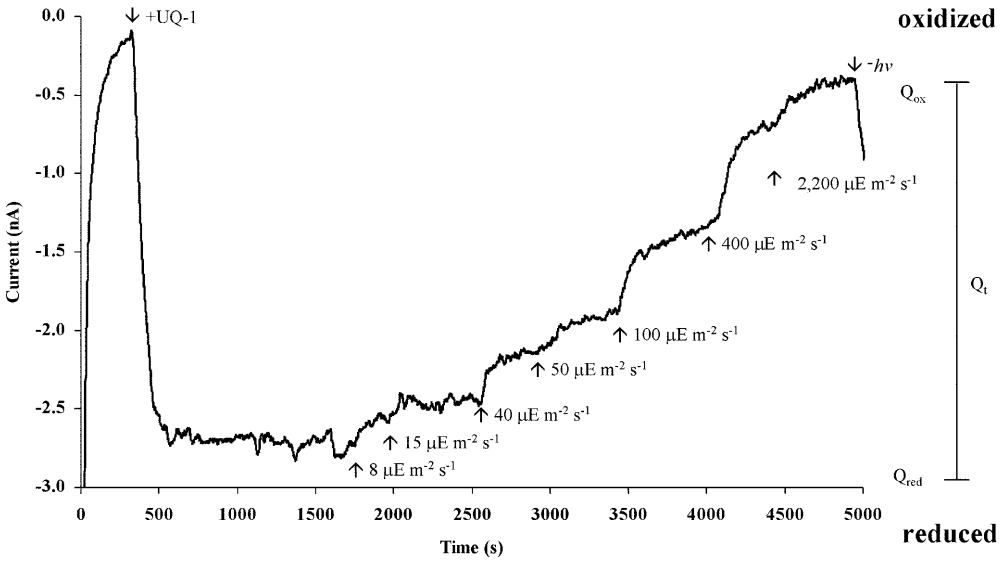

Fig. 2 Monitoring of the relative redox poise of the PQ pool at various light intensities in the PSII-less oxidase-less strain of *Synechocystis* sp. PCC 6803. UQ-1 was added where indicated, and the light intensities were sequentially increased at the time points indicated

of the PQ pool was determined by illumination at maximum intensity in the presence of DCMU. In those that contained respiratory oxidases Q_{red} was determined in the darkness in the presence of 200 μM KCN at the end of the assay. The relative reduction state of the quinone pool ($Q_{red/T}$) was determined by comparing the measured Q-electrode value under particular conditions to those found under fully reduced and fully oxidized conditions. Fully oxidized and fully reduced quinone pools were given the relative reduction values of 0 and 1, respectively. The long time intervals for the electrode to reach equilibrium after changing the light intensity (Fig. 2) is as expected for a redox mediator that does not serve as direct electron donor or acceptor and that equilibrates slowly with PQ in the membrane. An additional factor for the sluggish equilibration may be the relatively slow diffusion of UQ-1 in and out of the cells and membranes. In any case, this system is not suitable to determine the kinetics of PQ oxidation and reduction but can provide information regarding the steady-state PQ redox state as a function of illumination intensity.

The results for the strains assayed as described above are shown in Table 5. Quantitatively similar data were obtained when the fully oxidized state of the quinone pool was determined first and then the light intensity was decreased stepwise (data not shown). In darkness the PQ pool in the wild type was partially reduced, while in strains that lack CtaI it was completely reduced. Upon illumination the PQ pool became more oxidized in all strains and was nearly completely oxidized at a light intensity of 400 μmol photons m^{-2} s^{-1}. This is different than in chloroplasts of higher plants but is as would be expected for this cyanobacterium where PSI is 5- to 6-fold more abundant than PSII and where in darkness respiratory electron flow into the PQ pool can occur.

Low levels of illumination (2 μmol photons m^{-2} s^{-1}) were sufficient to produce a large change in the redox poise of the PQ pool in the CtaI-less strain. The redox poise of the PQ pool in this strain was very similar to that of wild type, except in darkness (Table 5); this is understandable as without CtaI in darkness there is no electron pathway out of the PQ pool in the thylakoid membrane. The similar redox poise of the PQ pool in the

Table 5 Relative reduction state of the quinone pool in various strains of *Synechocystis* sp. PCC 6803 grown in the presence of 5 mM glucose. Values given are means ± SD from at least three determinations

Light intensity (μmol photons m^{-2} s^{-1})	$Q_{red/T}$[a]			
	Wild type	CtaI-less	Oxidase-less	PSII-less, oxidase-less
0	0.57 ± 0.17	1.00 ± 0.01	1.00 ± 0.00	1.00 ± 0.00
2	0.55 ± 0.16	0.63 ± 0.12	0.95 ± 0.01	0.94 ± 0.04
8	0.51 ± 0.15	0.58 ± 0.08	0.84 ± 0.06	0.85 ± 0.08
15	0.48 ± 0.13	0.46 ± 0.05	0.78 ± 0.05	0.82 ± 0.03
25	0.41 ± 0.14	0.34 ± 0.08	0.65 ± 0.04	0.77 ± 0.14
50	0.20 ± 0.11	0.28 ± 0.06	0.44 ± 0.08	0.51 ± 0.13
100	0.14 ± 0.09	0.22 ± 0.05	0.30 ± 0.14	0.25 ± 0.13
400	0.07 ± 0.06	0.14 ± 0.05	0.07 ± 0.05	0.06 ± 0.05
2200	0.02 ± 0.01	0.14 ± 0.05	0.06 ± 0.01	0.00 ± 0.00
2200 + DCMU	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	N.D.[b]

[a] $Q_{red/T}$ was calculated as described in the text
[b] *N.D.* Not determined

light regardless the presence of CtaI indicates that under these conditions the rate of electron flow to PSI exceeds that to cytochrome oxidase.

In the oxidase-less strain, which lacks all three oxidases, the PQ pool was significantly more reduced than in the wild type at light intensities equal to or below 50 μmol photons m^{-2} s^{-1}. However, at light intensities of 100 μmol photons m^{-2} s^{-1} and above there is little difference in the redox poise of the PQ pool between the two strains (Table 5), again supporting the notion that the rate of respiratory electron flow is much smaller than that of photosynthetic electron transport at moderately high light intensity.

At any given light intensity there was very little difference in the redox state of the PQ pool between the oxidase-less strain and the PSII-less oxidase-less strain (Table 5). This fits with the concept of an abundance of PSI as compared to PSII in *Synechocystis* sp. PCC 6803: the number of electrons provided to the PQ pool by PSII appears to be much smaller than the capacity of PSI to take out electrons from the pool. Fluorescence emission spectra at 77 K showed no difference in the PSI/PSII ratio between the wild type and the oxidase-less mutant (data not shown), suggesting that this reasoning can be extended to the wild-type system as well.

Modes of growth

The PSII-less oxidase-less strain was unable to grow in unbuffered media; growth under these conditions resulted in a rapid decrease in the pH of the media (Fig. 3). Even when grown in the presence of 1 or 10 mM Tes-NaOH, a decrease in the pH of the media was observed when compared to the wild type, for which the pH of the media rose during growth (Fig. 3). This indicates that an acid was secreted from the cells, which may be the result of the absence of terminal oxidases as electron acceptors. Similar experiments with the oxidase-less strain resulted in a pH drop in the media similar to that seen with the PSII-less oxidase-less strain (data not shown). pH titrations of the media indicated that no compounds that were secreted had pK_A values in the range of 4–6, which are values one would typically expect for monovalent organic acids.

As both the oxidase-less and PSII-less oxidase-less strains grow without using or forming oxygen, attempts were made to grow these strains under anaerobic conditions (1% CO_2 in N_2); in strains with active PSII, however, oxygen is produced and cells are likely to grow microaerobically. Both the wild type and the oxidase-less strain grew well under such microaerobic conditions, with doubling times of approximately 16 h (data not shown). However, no strains that lacked PSII function (either by deletion of *psbB* or upon addition of atrazine to strains with functional PSII) were able to grow in liquid culture under these conditions, suggesting that a small amount of O_2 is needed for growth of *Synechocystis* sp. PCC 6803, even if the terminal oxidases are missing.

Discussion

A *Synechocystis* sp. PCC 6803 strain without oxygen evolution and uptake

Deletion of the respiratory oxidases and *psbB* from *Synechocystis* sp. PCC 6803 resulted in a viable strain

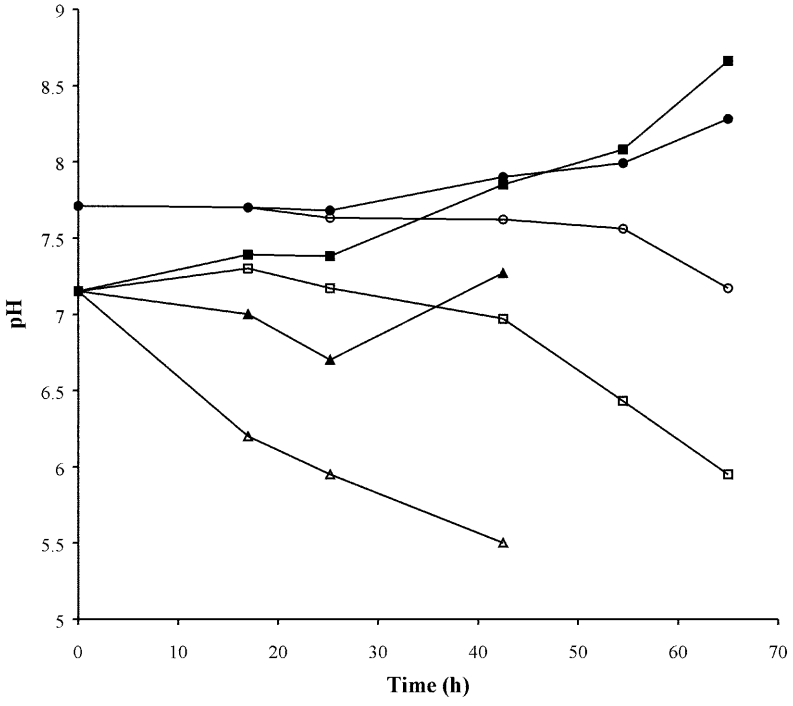

Fig. 3 Change of pH of the growth media during growth of the PSII-less oxidase-less and wild-type strains of *Synechocystis* sp. PCC 6803. Curves shown are from a single experiment. However, data from replicate experiments were quantitatively similar to those shown here. ● wild type (10 mM Tes-NaOH), ■ wild type (1 mM Tes-NaOH), ▲ wild type (unbuffered), ○ PSII-less oxidase-less (10 mM Tes-NaOH), ❑ PSII-less oxidase-less (1 mM TES-NaOH), △ PSII-less oxidase-less (unbuffered). All experiments were performed with 5 mM glucose in the media. For experiments in unbuffered media, cultures were inoculated with cells scraped from a plate, while for those with buffered media, cultures were inoculated with cells from a liquid culture containing 10 mM Tes-NaOH

that neither consumes oxygen by respiration nor evolves oxygen by photosynthesis, but that requires a fixed-carbon source for growth. The need for relatively strong buffering capacity suggests that growth of cells without oxidases results in acid production. The excreted product is not the primary product of fermentative pathways that have been described in other cyanobacteria as these pathways all produce organic acids with pK_A values in the range of 4–6 (for a review, see Stal and Moezelaar 1997). Possibly nitrate, sulfate, or other oxidized compounds could act as electron acceptors under these conditions.

Quinone electrode

The adaptation of the Q-electrode for use with whole cells of *Synechocystis* sp. PCC 6803 allows us to compare the relative reduction state of the PQ pool from different strains and under different conditions. The use of this technique has allowed us to rule out the possibility that the longer doubling times of the PSII-less oxidase-less strain at lower light intensities (Table 1) were simply due to over-reduction of the PQ pool caused by input from the stromal components via NAD(P)H dehydrogenases (Ogawa 1991; Berger et al. 1991) and/or succinate dehydrogenase (Cooley et al. 2000) being higher than the drain created by PSI activity.

It should be noted that the quinone reduction state as measured here is the relative, steady-state redox state of the exogenous UQ-1 added. Changes in this value will reflect similar changes in the steady-state redox state of the endogenous redox active PQ pool, but the redox state of the PQ pool may be different from that of the exogenous UQ-1 depending on the midpoint potential of UQ-1 relative to that of PQ. Unfortunately, in the literature there is little consensus on the precise midpoint redox potentials of these two compounds in biological membranes. If the UQ-1 midpoint redox potential were approximately 60 mV more positive than that of PQ (Rich and Bendall 1979), the results in Table 5 would overestimate how reduced the PQ pool actually is, but if the UQ-1 and PQ-9 midpoint potentials in situ are close together the $Q_{red/T}$ values have quantitative relevance. Because of this uncertainty, the results obtained with the Q-electrode will be discussed qualitatively in this section.

The Q-electrode data revealed a number of interesting features. Firstly, in the wild type the PQ pool is rather reduced in darkness when the cells are grown photomixotrophically. This is different than in thylakoids of higher plants, where the PQ pool is mostly oxidized in darkness. This difference is in line with the higher succinate dehydrogenase and NADPH dehydrogenase activity in cyanobacteria. Upon illumination of the cells the PQ pool becomes increasingly more oxidized with increasing intensity (Table 5), in line with the notion that PSI is very much overabundant relative to PSII in this system (Aizawa et al. 1992; Shen et al. 1993).

A second interesting observation made with the Q electrode was that in the CtaI-less strain the PQ pool is fully reduced in darkness (Table 5). This strain respires at wild-type rates (Schmetterer et al. 1994) presumably through the activity of a quinol oxidase of the cytochrome *bd* type (Cyd), which appeared to be exclusively on the cytoplasmic membrane under the growth conditions used (Howitt and Vermaas 1998). This finding supports the interpretation that Cyd is on the cytoplasmic membrane in our growth conditions, and implies that the Q-electrode is essentially "blind" to the redox state of the cytoplasmic membrane. Presumably the size of the PQ pool in the cytoplasmic membrane is so small in comparison to that in the thylakoid membrane that its contribution to the overall redox state of the total quinone pool is insignificant. The "blindness" of the Q-electrode to the redox state of the cytoplasmic membrane means that results can be interpreted more easily as, in first approximation, only redox reactions on the thylakoid membrane need to be taken into account.

A third observation worth noting was that the Q-electrode data for the oxidase-less strains with and without PSII were essentially the same (Table 5). Deletion of the oxidases results in significant changes to the PQ reduction state as compared to the wild type at lower light intensities, but additional deletion of PSII had no significant effect. This fits with the notion of a super-stoichiometric presence of PSI relative to PSII. However, this set of data also highlights the importance of respiratory oxidases in regulating the redox poise of the PQ pool at lower light intensities. Thus, the traditional view that respiration is present in cyanobacteria solely to provide energy for survival during periods of darkness may need to be revised to include a regulatory function with respect to the redox poise of the cell. Similarly, in plants, mitochondrial activity affects the redox poise of the chloroplast (Hoefnagel et al. 1998).

Slow PSI reduction is indicative of an oxidized PQ pool after strong illumination

Electron transfer reactions between PQ and PSI can be monitored by measuring $P700^+$ reduction kinetics after a high-intensity illumination. $P700^+$ reduction kinetics are slow, with a half-time in all three strains of 300–350 ms (Table 4). However, assuming a maximum electron transport rate of 300 μmol O_2 (mg Chl)$^{-1}$ h^{-1} and 150 chlorophylls per PSI in wild-type *Synechocystis* sp. PCC 6803, one can calculate that under steady-state conditions the $P700^+$ reduction rate should be about 50 s^{-1}. This corresponds to $P700^+$ reduction half-times that are an order of magnitude faster than those experimentally observed by us (Table 4) and others (for example, Jeanjean et al. 1999) in *Synechocystis* sp. PCC 6803, supporting the concept of a very oxidized PSI donor side (PQ pool, cytochrome b_6f complex, and plastocyanin/cytochrome c_{553}) after illumination causing a lack of electrons to donate to $P700^+$. The long $P700^+$

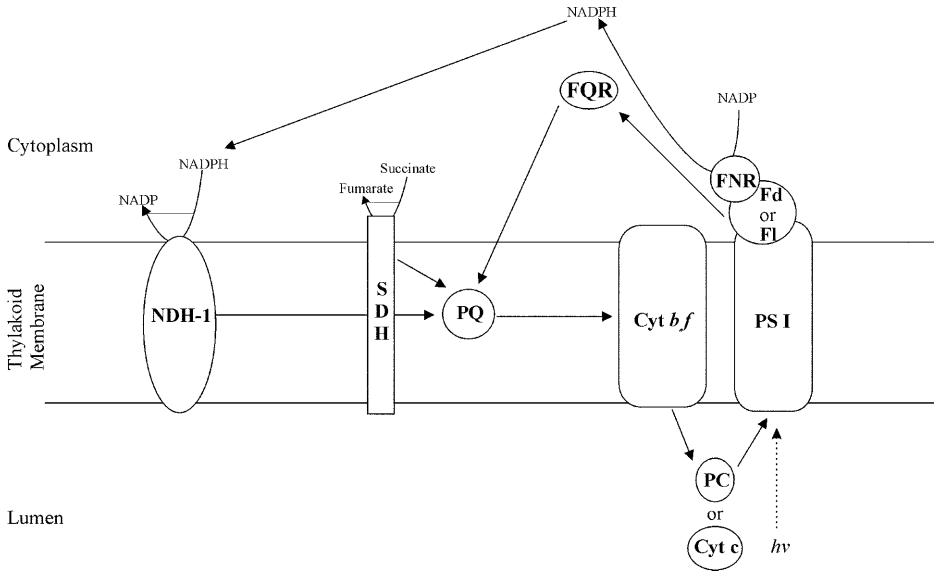

Fig. 4 Schematic representation of electron-transport chains in the PSII-less, oxidase-less strain of *Synechocystis* sp. PCC 6803. For simplicity, the Type-2 NADH dehydrogenases that presumably have limited metabolic activity in this system, have been omitted. It should be pointed out that evidence for ferredoxin plastoquinone oxidoreductase (*FQR*) activity is purely functional, and may not represent a distinct enzyme (complex). A purified enzyme with FQR activity has never been unequivocally demonstrated. Abbreviations: *Cyt* b_6f cytochrome b_6f complex, *Cyt c* cytochrome *c*, *Fd* ferredoxin, *Fl* flavodoxin, *FNR* ferredoxin $NADP^+$ oxidoreductase, *FQR* ferredoxin plastoquinone oxidoreductase, *NDH-1* Type-1 NAD(P)H dehydrogenase, *PC* plastocyanin, *PQ* plastoquinone, *PSI* photosystem I, *SDH* succinate dehydrogenase

reduction time after illumination is interpreted to reflect electron transfer into the PQ pool by pathways involving succinate dehydrogenase (Cooley et al. 2000), one of the NAD(P)H-oxidizing enzymes, or cyclic electron-transfer pathways around PSI. Obviously, the electron-donation capacity of these pathways is rather limited compared to the electron-accepting capacity of oxidized PSI, but the fact that $P700^+$ reduction kinetics are similar for all strains studied here (Table 4) implies that the capacity of electron-donating pathways has not been altered drastically by any of the mutations.

In line with this reasoning regarding the slow $P700^+$ reduction rates observed, the addition of DBMIB, an inhibitor of the cytochrome b_6f complex, slowed the half-time of reduction of $P700^+$ by less than a factor of 2 in all three strains examined (Table 4), indicating that cytochrome b_6f activity is not the rate-limiting step in $P700^+$ reduction. The limited effect of DCMU on $P700^+$ reduction rates again emphasizes the small electron-transfer capacity of PSII relative to that of PS I.

Cyclic electron transport around PSI

Deletion of PSII and the respiratory oxidases results in a strain that has vastly simplified electron-transport pathways as compared to the wild type. Aerobic respiratory electron-transport reactions on the cytoplasmic membrane can be discounted, as there is no major sink for electrons. On the thylakoid membrane, the only possible pathways of electron transport are (i) donation of electrons from stromal components to PSI via the intersystem chain or (ii) cyclic electron transport around PSI. Figure 4 illustrates some of the possible pathways but, in principle, any pathway that accepts electrons from PSI and donates them to the PQ pool without the intermediate use of ATP or other high-energy compounds could serve as a cyclic electron-transport pathway that generates a light-dependent proton gradient across the membrane. Therefore, the two possible pathways of electron transport (donation of electrons from stromal components to PSI via the PQ pool, and cyclic electron transfer around PSI) are not mutually exclusive.

The longer doubling times seen when the PSII-less oxidase-less strain is grown at light intensities below 25 μmol photons m^{-2} s^{-1} (Table 1) suggests that the growth rate of the cells is dependent on the activity of PSI and the ability to generate ATP via cyclic electron transport. The difficulty of growing the cells at 2 μmol photons m^{-2} s^{-1} indicates that this intensity is on the borderline of the minimum light intensity required to drive cyclic electron transport at a sufficient rate to generate enough ATP for cell maintenance. The results obtained with the quinone electrode indicate that the PQ pool is not over-reduced in these conditions, and that therefore potential over-reduction of the PQ pool is not a factor in the survival or growth rate of the PSII-less oxidase-less strain in the light. Instead, we interpret these data to indicate that cyclic electron transfer involving PSI aids the strain to produce sufficient ATP for metabolic processes. The current study does not allow a delineation of the pathway(s) by which PSI electrons return to the PQ pool, but generation and analysis of strains with additional mutations may address this important question in the future.

Stress tolerance

The apparent dependence of the PSII-less oxidase-less strain on cyclic electron transport was further confirmed by its greater sensitivity to sulfate limitation than the wild type (Table 2). This presumably results from insufficient ATP levels in the cell to allow both cell maintenance and active uptake of sulfate to occur. The fact that this effect is not seen when fixed nitrogen is limited to the same relative percentage compared to that in unmodified BG-11 is likely to reflect the fact that the N concentration in BG-11 is nearly 100-fold higher than that of S.

Pathways of cyclic electron transport around PSI (Tanaka et al. 1997; Jeanjean et al. 1998, 1999) and respiration (Fry et al. 1986; Molitor et al. 1986; Moser et al. 1991) are activated during salt stress. In *Synechocystis* sp. PCC 6803, the latter is thought to be the result of activation of CtaI (Jeanjean et al. 1993). Analysis of the salt-tolerance levels of the strains created (Table 3) reveals that despite the reported activation of respiration during adaptation to salt stress in cyanobacteria, deletion of the respiratory oxidases does not significantly change the salt tolerance of the strain as compared to the parental strain. The salt tolerance of the PSII-less oxidase-less strain is only slightly below that of the PSII-less strain, and is still 50% of that of wild type. From the results presented here it is clear that PSI plays a much greater role in determining salt tolerance than either respiration or PSII, as deletion of PSI significantly increases salt sensitivity. This illustrates the important role that PSI, and possibly cyclic electron transfer around this photosystem, plays in generating additional ATP.

In summary, the deletion of both PSII and the respiratory oxidases in *Synechocystis* sp. PCC 6803 has resulted in a strain with vastly simplified electron-transport pathways. The PSII-less oxidase-less strain, in which cyclic electron flow around PSI plays a relatively larger physiological role than in other strains and in which cyclic electron flow around PSI can be demonstrated to apparently provide additional ATP for cell processes, is expected to be a useful system to elucidate the molecular nature of cyclic electron-transfer pathways.

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