# Unexpected changes in photosystem I function in a cytochrome c 6 -deficient mutant of the cyanobacterium Synechocystis PCC 6803

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# Unexpected changes in photosystem I function in a cytochrome $c_6$-deficient mutant of the cyanobacterium *Synechocystis* PCC 6803

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**Abstract**

Cytochrome $c_6$, the product of the *petJ* gene, is a photosynthetic electron carrier in cyanobacteria, which transfers electrons to photosystem I and which is synthesised under conditions of copper deficiency to functionally replace plastocyanin. The photosystem I photochemical activity (energy storage, photoinduced P700 redox changes) was examined in a *petJ*-null mutant of *Synechocystis* PCC 6803. Surprisingly, photosystem I activity in the *petJ*-null mutant grown in the absence of copper was not much affected. However, in a medium with a low inorganic carbon concentration and with $NH_4^+$ ion as nitrogen source, the mutant displayed growth inhibition. Analysis showed that, especially in the latter, the *isiAB* operon, encoding flavodoxin and CP43′, an additional chlorophyll *a* antenna, was strongly expressed in the mutant. These proteins are involved in photosystem I function and organisation and are proposed to assist in prevention of overoxidation of photosystem I at its lumenal side and overreduction at its stromal side. 

*Keywords:* Cytochrome $c_6$; Flavodoxin; Photosystem I; P700; *isiAB*; *petJ*

## 1. Introduction

According to the Z scheme, photosynthetic electron flow in oxygenic photosynthesis needs the presence of soluble electron carriers located in the lumen of the thylakoids to connect the electron flow output from the cytochrome $b_6f$ complex to the photosystem I (PSI) reaction centre [1,2]. In cyanobacteria, these electron carriers are plastocyanin, if copper is available, and cytochrome $c_{553}$ (cytochrome $c_6$) when copper is lacking [3]. A mutant deleted in the *petJ* gene encoding cytochrome $c_6$ was generated in *Synechococcus* PCC 7942 [4] and in *Synechocystis* PCC 6803 [5]. Rather surprisingly, these mutants lacked severe impairments in the photosynthetic electron flow and grew practically at the same rate as the wild-type (WT) in a medium depleted of $Cu^{2+}$ ions [4,5].

In this work, we have examined cyclic electron flow around PSI and expression of a number of electron carriers in a *petJ*-null mutant growing in the absence of copper. The results indicate that the mutant compensated the lack of cytochrome $c_6$ by adaptive changes at the electron acceptor side of PSI.

## 2. Materials and methods

### 2.1. Strains and culture conditions

The WT *Synechocystis* PCC 6803 strain originates from the Pasteur Culture Collection. The *petJ*-null mutant (*petJ::Kmr*) was kindly provided by Dr H.B. Pakrasi [5]. Only one copy of the *petJ* gene is present in the genome of *Synechocystis* PCC 6803. The genotype of the *petJ::Kmr* mutant was routinely checked by polymerase chain reaction (PCR). WT and mutant cells were cultivated under continuous illumination at a photon flux density of about 50 μmol photons $m^{-2}$ $s^{-1}$ at 34°C on a rotary shaker (170 rpm), in modified Allen's mineral medium (12 mM

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$HCO_3^-$). Copper (1 μM) was added to this medium (normal growth conditions) or copper was omitted ($Cu^{2+}$-depleted growth conditions). For high salt concentration, the $Na^+$ ion concentration was raised to 550 mM by addition of NaCl [6]. Cells used for the experiments were harvested in the exponential phase. A series of experiments were carried out in BG11 medium [7] supplemented with 5 mM HEPES-Na (pH 7.5) medium, with or without $Cu^{2+}$, and under different nitrogen regimes ($NO_3^-$ or $NH_4^+$ as nitrogen source). The BG11 medium represents a low inorganic carbon medium in comparison to the Allen medium.

### 2.2. Biological preparations

Cells were harvested by centrifugation, rinsed, and suspended in 50 mM HEPES-NaOH (pH 7.5), 0.8 M sorbitol, 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride at 4°C. Cells were subsequently broken either by shaking with glass beads in a cooled Vibrogen-Zellmühle (E. Bühler) or by two passages through a pre-cooled French press. Soluble proteins were prepared as in [6].

### 2.3. Photoacoustic spectroscopy

Energy storage by cyclic electron flow around PSI was measured in vivo using the photoacoustic technique, as described in [8–11].

### 2.4. Redox state of P700

Changes in the redox state of reaction centre P700 of PSI were monitored via absorbance changes around 820 nm [12] with a modulated fluorescence measurement system MKII ($P700^+$ kit) from Hansatech Instruments coupled to a computer.

### 2.5. Chlorophyll fluorescence emission

Room temperature chlorophyll fluorescence was measured with a PAM-2000 fluorimeter (Walz, Effeltrich). Fluorescence was excited with dim red light modulated at 600 Hz. Cell suspensions (10 μg chlorophyll *a* $ml^{-1}$) were kept for 10 min in darkness prior to illumination with white light for determination of the steady-state level of chlorophyll fluorescence ($F_s$). White light was provided by a Schott KL1500 light source, the fluence rate was adjusted by neutral density filter. The maximal fluorescence level ($F_m$) was obtained by adding an 800-ms pulse of intense white light (1500 μmol photons $m^{-2}$ $s^{-1}$) to the actinic white light. The photochemical quenching coefficient $q_p$ was determined as $(F_m-F_s)/(F_m-F_o)$. The term $1-q_p$ is non-linearly related to the fraction of closed PSII reaction centres, it reflects the fraction of PSII centres with the primary (quinone) electron acceptor ($Q_A$) in the reduced state [13].

### 2.6. SDS–PAGE and immunoblotting

Proteins (80–100 μg according to Bradford [14]) present in the supernatant after ultracentrifugation were separated using SDS–PAGE according to standard procedures. Proteins were blotted semi-dry onto a nitrocellulose membrane (Schleicher and Schuell). Immunodecoration was done with a polyclonal antibody raised against *Synechocystis* PCC 6803 flavodoxin (Dr. M. Hagemann, Rostock, Germany). Binding of the antibodies was visualised with a secondary antibody with conjugated horseradish peroxidase and the ECL kit (Amersham Buchler). Haem staining was carried out according to [15].

### 2.7. RNA manipulations (RT-PCR)

Post harvest, cells were treated by RLT+ mercaptoethanol buffer (Qiagen). Several phenol–chloroform extractions removed proteins, all the nucleic acids were recovered after precipitation by isopropanol. RNA was obtained after DNase treatment, followed by precipitations with LiCl. The absence of DNA was checked either by reverse transcriptase (RT)-PCR after RNase treatment or by routine PCR. Experiments were carried out with the kit Superscript one-step RT-PCR from Gibco-BRL (Invitrogen). Primers for the *isiA* gene were chosen in the coding sequence (5′ primer: GCAAACCTATGGCAACGACACCG, 3′ primer: CCGTTTGGGTGGTGGCGTCGTA) to obtain a RT-PCR fragment of 0.5 kb. Primers for the *petE* gene were located 41 bp downstream of the start codon (5′ primer: GGTCTCCAGTTTCTTTTTATC) and 37 bp upstream of the last codon (3′ primer: TGGGGTTCACAGTAGTAGGT) allowing the synthesis of an RT-PCR fragment of 0.3 kb.

## 3. Results and discussion

### 3.1. PSI function in the WT and the petJ-null mutant

We checked by haem staining that growth of WT *Synechocystis* PCC 6803 in the copper-depleted medium induced the expression of the *petJ* gene (Fig. 1A). Concomitantly, RT-PCR analyses showed that transcription of the *petE* gene encoding plastocyanin was practically turned off in the absence of copper in the WT and in the *petJ*-null mutant (Fig. 1B).

*Synechocystis* PCC 6803 cells were transiently illuminated with far-red light, causing changes in their absorbance at c. 820 nm. Those changes reflect the dynamic P700 redox state. In cultures with $Cu^{2+}$, no significant differences was observed between the WT and the mutant in the kinetics of photo-induced oxidation of P700 and its reduction in darkness (Fig. 1C). After growth in $Cu^{2+}$-depleted medium, the P700 oxidation time strongly increased in the *petJ*-null mutant (Fig. 1D). Such a slow

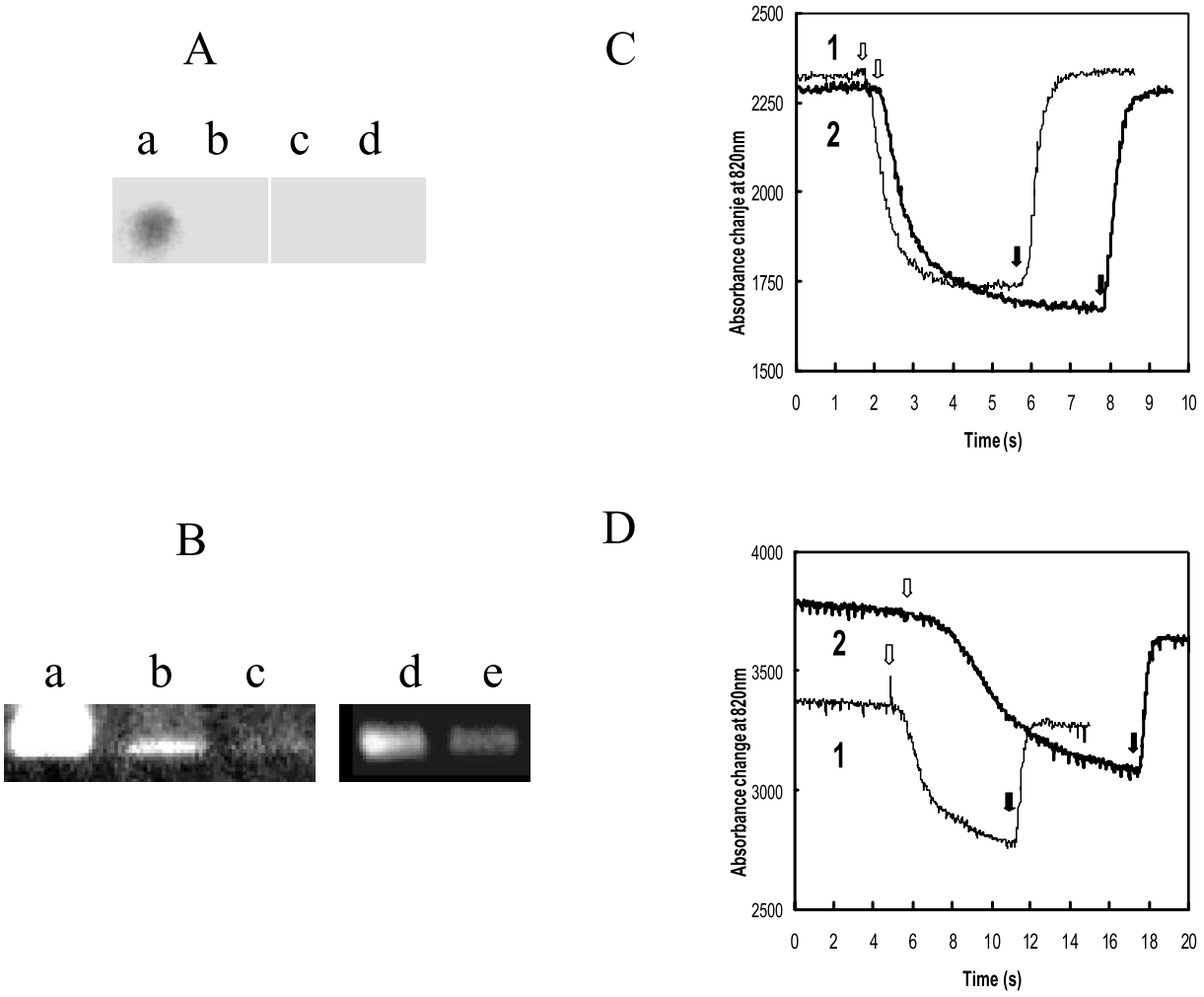


Fig. 1. A: Expression of the *petJ* gene, encoding cytochrome $c_6$, in *Synechocystis* PCC 6803 detected by haem staining on soluble protein extracts (100 μg): lane a, WT grown without copper; lane b, WT grown with 1 μM of copper; lane c, *petJ*-null mutant grown without copper; lane d, *petJ*-null mutant grown with 1 μM of copper. B: Transcription of the *petE* gene, encoding plastocyanin, in WT *Synechocystis* PCC 6803 detected by RT-PCR on RNA (1.5 μg): lane a, DNA (1 μg); lanes b and d, WT cells grown with 1 μM of copper; lane c, WT cells grown without copper; lane e: *petJ*-null cells grown without copper. C: Far-red light (FRL: 30 μmol photons $m^{-2}$ $s^{-1}$) induced P700 redox changes in WT and in *petJ*-null. WT and mutant grown with $Cu^{2+}$ (1 μM final), WT (curve 1) and *petJ*-null mutant (curve 2). D: The same without $Cu^{2+}$, WT (curve 1), and *petJ*-null mutant (curve 2). Open arrows: FRL on, black arrows: FRL off.

oxidation could be related to either slower respiration or a fast recycling of the electrons from the stromal PSI acceptor side to P700. The dark oxygen consumption was similar in both strains (data not shown). Rather, Table 1 shows that the rate of dark reduction of $P700^+$ in the *petJ* mutant was indeed slightly accelerated as compared to the reduction rate in the WT (Table 1). Photochemical energy storage in the *petJ*-null mutant grown with $Cu^{2+}$ was identical to that of the WT but it was slightly decreased relative to the WT when growth proceeded in a $Cu^{2+}$-depleted medium (Table 1). The P700 dark reduction data, which showed a faster rate in the mutant from $Cu^{2+}$-free medium, and the observation that energy storage in the mutant was somewhat lower point to changes in the electron transfer and energy conservation characteristics of PSI.

The physiological data presented above indicated that electron transfer to PSI could take place in the absence of

Table 1
PSI activity determined in the WT and in the *petJ*-null mutant (grown on Allen medium, plus or minus copper)

| | WT plus $Cu^{2+}$ | WT minus $Cu^{2+}$ | *petJ*-null plus $Cu^{2+}$ | *petJ*-null minus $Cu^{2+}$ |
|---|---|---|---|---|
| Energy storage | 13 ± 1.3 (3) | 12.5 ± 0.6 (3) | 13 ± 0.5 (3) | 10.4 ± 0.5 (3) |
| P700 reduction half-life | 330 ± 30 (4) | 300 ± 30 (7) | 330 ± 50 (4) | 260 ± 40 (7) |

Energy storage (arbitrary units) was estimated by photoacoustic spectroscopy in far-red light (90 μmol photons $m^{-2}$ $s^{-1}$). P700 redox changes were monitored under far-red light (30 μmol photons $m^{-2}$ $s^{-1}$) as described in Section 2. P700 reduction half-life was expressed in milliseconds.

plastocyanin and cytochrome $c_6$, suggesting the existence of alternative PSI electron donors. In the sequenced genome of *Synechocystis* PCC 6803, cytochrome *M* [16] could be a potential candidate to replace cytochrome $c_6$, although recent studies [17] have revealed in vitro a redox potential that would be too low for such a function in our present understanding of electron transfer from plastoquinone (PQ) to the PSI reaction centre. The question on how electrons transfer to PSI in *petJ* mutant grown in the absence of copper ion remains to be answered in detail.

### 3.2. Overexpression of the isiAB operon in the petJ-null mutant

We have examined the possibility that electron carriers are expressed or overexpressed in response to the absence of cytochrome $c_6$ in the *petJ* mutant. The mutant grown in the absence of $Cu^{2+}$ accumulated high amounts of flavodoxin whereas flavodoxin was hardly detectable in WT (Fig. 2A). In control experiments, flavodoxin accumulated under salt stress conditions, both in WT and in the *petJ*-null mutant (Fig. 2B). Flavodoxin is a PSI electron acceptor [18] encoded by the *isiB* gene, which has been shown to be derepressed under a range of different stress conditions: iron starvation, heat shock and growth in high salt medium [11,18–24]. In the latter case, flavodoxin has been proposed to contribute to the observed increase in cyclic electron flow [6,10,11]. The *isiB* gene is located downstream of the *isiA* gene, and these two genes constitute an operon [25]. Transcripts of the *isiA* gene were detected at the mRNA level in the *petJ*-null mutant using the technique of RT-PCR (Fig. 2C). In the WT, *isiA* transcripts were detected in comparatively low amounts (Fig. 2C). The *isiA* gene encoding a chlorophyll–protein complex is expressed during iron starvation and was originally assumed to function with PSII according to the homology with CP43 [20,26,27]. Recent studies have clearly demonstrated that the product of the *isiA* gene could work as an antenna to PSI [28,29] and could play a role in thermal dissipation of the absorbed light energy [26].

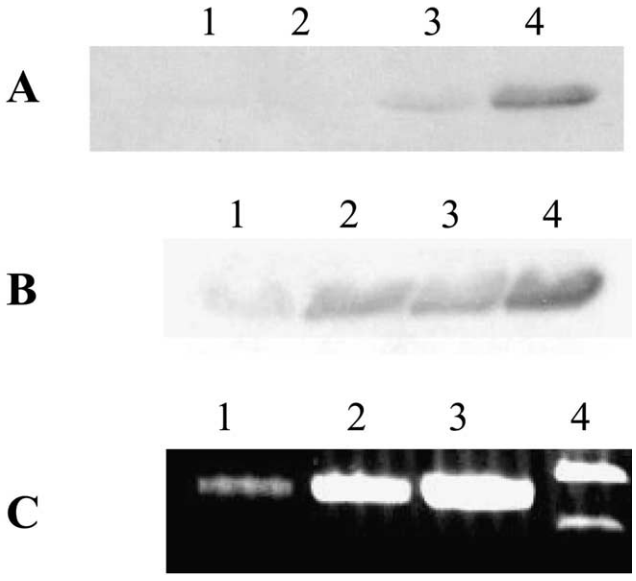


Fig. 2. Expression of the *isiA* and *isiB* genes in WT and the *petJ*-null mutant, effects of presence and absence of copper ions in the growth medium, and effect of NaCl stress exposure. A: Immunodetection of the *isiB* transcript flavodoxin in an extract of soluble proteins (90 μg final loading) from WT (lane 1) and *petJ*-null mutant (lane 2), both grown with 1 μM $Cu^{2+}$ and the same grown without $Cu^{2+}$, WT (lane 3) and mutant (lane 4). Decoration was with polyclonal antibodies raised against flavodoxin. B: Immunodetection of flavodoxin in extracts of soluble proteins (80 μg, final loading). Lane 1, WT minus copper ion, minus NaCl; lane 2, the *petJ*-null mutant grown minus $Cu^{2+}$ and minus NaCl; lanes 3 and 4, WT and *petJ*-null mutant grown minus $Cu^{2+}$ and plus 0.55 M NaCl, respectively. C: Presence of the *isiA* transcript in the mRNA pool from WT and *petJ*-null mutant grown without $Cu^{2+}$ as revealed by RT-PCR. Lane 1: mRNA from WT; lane 2: mRNA from *petJ*-null mutant; lane 3: DNA from WT; lane 4: DNA markers (0.6 and 0.4 kb). RT-PCR was performed with 1.2 μg of RNA.

Our results indicate that the *isiAB* operon was weakly expressed in the absence of $Cu^{2+}$ in WT and that its transcription was strongly increased in the *petJ*-null mutant. Though originally related to specific stress, like iron limitation or salt stress, the derepression of the expression of the *isiA* and *isiB* genes now appeared to coincide with other stress conditions such as in the *petJ*-null mutant in the absence of $Cu^{2+}$. It is suggested that mutant cells in the absence of $Cu^{2+}$ experienced permanent stress that provoked a stress response, derepression of the *isiAB* operon, also known from other stress conditions.

### 3.3. Growth conditions requiring the presence of cytochrome $c_6$

The usual growth conditions (high inorganic carbon concentration, with nitrate and in the presence of $Cu^{2+}$) allowed the electron flux coming out from PSI to be consumed in the Calvin cycle and in the nitrate assimilation pathway while the excess could be channelled to flavodoxin which likely mediates a cyclic electron pathway around PSI [11]. The replacement of $NO_3^-$ by $NH_4^+$, which suppressed the influx of electrons to the nitrate reduction machinery, induced a delay in the growth of the *petJ*-null mutant cultivated in $Cu^{2+}$-depleted medium and in the presence of a high inorganic carbon concentration (Fig. 3A). The effect was more drastic when mutant cells were grown in $Cu^{2+}$-depleted BG11 mineral medium, a medium which contains relatively less $HCO_3^-$ than Allen's medium, in which nitrate was replaced by $NH_4^+$. In that case, growth ceased practically after 40 h (Fig. 3B). Under those conditions, P700 was strongly oxidised in the *petJ*-null mutant compared to WT (Fig. 3D). The light saturation curves displayed a different pattern in the P700 oxidation level between WT and the *petJ*-null mutant when cells were incubated with $NH_4^+$ (Fig. 3E). This overoxidation of P700 was correlated to a more reduced state of the PQ pool, as was shown by the fluorimetric determination of

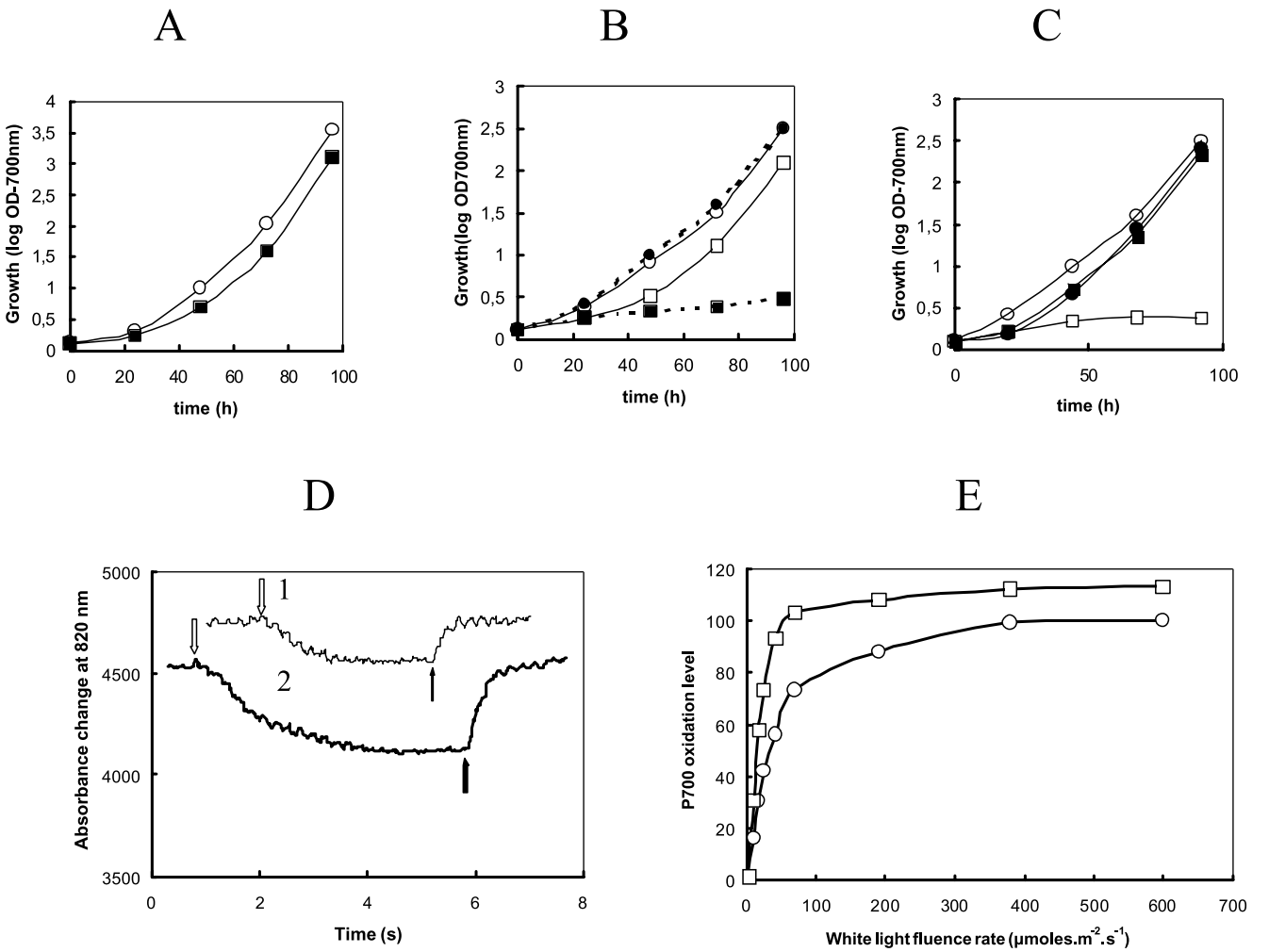


Fig. 3. Growth conditions requiring the presence of the *petJ* gene. The cells were previously cultivated in Allen's medium (12 mM $CO_3H^-$) plus $Cu^{2+}$ (Section 2), centrifuged, rinsed and suspended in the different media as indicated. A: Growth of WT (open circles) and *petJ*-null mutant cells (black squares) without $Cu^{2+}$ in modified Allen's medium (12 mM $HCO_3^-$, 5 mM $NH_4^+$ as nitrogen source). B: Growth of WT and the *petJ*-null mutant in BG11 medium minus $Cu^{2+}$ with $NO_3^-$ (WT, open circles; *petJ*-null, open squares) or $NH_4^+$ (WT, black circles; *petJ*-null, black squares) as nitrogen source. C: Growth of WT (circles) and the *petJ*-null mutant (squares) in the $NH_4^+$ BG11 medium minus $Cu^{2+}$ without NaCl stress (open symbols) or with 550 mM NaCl stress (black symbols). D: P700 oxidation changes in WT (curve 1) and *petJ*-null mutant (curve 2) under white light (WL, 20 µmol photons $s^{-1}$ $m^{-2}$). Open arrows, WL is turned on; black arrows, WL is switched off. E: P700 oxidation level versus white light fluence rate displayed by WT (circles) and *petJ*-null mutant (squares) grown in the BG11 medium minus $Cu^{2+}$ and with $NH_4^+$ as nitrogen source. The experiments were carried out 50 h after the exchange of the growth medium. The P700 oxidation level is expressed in % of the P700 maximal level reached in WT at maximum white light intensity.

the state of reduction of $Q_A$(Table 2). Fluorescence was emitted from PSII which in that way responds to the relatively reduced state of the electron carriers in the interphotosystem chain. Obviously with a less productive PSII, the growth rate declines. Growth of the *petJ*-null mutant could be restored in a BG11-$NH_4^+$ medium by raising the salt concentration up to 550 mM NaCl (Fig. 3C). As previously reported, cyclic electron flow around PSI is stimulated by high salt growth conditions in *Synechocystis* [6,10,11], and the expression of flavodoxin as well as the overexpression of Ferredoxin-$NADP^+$-Reductase (FNR) [11,30] were involved in this phenomenon. One can conclude from the responses of the *petJ*-null mutant to differently defined growth media that the changes at the PSI electron acceptor side and an alternative way of electron flow around PSI help to balance the redox state of P700 and the interphotosystem chain of electron carriers.

### 3.4. Conclusion

The earlier observed absence of an apparent phenotype for the *petJ*-null mutant in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 has led to the assumption that the presence of the *petJ* gene product, cytochrome $c_6$, was dispensable even after growth in a $Cu^{2+}$-free medium. In the latter case, conditions would demand the presence of cytochrome $c_6$ as the functionally equivalent copper protein plastocyanin would be absent as well. However, the results reported here show that in cases of an overly reduced cytoplasm the mutant has problems growing. Also,

Table 2
Effect of the growth conditions on the extent of reduction of $Q_A$ in the WT and in the *petJ*-null mutant

| | WT | *petJ*-null mutant |
|---|---|---|
| HC medium plus $Cu^{2+}$, $NO_3^-$-grown cells | 0.29 ± 0.03 (3) | 0.28 ± 0.03 (3) |
| HC medium minus $Cu^{2+}$, $NO_3^-$-grown cells | 0.26 ± 0.02 (3) | 0.34 ± 0.03 (3) |
| HC medium minus $Cu^{2+}$, $NH_4^+$-grown cells | 0.25 ± 0.03 (3) | 0.35 ± 0.03 (3) |
| BG11 medium minus $Cu^{2+}$, $NH_4^+$-grown cells | 0.24 ± 0.03 (5) | 0.45 ± 0.05 (5) |

Cells were grown in the modified Allen's medium (containing 12 mM $CO_3H^-$, HC: high carbon medium) plus or minus copper and in the BG11 medium (minus copper) with nitrate or ammonium as nitrogen source. Cells growing in Allen's medium plus $Cu^{2+}$ were centrifuged, washed and suspended in the different media. The extents of $Q_A^-$ reduction estimated by $1-q_p$ in WT and in the *petJ*-null mutant were measured at a photon flux density of 20 μmol photons $s^{-1}$ $m^{-2}$ (white light) as described in Section 2. The measurements on the *petJ*-null mutant incubated in the BG11 medium plus ammonium and minus copper were carried out after 50 h of culture.

in the *petJ*-null mutant, in marked contrast to the WT, the *isiA* and *isiB* genes, present in one operon, were derepressed. Those genes encode a chlorophyll–protein antenna and flavodoxin. The chlorophyll–protein antenna may protect PSI against photoinhibition via release of excitation energy as heat [29]. Flavodoxin is known to participate in the electron flux at the stromal side of PSI [18], in conjunction with ferredoxin. Here, an own role for flavodoxin envisages its contribution to electron flow away from the stromal PSI acceptors and back to P700 via PQ or via an alternative non-energy-conserving pathway that may incorporate cytochrome *M*. As indicated, cytochrome *M* would not be the best acceptor in normal linear flow since it is rather believed to be an electron donor to the cytochrome *c* oxidase [31]. Given conditions, PQ was found to be overly reduced in the mutant and not in the WT. These observations indicated that the mutant experienced problems with excess electrons at the stromal side of the thylakoid membranes rather than at the actual working side of the deleted cytochrome $c_6$, the lumen. The derepression of the *isiAB* operon compensates for the cytochrome $c_6$ deficiency by rapid recycling of electrons around PSI, which protects PSI from photoinhibition by preventing overoxidation of P700 at its lumenal side. *Fur* boxes have been found upstream of *isiA* and *isiB* in *Synechocystis* PCC 6803 and consequently those genes are thought to belong to the Fur-regulated gene family. The expression of the *isiA* and *isiB* genes after heat shock and salt stress [11,22] suggests that they could also be regulated by other transcription factors, or by different stress which might induce the same general response that could be judged in response to oxidative stress. In *Escherichia coli*, the expression of the *fldA* gene encoding flavodoxin is regulated by two transcription factors, one is *Fur* (Fur-regulated genes derepressed under iron limitation) and the other is *SoxS* which is involved in the response to oxidative stress [32].

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