

2-26-2013

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Recommended Citation

Summerfield, Tina C.; Crawford, Tim S.; Young, Roger; Chua, Jocelyn PS; Macdonald, Rebecca; Sherman, Louis A.; and Eaton-Rye, Julian J., "Environmental pH Affects Photoautotrophic Growth of *Synechocystis* sp. PCC 6803 Strains Carrying Mutations in the Lumenal Proteins of PSII." (2013). *Department of Biological Sciences Faculty Publications*. Paper 48.
<http://dx.doi.org/10.1093/pcp/pct036>

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Running title: Environmental pH controls growth of Photosystem II mutants

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Bioenergetics and Photosynthesis – Associate Editor Neil Baker

Environmental pH controls photoautotrophic growth of *Synechocystis* sp. PCC 6803 strains carrying mutations in the lumenal proteins of Photosystem II

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Funding support was provided by a New Zealand Marsden Grant (09-UOO-118) to TCS and a grant from the US Department of Energy (DE-FG02-99ER20342) to LAS.

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ABSTRACT

Synechocystis sp. strain PCC 6803 grows photoautotrophically across a broad pH range but wild-type cultures reach a higher density at elevated pH; however, photoheterotrophic growth is similar at high and neutral pH. A number of Photosystem II (PS II) mutants each lacking at least one luminal extrinsic protein, and carrying a second PS II mutation in a luminal domain, are able to grow photoautotrophically in BG-11 medium at pH 10.0, but not pH 7.5. We investigated the basis of these pH effects and observed no pH-specific change in variable fluorescence yield from PS II centers of the wild type, Δ PsbO: Δ PsbU, or Δ PsbV: Δ PsbQ strains. However, 77 K fluorescence emission spectra indicated increased coupling of the phycobilisome (PBS) antenna at pH 10.0 in all mutant strains. DNA microarray data showed a cell-wide response to transfer from pH 10.0 to pH 7.5, including decreased mRNA levels of a number of redox or oxidative stress responsive transcripts [Hihara Y, Sonoike K, Kanehisa M, Ikeuchi M (2003) J Bacteriol 185: 1719-1725; Hihara Y, Kamei A, Kanehisa M, Kaplan A, Ikeuchi M (2001) Plant Cell 13: 793-806]. We hypothesize this transcriptional response led to increased scavenging for reactive oxygen species and increased photosynthetic oxygen evolution at pH10. These changes enable photoautotrophic growth of the PS II mutants at pH 10.0.

Cyanobacteria are known to be alkaliphilic microbes (Pikuta et al., 2007), although many strains are able to grow over a wide pH range. Nonetheless, a large body of research with model organisms, such as *Synechocystis* sp. Strain PCC 6803 (hereafter *Synechocystis* 6803) has been conducted in buffered growth media typically around pH 7.5, which might not represent the most natural condition. Unexpectedly, several Photosystem II (PS II)-specific mutations introduced into *Synechocystis* 6803 were found to create strains unable to grow at pH 7.5, whereas photoautotrophic growth could be restored in these mutants at pH 10.0 (Eaton-Rye et al., 2003; Summerfield et al., 2005). The pH-sensitive strains are all able to grow photoautotrophically in medium buffered at pH 10.0, but not at pH 7.5, and all strains carried mutations targeting the luminal proteins of PS II. These include two double mutants, $\Delta\text{PsbO}:\Delta\text{PsbU}$ and $\Delta\text{PsbV}:\Delta\text{CyanoQ}$ and a third strain lacking PsbV and carrying a short segment deletion between Arg384 and Val392 in CP47 (Eaton-Rye et al., 2003; Summerfield et al., 2005). Additionally, mutation of the CP47 residue Glu364 to Gln combined with the removal of PsbV created a pH-sensitive strain (Eaton-Rye et al., 2003).

Photosystem II is the light-driven water-plastoquinone oxidoreductase of oxygenic photosynthesis (Wydrzynski and Satoh, 2005; Renger, 2012). The photosystem includes a core complex of the PsbA (D1) and PsbD (D2) polypeptides that bind the P680 reaction center chlorophylls, the Mn_4CaO_5 oxygen-evolving complex (OEC) and the majority of the remaining redox cofactors (Nixon et al., 2005; Kawakami et al., 2011; Müh et al., 2012). The PS II core is flanked by PsbB (CP47) and PsbC (CP43) which are chlorophyll-binding proteins that serve as a proximal antenna transferring excitation energy from the peripheral phycobilisome (PBS) antenna to P680 (Eaton-Rye and Putnam-Evans, 2005; Mullineaux, 2008). Hydrophilic domains of CP47 and CP43, together with the extrinsic proteins, provide a cap over the OEC (Bricker et al., 2012). In cyanobacteria, additional lipoproteins are associated with the luminal face of PS II; however, these proteins are not present in the available PS II crystal structures from either *Thermosynechococcus elongatus* or *T. vulcanus* (Fagerlund and Eaton-Rye, 2011).

The luminal PS II extrinsic proteins play a role in protecting the OEC from bulk reductants and contribute to putative channels associated with the access of substrate H_2O and the egress of O_2 and H^+ (Gabdulkhakov et al., 2009; Umena et al., 2011). The X-ray crystallographic structures from *T. elongatus* and *T. vulcanus* have confirmed the presence of PsbO, PsbU and PsbV as hydrophilic luminal extrinsic proteins (Ferreria et al., 2004; Loll et al., 2005; Umena et al., 2011). In addition, the lipoprotein CyanoQ has been shown to be associated with highly active PS II preparations from *Synechocystis* 6803 (Roose et al., 2007).

In cyanobacteria, the PsbO protein contributes to the stability of the PS II dimeric supercomplex through interactions with loop A and loop E of CP47 (De Las Rivas and Barber, 2004; Bentley and Eaton-Rye, 2008). Removal of PsbO increases sensitivity of PS II to photoinactivation and results in a strict dependence on Ca^{2+} and Cl^- for photoautotrophic growth (Bockholt et al., 1991; Burnap and Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991; Burnap et al., 1996). A similar light sensitivity and a dependence on Ca^{2+} and Cl^- for photoautotrophy has been observed when the *psbV* gene is deleted (Shen et al., 1995; Morgan et al., 1998; Shen et al., 1998).

Susceptibility to photoinactivation is also observed when the PsbU subunit is removed (Clarke and Eaton-Rye, 1999; Inoue-Kashino et al., 2005; Abasova et al., 2011). Removal of PsbU results in slower photoautotrophic growth in the absence of Ca^{2+} and Cl^- (Shen et al., 1997): and, in the absence of both ions, the ΔPsbU mutant is not photoautotrophic (Inoue-Kashino et al., 2005; Summerfield et al., 2005). In *Synechocystis* 6803, absence of PsbU affected both energy transfer and electron transfer in the PBS/PS II complex (Veerman et al., 2005). In addition, in the *Synechococcus* sp. PCC 7942 mutant lacking PsbU, cells exhibited an elevated resistance to oxidative stress (Balint et al., 2006; Abasova et al., 2011). Furthermore the PsbO, PsbU and PsbV proteins are required for the thermal stability of PS II and for the development of cellular thermotolerance (Nishiyama et al., 1999; Kimura et al., 2002). It is therefore apparent the extrinsic proteins enable cyanobacteria to adjust to a number of variable environmental parameters.

Transfer of wild-type *Synechocystis* 6803 from pH 7.5 to pH 10.0 increased mRNA levels of genes encoding several low-molecular-weight intrinsic subunits as well as extrinsic proteins of PS II, including *psbO* and *psbU* (Summerfield and Sherman, 2008). This was part of a cell wide response that included mechanisms to maintain pH homeostasis in the cytosol such as the presence of monovalent cation/proton antiporters (Padan et al., 2005). Cyanobacteria exhibit additional complexity, compared to non-photosynthetic organisms, in maintaining a pH in the thylakoid lumen that is approximately 2 pH units more acid than the cytosol. Moreover, cyanobacterial cytoplasmic and lumen pHs are altered by the environmental pH with an increase of 2 pH units in the external pH resulting in an internal increase of approximately 0.2 pH units (Belkin and Packer, 1988; Ritchie, 1991).

It appears that the external pH is able to influence the growth of mutants with an altered complement of PS II extrinsic proteins, even though these proteins are located in the acidic thylakoid lumen. To investigate how the different combinations of the extrinsic proteins influence the ability of *Synechocystis* 6803 cells to adapt to different pHs, we have

investigated PS II activity *in vivo* using non-invasive measurements of variable chlorophyll fluorescence yield. We have combined these PS II activity measurements with pH-dependent changes in global gene expression in the wild type and the pH-sensitive Δ PsbO: Δ PsbU mutant to investigate how modification of PS II activity by the presence or absence of specific lumenal extrinsic proteins can influence gene expression and facilitate photoautotrophic growth.

MATERIALS AND METHODS

Cyanobacterial Strains and Growth Conditions

The glucose tolerant variant of *Synechocystis* 6803 (Williams, 1988) was used in this study. Cultures were maintained on BG-11 plates containing 5 mM glucose and 20 μ M atrazine and appropriate antibiotics. In both solid and liquid media chloramphenicol was present at a concentration of 15 μ g/mL and erythromycin, kanamycin, and spectinomycin were present at 25 μ g/mL. The BG-11 solid media were supplemented with 10 mM TES-NaOH (pH 8.2) and 0.3% sodium thiosulfate. Liquid cultures were grown in BG-11 media containing either 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5) or 25 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 10.0); photomixotrophic and photoheterotrophic cultures contained 5 mM glucose and appropriate antibiotics, in addition, photoheterotrophic grown cultures contained 20 μ M atrazine. Cultures were maintained at 30°C under constant illumination at 30 μ mol photons $\text{m}^{-2} \text{s}^{-1}$ in a MLR-350 growth cabinet (Sanyo Electric Biomedical Co. Ltd., Tokyo).

Mutants lacking PsbO and/or PsbU were produced as described in (Eaton-Rye et al., 2003), these strains were constructed in the background of a control strain that has a kanamycin-resistance cassette located downstream of the *psbB* gene (Eaton-Rye and Vermaas, 1991) which has a phenotype otherwise indistinguishable from wild type. Strains lacking PsbV and/or CyanoQ were produced as described in (Summerfield et al., 2005). For DNA micorarray experiments, cultures were grown in BG-11 at pH 10.0 until an $\text{OD}_{730 \text{ nm}} \sim 0.2$ (corresponding to approximately 8×10^7 cells mL^{-1}) and transferred to BG-11 medium buffered at pH 7.5: cells were harvested at 0 h, 1 h and 2 h following transfer to pH 7.5; cells from the 0 h and 2 h time points were used for microarray experiments.

77 K Fluorescence Emission Spectra

Samples were assayed for fluorescence emission at 77 K with a modified Perkin-Elmer MPF-3L fluorescence spectrophotometer (Perkin-Elmer, Waltham, MA) equipped with a custom-built silver Dewar. Samples for analysis were prepared from mixotrophic cultures grown to mid-logarithmic phase in BG-11 at the appropriate pH. Cells were washed three times in their respective media to remove glucose and resuspended to a chlorophyll *a* concentration of 2 μ g Chl mL^{-1} , and left to stabilize for 30 min at 30°C and 50 μ mol photons

$\text{m}^{-2} \text{s}^{-1}$ light. One millilitre of cells were loaded into EPR tubes and quickly frozen by plunging into liquid nitrogen, and were kept in liquid nitrogen until emission spectra were collected.

77 K fluorescence emission spectra were measured using two different excitation energy wavelengths for each sample: 440 nm to specifically excite chlorophyll *a* and 580 nm to specifically excite the PBS. The excitation slit wavelength was set at 12 nm and 10 nm for 440 nm and 580 nm excitation, respectively; the emission slit wavelength was set at 4 nm. Spectra were normalized to the emission peak at 725 nm arising from PS I.

Variable Chlorophyll *a* Fluorescence Yield Measurements

Cells were grown and washed as described for 77 K fluorescence emission spectra measurements but were resuspended at $6 \mu\text{g Chl mL}^{-1}$. After 30 min at 30°C and a light intensity of $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, cells were diluted to $3 \mu\text{g Chl mL}^{-1}$ in 40 mL dark flasks, and dark adapted for 15 min at 30°C with gentle shaking.

Chlorophyll *a* fluorescence induction measurements were made using an FL-3500 fluorometer (Photon Systems Instruments, Brno, Czech Republic) in 4 mL quartz cuvettes with a 1 cm optical path containing a 2 mL sample volume. Fluorescence induction was induced by a 455 nm constant actinic light applied at $2800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 5 s. Fluorescence was measured using weak 455 nm probing flashes. When chlorophyll *a* fluorescence decay measurements were made a 455 nm actinic saturating flash was given using the FL-3500 fluorometer and the decay followed by either weak red (625 nm) or blue (455 nm) measuring flashes.

RNA extraction

Total RNA was extracted and purified using phenol-chloroform extraction and CsCl gradient purification as previously described (Reddy et al., 1990; Singh and Sherman, 2002).

Microarray Design

The DNA microarray platform and construction was as described in Postier et al. (2003) and the cDNA labeling, prehybridization and hybridization protocols are described in

detail in Singh et al. (2003). The microarray experiment involved a loop design that compared the wild type and the Δ PsbO: Δ PsbU strain in BG-11 at pH 10.0 and 2 h following a transition to BG-11 at pH 7.5 by using an analysis of variance (ANOVA) model (Singh et al., 2003; Li et al., 2004).

Data acquisition included the ANOVA model approach to test the null hypothesis that a particular gene's expression level did not differ between the treatments and to calculate a p-value (Singh et al., 2003). This experiment contained two genotypes (wild type and the Δ PsbO: Δ PsbU strain) and two stimuli (growth at pH 10.0 and transition to medium at pH 7.5) for a total of four treatment combinations. The effects of the absence of PsbO and PsbU and the transition from pH 10.0 to pH 7.5 were examined in the ANOVA design essentially as described in (Kerr and Churchill, 2001a, 2001b; Singh et al., 2003; Li et al., 2004). We used the false discovery rate (FDR) of 5% to control the proportion of significant results that are Type I errors (false rejection of the null hypothesis) as described in (Summerfield and Sherman, 2007). Genes with an FDR = 0.05 (corresponding to 5% expected false positives) and that exhibited a change of at least 1.5-fold were considered interesting and retained for further analysis. The p-value of these genes ranged from 2.7×10^{-2} to 6.3×10^{-17} .

Semi Quantitative RT-PCR

DNase I treatment and reverse transcription were performed as described in (Summerfield et al., 2008). PCR was carried out using 94°C 1 min, followed by 20-30 cycles of: 94°C 30 s, 52°C 30 s and 68°C 30 to 120 s (depending on amplicon size), to amplify regions of the genes listed below.

Semi-quantitative RT-PCR was used to examine mRNA levels in the wild type and the Δ PsbV: Δ PsbQ strain. The genes amplified, the primers used and PCR product size and number of PCR cycles were: sll0306 (F 5'- gactacctcagtctgtcg, R 5'- ctgatgttgagatgctgg, 260 bp, 25 cycles); sll0789 (F 5'- tacgtagtgtattgggtgc, R 5'- gcatctaaaccctcaacc, 212 bp, 25 cycles); sll1514 (F 5'- gataattccagcagcag, R 5'- gtcaaagtaggataccg, 350 bp, 25 cycles); sll1577 (F 5' - ttccaagctgatgctcg, R 5' - agcactgcaatcaccacg, 439 bp, 20 cycles), and *rnpB* (F 5'-tgtcacagggaaatctgagg, R 5'-gagagagttagtcgtaagc, 405 bp, 25 cycles). The *rnpB* gene was included as this transcript is frequently used as a constitutively expressed control for gene expression. Amplification products were separated on a 2% agarose Tris-acetate-EDTA or TAE gel.

RESULTS

Photoautotrophic but not Photoheterotrophic Growth is Increased in Growth Medium Buffered at pH 10.0 Compared to pH 7.5

It has previously been observed that wild-type cultures grown at pH 10.0 and pH 7.5 have similar initial doubling times, oxygen evolution rates and number of assembled PS II centers (Eaton-Rye et al., 2003). However, following 100 h of photoautotrophic growth, wild-type cultures reached an $OD_{730\text{ nm}} \sim 3$ in pH 10.0 medium, compared to an $OD_{730\text{ nm}} < 2$ in medium buffered at pH 7.5 (Fig. 1A). In contrast, photoheterotrophic growth of the wild type was similar in BG-11 buffered at either pH 10.0 or pH 7.5 (Fig. 1A). Furthermore, a strain lacking both the PsbO and PsbU proteins grew photoautotrophically at pH 10.0 but not pH 7.5 (Eaton-Rye et al., 2003), whereas photoheterotrophic growth was similar at pH 7.5 and pH 10.0 (Fig. 1B). These growth characteristics indicate the pH of the growth medium has a PS II-specific effect; this is supported by observations of additional PS II mutants that exhibit photoautotrophic growth at pH 10.0 but not pH 7.5 (Eaton-Rye et al., 2003; Summerfield et al., 2005). To investigate the impact of extracellular pH on PS II activity and assembly, variable chlorophyll *a* fluorescence yield and 77 K fluorescence emission spectra were measured.

Absence of Extrinsic Proteins of PS II Alters Chlorophyll *a* Variable Fluorescence from PS II in strains grown at both pH 7.5 and pH 10.0

Chlorophyll *a* variable fluorescence arises from PS II and can be induced by actinic light applied to dark-adapted cells (Papageorgiou et al., 2007; Stamatakis et al., 2007; Kaňa et al., 2012). Fluorescence induction for wild type and mutants lacking extrinsic proteins grown at pH 7.5 and pH 10.0 is presented in Fig. 2. The control strain exhibited a fluorescence induction curve typical of *Synechocystis* 6803 cells with a pronounced O to J rise reflecting the photochemical reduction of Q_A to form Q_A^- , followed by a slow J to I thermal phase on a ms time scale before undergoing a large I to P rise at around 0.5 to 1 s (Kaňa et al., 2012) (Fig. 2A). Removal of PsbU resulted in a reduction of the O to J rise but a substantial I to P rise remained; in contrast, removal of PsbO resulted in a reduced J level and prevented any I to P rise (Fig. 2A). The $\Delta\text{PsbO}:\Delta\text{PsbU}$ strain and a $\Delta\text{PsbO}:\Delta\text{PsbU}$ pseudorevertant strain, that

grew photoautotrophically at pH 7.5 (Summerfield et al., 2007), exhibited fluorescence induction curves similar to that observed with Δ PsbO cells (Fig 2C). For each of the five strains the fluorescence induction curves of cells grown at pH 10.0 and pH 7.5 were similar, except for a slight increase in the O to J rise at pH 10.0 (Fig. 2A-D).

Fluorescence induction was investigated in the pH-sensitive Δ PsbV: Δ CyanoQ mutant (Fig. 2E, F) as this strain exhibited a similar phenotype to the Δ PsbO: Δ PsbU strain (Summerfield et al., 2005). Wild type and the Δ CyanoQ strains possessed a similar O-J-I-P transient, whereas the Δ PsbV and Δ PsbV: Δ CyanoQ strains exhibited suppressed fluorescence induction. Results in Fig. 2 indicate the absence of an O-J-I-P transient does not preclude photoautotrophic growth and the ability of the Δ PsbO: Δ PsbU or Δ PsbV: Δ CyanoQ strains to grow at pH 10.0 was not accompanied by restoration of an I to P rise. In all strains the O to J rise was slightly enhanced at pH 10.0: since this might correlate with an increased absorption cross section for energy transfer to PS II from the peripheral antenna or PBS, this was investigated using 77 K fluorescence emission spectra.

Growth medium pH alters 77 K Fluorescence emission spectra of PS II mutants

We measured 77 K fluorescence emission using 580 nm excitation of the PBS. Compared to the control strain, the Δ PsbU and Δ PsbO strains had increased emission at 685 nm suggesting an enhanced emission from the terminal emitter of the PBS in cultures grown at pH 7.5 (Fig. 3A). Increased fluorescence yield was previously reported for the Δ PsbU strain grown in unbuffered BG-11 (Veerman et al., 2005). Emission at 695 nm was not increased in either the Δ PsbU or Δ PsbO strains supporting the interpretation that the increased fluorescence results from the terminal emitters of the PBS and not the CP43 chlorophyll *a* core antenna which also emits at 685 nm. The Δ PsbO: Δ PsbU strain had an emission spectra similar to the Δ PsbO strain, whereas, the pseudorevertant Δ PsbO: Δ PsbU strain had decreased emission at ~685 nm compared to the Δ PsbO and Δ PsbO: Δ PsbU strains but this was still increased compared to the wild type (Fig. 3C). The fluorescence emission at 685 nm was markedly decreased in strains lacking PsbO and PsbU when grown at pH 10.0 (Fig. 3B, D).

At pH 7.5, compared to the wild type, the Δ CyanoQ strain showed slightly increased emission at 648 nm, 665 nm and 685 nm corresponding to PC, APC and the terminal emitter of the PBS, respectively (Fig. 3E). The Δ PsbV and Δ PsbV: Δ CyanoQ strains exhibited an increased emission at 685 nm when grown at pH 7.5 (Fig. 3E), and this was reversed when

these strains were grown at pH 10.0 (Fig. 3F), similar to the Δ PsbU, Δ PsbO and Δ PsbO: Δ PsbU strains.

Confirmation that the elevated fluorescence resulted from excitation of the PBS and not the core antenna pigments, was obtained by measuring the F_o fluorescence level when probed with a red light (625 nm) compared to blue light (455 nm) at pH 7.5. The F_o fluorescence emission was elevated following excitation with red light which excited the PBS compared to when blue light (455 nm) was used to directly excite the core antenna pigments (Fig. 4A). At pH 10.0, the F_o fluorescence level probed with red light or blue light were similar (Fig. 4B), consistent with quenching of fluorescence from the PBS at pH 10.0. In *Synechocystis* 6803, non-photochemical quenching (NPQ) involves interaction of the orange carotenoid protein (OCP) with the PBS to increase energy dissipation, therefore decreasing the amount of energy arriving at PS II (Wilson et al., 2006). To assess whether the decreased fluorescence at pH 10.0 was due to increased NPQ, we induced NPQ in the wild type and Δ PsbO: Δ PsbU strains grown at both pH 7.5 and pH 10.0. Induction of NPQ using blue light, resulted in a small decrease in fluorescence at 648 nm, 665 nm and 685 nm but did not substantially reduce the elevated fluorescence at 685 nm in the Δ PsbO: Δ PsbU strain grown at pH 7.5 (Supplemental Fig. 1A, B). This demonstrates NPQ is not the cause of fluorescence quenching in the PS II mutant strains at pH 10.0 and is consistent with increased coupling of the PBS to PS II at pH 10.0.

Our measurements of 77 K fluorescence emission, using excitation at 580 nm, in Δ PsbO: Δ PsbU cells transferred from pH 7.5 to pH 10.0 also showed decreased fluorescence did not occur until several hours after transfer to elevated pH (Supplemental Fig. 1C). In addition, cells grown at pH 10.0 and transferred to pH 7.5 showed no increase in fluorescence at 685 nm even 12 h after transfer to the lower pH (Supplemental Fig. 1D). The kinetics of these changes in fluorescence emission suggest the altered coupling of the PBS to PS II was not a primary response to the change in pH of the growth medium. Furthermore, the pH-dependent coupling of the PBS by itself does not explain the photoautotrophic growth of the Δ PsbO: Δ PsbU and Δ PsbV: Δ PsbQ strains at pH 10.0, but not pH 7.5, as the Δ PsbO, Δ PsbU and Δ PsbV strains showed similar increased fluorescence emission at pH 7.5 and grew photoautotrophically at this pH. To identify mechanisms involved in recovery of photoautotrophic growth of pH-sensitive mutants at pH 10.0, we investigated the cell wide response to altered growth medium pH by examining the transcriptional response following the transition from pH 10.0 to pH 7.5 in the wild type and the Δ PsbO: Δ PsbU strain.

Global Transcriptional Response to Transition from pH 10.0 to pH 7.5 in the Wild Type and Δ PsbO: Δ PsbU strain

Synechocystis 6803 cultures were grown in continuous light in BG-11 medium buffered at pH 10.0. Samples were taken for RNA isolation at 0 h (t0), and 2 h (t2) following transfer from pH 10.0 to pH 7.5. As indicated in the Materials and Methods, we considered genes to be differentially regulated if they showed a fold change of ≥ 1.5 with an FDR = 0.05. A similar number of genes met these criteria in the two strains; 209 and 242 genes in the wild type and Δ PsbO: Δ PsbU strain, respectively. Genes were divided into functional categories according to the Cyanobase designation (<http://bacteria.kazusa.or.jp/cyaobase/cyano.html>), and the number of differentially expressed genes in each category is shown in Table I. Transcript level changes across a range of different functional categories indicated a cell-wide response to external pH; categories containing large numbers of genes exhibiting differential transcript abundance included: photosynthesis and respiration (20 and 42 genes, in wild type and Δ PsbO: Δ PsbU, respectively), transport and binding proteins (36 and 28 genes in wild type and 37 Δ PsbO: Δ PsbU, respectively); regulatory functions (20 genes, in both strains); and cellular processes (14 and 17 genes, in wild type and Δ PsbO: Δ PsbU, respectively). There were 198 genes up-regulated both on transfer from pH 7.5 to pH 10.0 and on transfer from pH 10.0 to pH 7.5: these had previously been identified and designated pH-independent (Summerfield and Sherman, 2008) and these were not included in Table I. All except four of these genes were similarly regulated or unchanged in the Δ PsbO: Δ PsbU strain on transition from pH 10.0 to pH 7.5.

Differences in the Transcript Level Response to Transfer from pH 10.0 to pH 7.5 in the Wild Type and the Δ PsbO: Δ PsbU Strain

Large numbers of genes involved in photosynthesis and respiration were increased in the Δ PsbO: Δ PsbU strain but not the wild type at pH 7.5. These included genes encoding cytochrome *c* oxidase (slr1136-8), this is the major terminal oxidase in *Synechocystis* 6803 with a role in both the thylakoid and plasma membranes (Howitt and Vermaas, 1998). This up-regulation likely reflects increased respiration in this strain due to the inability of the mutant to grow photoautotrophically at pH 7.5. In addition, genes encoding several components of the photosynthetic electron transport chain had increased transcript abundance in the Δ PsbO: Δ PsbU mutant at pH 7.5 compared to pH 10.0, but were unchanged in the wild

type. This included transcripts encoding: PS I components (PsaL, PsaK1 and PsaE); core subunits of NADH dehydrogenase (NdhB and NdhD1); the cytochrome *b*₅₅₉ subunits together with other low-molecular-weight PS II proteins from the same operon (PsbEFLJ), and three genes (*ssl0020*, *sll1584* and *slr1828*) encoding ferredoxin or ferredoxin-like proteins, including the most highly expressed ferredoxin gene, *ssl0020*, that is essential for viability (Poncelet et al., 1998) (Table II). Ferredoxin (Fd) is the final electron acceptor of the photosynthetic electron transport chain, in addition, Fd transfers electrons to other proteins; interacting with both regulatory and metabolic polypeptides (Hanke et al., 2011).

Oxidative Stress Responsive Genes Exhibit Decreased Transcript Abundance in the Wild Type and the Δ PsbO: Δ PsbU Strain on Transfer to pH 7.5

Several genes with increased mRNA levels following exposure to hydrogen peroxide had decreased mRNA levels at pH 7.5, conversely genes with decreased mRNA levels following hydrogen peroxide treatment were increased at pH 7.5 (Table II). Genes that exhibited decreased transcript abundance following transfer from pH 10.0 to pH 7.5 in the wild-type strain, included *dnaK2*, *dnaJ*, *hspA* and *htpG* (Table II) (cf. Li et al., 2004; Kanasaki et al., 2007). Two of these genes (*htpG* and *hspA*) had decreased mRNA levels in the mutant strain at pH 7.5. Increased transcript levels of these genes form part of a global response to numerous environmental factors including: oxidative, heat, UV light, high light, and osmotic stresses (Hihara et al., 2001; Li et al., 2004; Singh et al., 2006; Rupprecht et al., 2007).

Genes with roles in scavenging reactive oxygen species (ROS) exhibited decreased transcript levels at pH 7.5 compared to pH 10.0 in both strains. This included: *trxQ* (*slr0233*) encoding one of the four thioredoxins in *Synechocystis* 6803 (Perez-Perez et al., 2009a) and *ntr* (*slr0600*) encoding a putative NADP⁺ thioredoxin reductase (NTR) and it has been suggested that NTR donates electrons to TrxQ (Perez-Perez et al., 2009a). Deletion of either *trxQ* or *ntr* increased sensitivity to oxidative stress (Hishiya et al., 2008; Perez-Perez et al., 2009a). One of the two genes encoding glutaredoxin (*grxI*, *slr1562*) showed decreased transcript abundance at pH 7.5 in the wild type: the Grx1 protein has been shown to accept electrons from NTR (Marteyn et al., 2009). In the Δ PsbO: Δ PsbU strain, the detoxification gene *tpx* (*sll0755*) encoding a peroxiredoxin shown to accept electrons from thioredoxin showed decreased mRNA levels at pH 7.5, this gene is also up-regulated under high light, and heat exposure (Perez-Perez et al., 2009b). Similarly, the gene *ahpC* (*sll1621*) exhibited

decreased mRNA levels at pH 7.5 in the Δ PsbO: Δ PsbU strain; this gene encodes a protein with sequence identity to type 2 peroxiredoxin-like hypothetical proteins, and may play a critical role in coping with photooxidative stress (Kobayashi et al., 2004).

Oxidative-stress-responsive genes with roles in maintaining photosynthetic performance under stress conditions had decreased mRNA levels in both strains at pH 7.5. These included slr0947, down-regulation of this gene has been shown to decrease energy transfer from the PBS to PS II (Ashby and Mullineaux, 1999). A decreased mRNA level of this gene at pH 7.5 is consistent with the decoupling of the PBS observed at pH 7.5 (Fig. 3). Under high light conditions, Slr0947 is involved in up-regulation of the stress-responsive *hliB* gene (Kappell and Van Waasbergen, 2007). The *hli* genes encode high-light-inducible polypeptides (HLIPs), also known as small CAB (chlorophyll *a/b* binding)-like proteins or SCPs, (Dolganov et al., 1995; Funk et al., 1999) that are up-regulated under various stress conditions, and are thought to maintain photosynthetic performance by absorbing excess excitation energy or enabling the cells to cope with elevated reactive oxygen species (ROS) (He et al., 2001). Two of the four *hli* genes: *hliA* and *hliB* had decreased mRNA levels at pH 7.5 and were up-regulated following hydrogen peroxide treatment. The HLIPs interact with Slr1128, a protein of unknown function (Wang et al., 2008): slr1128 transcript levels are decreased at pH 7.5 in both strains. The gene encoding OCP is induced following hydrogen peroxide treatment and has decreased mRNA levels in wild type at pH 7.5.

Several genes involved in photosynthesis that exhibited altered transcript levels following exposure to hydrogen peroxide were inversely effected by transfer to pH 7.5 (Table II). In the wild type, decreased mRNA levels were observed for sll0550, encoding a flavoprotein (Flv3) involved in the Mehler reaction, and accepting electrons from PS I (Helman et al., 2003). The genes encoding NADH dehydrogenase subunits NdhD4 and NdhF4 had increased mRNA levels in both strains at pH 7.5, these were decreased following hydrogen peroxide treatment. These subunits are involved in CO₂ uptake (Ogawa et al., 2000) and PS I-mediated cyclic electron flow (Bernat et al., 2011). The gene encoding PS II extrinsic protein (PsbV) had increased mRNA levels at pH 7.5 and decreased mRNA levels following hydrogen peroxide treatment, and the *psb28-2* gene which has similarity to *psb28* although its function is not known, had decreased mRNA levels at pH 7.5 and was up-regulated under stress including exposure to hydrogen peroxide, UV-light and osmotic stress (Table II) (Huang et al., 2002; Li et al., 2004; Paithoonrangsarid et al., 2004).

Increased transcript levels of PBS structural genes (*apcABC* and *cpcBA*), and decreased transcription of genes *nblA1*, *nblA2* and *nblB*, that encode proteins involved in PBS

degradation, were observed at pH 7.5 in the wild type and to a greater extent in the Δ PsbO: Δ PsbU strain (Table II). These PBS structural genes are repressed by hydrogen peroxide treatment while the *nblA* and *nblB* genes are induced. Response regulator RppA (Sll0797) is involved in regulation of the PBS structural genes and the *nblA* genes (Li and Sherman, 2000). At pH 7.5, genes encoding this response regulator and neighbouring histidine kinase (*sll0798*, *rppB*) exhibited increased transcript levels.

Genes involved in transcriptional regulation that were induced by hydrogen peroxide treatment exhibited decreased mRNA levels in both strains at pH 7.5: *hik34* encoding a histidine kinase and *sigB* encoding sigma factor. In addition, the sigma factor encoding gene, *sigC*, had decreased mRNA levels in the wild type. The gene cluster *sll0788*–*sll0790* encodes a hypothetical protein, a response regulator (Rre34) and a histidine kinase (Hik31), respectively. Two of these genes, *sll0789* and *sll0788*, were repressed by hydrogen peroxide treatment and exhibited decreased transcript levels at pH 7.5 in both strains. The gene encoding Hik31 was decreased in the mutant but not the wild type at pH 7.5, and this gene was repressed by hydrogen peroxide treatment (Table II). Hik31 is involved in glucose metabolism, (Kahlon et al., 2006) and has been shown to be involved in acclimation to low oxygen conditions, where it plays a role in down-regulating transcripts involved in photosynthesis (including the PBS components), chaperones and ribosomal proteins (Summerfield et al., 2011). Regulatory gene *sll1392* (*pfsR*), which is involved in photosynthesis and a Fe-homeostasis stress response, had decreased mRNA levels at pH 7.5. Transcriptional changes following transition from pH 10.0 to pH 7.5 indicated growth at pH 10.0 stimulated a response with numerous similarities to the oxidative stress response in both strains.

Expression of Stress-Responsive Genes is Decreased in the Δ PsbV: Δ CyanoQ Strain at pH 7.5

To determine whether decreased mRNA levels of the stress responsive genes were shared by other pH-sensitive mutants, semi-quantitative RT-PCR was performed on RNA extracted from the Δ PsbV: Δ CyanoQ strain. The mRNA levels of a subset of genes that showed differential transcript abundance in the wild type and the Δ PsbO: Δ PsbU strain were examined. Genes encoding the heat shock protein HspA and sigma factor SigB are up-regulated following peroxide stress and these genes showed decreased mRNA levels in the Δ PsbV: Δ CyanoQ strain on transfer from pH 10.0 to pH 7.5 (Fig. 4C). The gene encoding

Rre34 that is in an operon with the gene encoding the histidine kinase Hik31 had decreased transcript levels at pH 7.5, whereas sll1577 encoding a PBS subunit was unchanged. This is similar to the response for the wild type and Δ PsbO: Δ PsbU strain. Hence, the wild type and the Δ PsbO: Δ PsbU and Δ PsbV: Δ CyanoQ mutants showed decreased mRNA levels of stress responsive genes following transfer to pH 7.5. Based on our observations, we hypothesized that increased transcript abundance of oxidative stress responsive genes at pH 10.0 improves resistance to oxidative stress.

The Wild Type is More Sensitive to Rose Bengal when Grown in BG-11 Medium at pH 7.5 Compared to pH 10.0

To test whether the cells have increased resistance to oxidative stress at pH 10.0 compared to pH 7.5, we exposed wild-type cultures grown at pH 10.0 and at pH 7.5 to the singlet oxygen generator rose bengal. Photoautotrophic wild-type cultures at pH 7.5 exhibited increased sensitivity to rose bengal compared to cultures grown at pH 10.0 (Fig. 5A). The addition of either 5 μ M or 2.5 μ M rose bengal to BG-11 medium at pH 7.5 prevented photoautotrophic growth of the wild type (Fig. 5A). However, addition of 2.5 μ M rose bengal to the wild type at pH 10.0 had no impact on doubling time, and the presence 5 μ M rose bengal only slightly decreased the growth rate (Fig. 5A). Absorbance at 543 nm was used to demonstrate the stability of rose bengal, this was similar in BG-11 at pH 7.5 and pH 10.0 (Fig. 5B). Photomixotrophic growth of the wild type in BG-11 medium buffered at pH 7.5 or pH 10.0 in the presence of 5 mM glucose was similar for the first 50 h, but by 100 h the pH 10.0 culture had reached a much higher OD_{730 nm} than the pH 7.5 culture (almost 5 compared to ~2.7), indicating a pH effect similar to that observed in photoautotrophically grown cultures (cf. Fig. 1A and Fig. 6A). Photomixotrophically grown cultures also showed pH-dependent sensitivity to rose bengal with 2.5 μ M rose bengal preventing photomixotrophic growth of the wild type at pH 7.5 but not pH 10.0 (Fig. 6A).

The Δ PsbO: Δ PsbU and Δ PsbV: Δ CyanoQ Strains are More Sensitive to Rose Bengal than the Wild Type and are Unable to Grow Photomixotrophically in the Presence of Rose Bengal in BG-11 Medium at pH 7.5

The Δ PsbO: Δ PsbU strain grew photomixotrophically at both pH 10.0 and pH 7.5 although cultures grew faster at pH 10.0 (Fig. 6B). The mutant was more sensitive to rose

bengal than wild type with the mutant showing no growth in the presence of 2.5 μM rose bengal at pH 10.0 or pH 7.5 (data not shown). The presence of 1 μM rose bengal prevented growth of the $\Delta\text{PsbO}:\Delta\text{PsbU}$ strain at pH 7.5 but not pH 10.0, although growth was reduced at pH 10.0 (Fig. 6B). Addition of rose bengal had a similar impact on photomixotrophic growth of the $\Delta\text{PsbV}:\Delta\text{CyanoQ}$ strain, with no growth of this strain at pH 7.5 and decreased growth at pH 10.0 (Fig. 6C). This strain appeared more sensitive to the presence of rose bengal at pH 10.0 than the $\Delta\text{PsbO}:\Delta\text{PsbU}$ strain (Fig. 6B, C).

The $\Delta\text{PsbO}:\Delta\text{PsbU}$ pseudorevertant grew photomixotrophically at both pH 10.0 and pH 7.5 with a similar doubling time for the first ~ 50 h, similar to wild type (Fig. 6D). However, this strain was unable to grow photomixotrophically in the presence of 1 μM rose bengal at pH 7.5 (Fig. 6D). Growth was retarded under photomixotrophic conditions at pH 10.0 in the presence of 1 μM rose bengal to a similar extent to the $\Delta\text{PsbO}:\Delta\text{PsbU}$ strain (Fig. 6B, D). The pseudorevertant's increased sensitivity to rose bengal at pH 7.5 compared to pH 10.0 indicates that the mechanism enabling photoautotrophic growth of the pseudorevertant at pH 7.5 is not sufficient to confer resistance to rose bengal and is not the same mechanism enabling growth of the mutants at pH 10.0.

DISCUSSION

At elevated pH, we observed improved growth of the wild-type strain and growth of PS II mutants under photoautotrophic conditions, but there was no pH effect on photoheterotrophic growth, indicating a PS II-specific pH effect. Fluorescence induction measurements showed the PS II mutants differed from the wild type, but growth under elevated pH did not restore a typical O-J-I-P transient as observed with wild-type cells. The 77 K fluorescence showed an increased emission at ~685 nm in cells grown at pH 7.5, this may reflect partial decoupling of the PBS resulting in increased fluorescence from the terminal phycobilin emitters in the PS II mutants and to a lesser extent in the wild type. At pH 10.0, this fluorescence emission is reduced, this was not due to NPQ and is likely to be due to improved energy transfer from the PBS to PS II, and this may account in part for the pH-induced recovery of photoautotrophy in these strains. However, increased coupling of the PBS to PS II at pH 10.0 is not sufficient to fully explain the restoration of photoautotrophic growth in the PS II mutants as the Δ PsbO and Δ PsbV mutants exhibited increased fluorescence emission at pH 7.5, but grew photoautotrophically at this pH. Furthermore, the time course experiment indicated the decoupling of the PBS at pH 7.5 was a secondary effect.

Cell wide transcriptional changes were observed in both the wild type and the Δ PsbO: Δ PsbU mutant strains on transfer from growth medium buffered at pH 10.0 to pH 7.5. These changes were consistent with the results of a previous pH 7.5 to pH 10.0 transition experiment with wild type (Summerfield and Sherman, 2008); reflecting maintenance of cellular homeostasis, as genes with altered mRNA levels were involved in osmotic, pH and ion homeostasis and included increased transcript abundance of stress-responsive genes at pH 10.0. A major difference in the response of the two strains was increased transcript abundance of photosynthesis genes in the Δ PsbO: Δ PsbU mutant, these changes may result from perturbation of the photosynthetic electron transport chain due to the absence of PsbO and PsbU. The removal of PsbU in *Synechocystis* 6803 was shown to increase PBS fluorescence and primary photochemistry of PS II (Veerman et al., 2005); and we have demonstrated a similar increase in PBS fluorescence in the absence of PsbO and PsbV but not CyanoQ. These findings are suggestive of a role for the extrinsic proteins in moderating light entering PS II and are consistent with the decreased *psbO* and *psbU* transcript levels observed by Tucker et al. (2001) in the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142 in the dark; and the hypothesis that alterations on the oxidizing side of PS II mediate PS II activity

as *Cyanothece* sp. ATCC 51142 proceeds through a 12 h light-12 h dark diurnal cycle (Meunier et al., 1998).

A proposed mechanism for the recovery of the pH-sensitive Δ PsbO: Δ PsbU and Δ PsbV: Δ CyanoQ mutants at pH 10.0 involves increased abundance of transcripts encoding oxidative stress-responsive genes at pH 10.0 (Fig. 7). In the mutant strains, increased mRNA levels of stress-responsive genes protect the impaired PS II centers, as well as the rest of the cell, from excessive ROS damage and facilitate photoautotrophic growth. Many genes exhibiting increased transcript abundance under oxidative stress also showed increased mRNA levels in the wild type following transfer from pH 7.5 to pH 10.0 (Summerfield and Sherman, 2008).

All strains exhibited decreased sensitivity to rose bengal when grown at pH 10.0 compared to pH 7.5, thus demonstrating the increased resistance of these cells to oxidative stress. . This pH 10.0 acclimation to rose bengal is similar to the acclimation response to low levels of singlet oxygen observed in *Chlamydomonas reinhardtii* (Ledford et al., 2007) where exposure to low levels of rose bengal resulted in increased transcript abundance of genes involved in the oxidative stress response and led to increased tolerance to exposure to higher levels of rose bengal. In our model, at pH 10.0 the increased endogenous singlet oxygen production in the Δ PsbO: Δ PsbU and Δ PsbV: Δ CyanoQ strains (and also wild type) leads to the increased resistance to exogenous singlet oxygen from rose bengal through changes in transcript levels of stress-responsive genes (Fig. 7).

Moreover, in the Δ PsbO: Δ PsbU pseudorevertant at pH 7.5, a subset of oxidative-stress-responsive genes that have increased mRNA levels at pH 10.0 exhibited increased transcript levels compared to the Δ PsbO: Δ PsbU strain (Summerfield et al., 2007; Fig. 7), and we suggest these transcripts may be involved in photoautotrophic growth of the pseudorevertant at pH 7.5. The fact that only a subset of the stress-responsive genes that had increased transcript levels in the wild type and Δ PsbO: Δ PsbU at pH 10.0 were increased in the pseudorevertant at pH 7.5 may account for the ability of this strain to grow photoautotrophically but still exhibit increased sensitivity to rose bengal at pH 7.5. Furthermore, additional stress responsive genes such as *tpx* and *sl11615* exhibited elevated transcript levels at pH 10.0 compared to pH 7.5 in the Δ PsbO: Δ PsbU mutant and these were not changed in the wild type and were not elevated in the pseudorevertant compared to the Δ PsbO: Δ PsbU strain at pH 7.5 (Fig. 7).

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FIGURE LEGENDS

Figure 1. Growth of *Synechocystis* sp. PCC 6803 strains in BG-11 medium as measured by the optical density at 730 nm. A. Wild type: photoautotrophic growth in media buffered at pH 7.5 (open circles) and in media buffered at pH 10.0 (closed circles); photoheterotrophic growth in the presence of atrazine and glucose in media buffered at pH 7.5 (open squares) and media buffered at pH 10.0 (closed squares). B. Δ PsbO: Δ PsbU mutant: photoautotrophic growth in media buffered at pH 7.5 (open triangles) and in media buffered at pH 10.0 (closed triangles); photoheterotrophic growth in the presence of atrazine and glucose in media buffered at pH 7.5 (open diamonds) and media buffered at pH 10.0 (closed diamonds). In A and B the data are the average \pm the standard error of three independent experiments. Error bars not visible are smaller than the symbols.

Figure 2. Fluorescence induction kinetics of *Synechocystis* sp. PCC 6803 strains grown in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). Chlorophyll *a* fluorescence was induced with a 455 nm, 2800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ actinic light and probed using a non-actinic measuring light of the same wavelength. A-D: Control (squares); Δ PsbO (circles); Δ PsbU (triangles); Δ PsbO: Δ PsbU (diamonds); Δ PsbO: Δ PsbU pseudorevertant (inverted triangles). Panels E, F: wild type (squares); Δ CyanoQ (triangles); Δ PsbV (circles); Δ CyanoQ: Δ PsbV (diamonds). Traces were normalized to $(F-F_0)/F_0$.

Figure 3. 77 K fluorescence emission spectra of *Synechocystis* sp. PCC 6803 strains grown in BG-11 medium buffered at pH 7.5 (left) or pH 10.0 (right). Spectra were collected using excitation at 580 nm and normalized to a PS I emission peak at 725 nm. A, B: Control (black); Δ PsbO (red); Δ PsbU (green). C, D: Δ PsbO: Δ PsbU (blue); Δ PsbO: Δ PsbU pseudorevertant (orange). E, F: wild type (black); Δ CyanoQ (red); Δ PsbV (blue); Δ PsbV: Δ CyanoQ (orange).

Figure 4. pH dependent effects on Photosystem II mutants lacking the PsbV and CyanoQ proteins. A. Comparison of using red (625 nm) and blue (455 nm) measuring flashes on the level of F_0 and the variable fluorescence decay following a single turnover actinic flash in wild type (squares) and the Δ PsbV strain (circles) at pH 7.5. Open symbols, 625 nm measuring flash; closed symbols, 455 nm measuring flash. The actinic flash (455 nm) was

fired after the second measuring flash. The CyanoQ mutant was similar to the wild type and the Δ PsbV: Δ CyanoQ strain was similar to the Δ PsbV strain (data not shown). B. Same as Panel A but at pH 10.0. C. Wild type and Δ PsbV: Δ CyanoQ cells were grown in BG-11 at pH 10.0 and samples harvested at t0, then remaining cells were transferred to pH 7.5 and samples were harvested at 1 and 2 h following transfer, as indicated above the lane. Equal amounts of RNA were used for each time point. Transcripts amplified were: *sll0306*, *sigB*; *sll0789*, *rre34*; *sll1514*, *hspA*; *sll1577*, *cpcB* and *rnpB*.

Figure 5. A. Photoautotrophic growth of the wild-type *Synechocystis* sp. PCC 6803 strains in BG-11 medium as measured by the optical density at 730 nm. Growth of the wild-type strain plus 5 μ M rose bengal (squares) and 2.5 μ M rose bengal (circles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). B. Absorbance at 543 nm of 5 μ M rose bengal in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols).

Figure 6. Photomixotrophic growth of the *Synechocystis* sp. PCC 6803 strains in BG-11 medium in the presence of 5 mM glucose as measured by the optical density at 730 nm. A. Growth of the wild-type strain (diamonds), plus 2.5 μ M rose bengal (circles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). B. Growth of the Δ PsbO: Δ PsbU strain (diamonds) and with the addition of 1 μ M rose bengal (triangles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). C. Growth of the Δ PsbV: Δ CyanoQ strain (diamonds) and with the addition of 1 μ M rose bengal (triangles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). D. Growth of the Δ PsbO: Δ PsbU pseudorevertant strain (diamonds) and with the addition of 1 μ M rose bengal (triangles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols).

Figure 7. Model of the response to transition from pH 10.0 to pH 7.5 of *Synechocystis* sp. PCC 6803 wild type (WT) and a Δ PsbO: Δ PsbU mutant that is unable to grow photoautotrophically at pH 7.5, and comparison of a Δ PsbO: Δ PsbU mutant with a Δ PsbO: Δ PsbU pseudorevertant strain that is able to grow photoautotrophically at pH 7.5. Selected differentially abundant genes (1.5 fold change or greater) at pH 10.0 compared to pH 7.5 in the wild type and the Δ PsbO: Δ PsbU strain are listed. The results are presented in the form of a Venn diagram that highlights the overlap among oxidative stress induced genes and

genes involved in scavenging reactive oxygen species that exhibit increased mRNA levels at pH 10.0. In addition, genes with increased transcript levels in the pseudorevertant compared to the Δ PsbO: Δ PsbU strain at pH 7.5 are shown (Summerfield et al., 2007). Genes designated as¹ were reported previously to exhibit elevated transcripts following exposure to oxidative stress and genes designated² exhibited decreased transcripts following exposure to oxidative stress (Li et al. 2004; Kanesaki et al. 2007). We suggest global stress-induced gene expression changes, are sufficient to account for restoration of photoautotrophic growth in the Δ PsbO: Δ PsbU strain and the increased resistance to rose bengal observed in all strains at pH 10.0 and directly or indirectly led to changes in light harvesting.

Table I. Functional categories of pH-responsive genes^a in wild-type and Δ PsbO: Δ PsbU *Synechocystis* sp. PCC 6803 strains

General pathways	Number of genes	Differentially regulated genes			
		WT (Up) ^c	OU (Up)	OU/WT pH 7.5	OU/WT pH 10.0
Amino acid biosynthesis	97	17 (10)	11 (6)	11 (4) ^d	5 (1) ^d
Biosynthesis of cofactors, prosthetic groups, and carriers	124	13 (5)	15 (7)	13 (6)	8 (4)
Cell envelope	67	7 (6)	19 (19)	15 (15)	2 (1)
Cellular processes	76	14 (2)	17 (10)	11 (8)	9 (9)
Central intermediary metabolism	31	4 (3)	4 (3)	1 (1)	1 (0)
DNA replication, restriction, recombination, and repair	60	5 (2)	6 (4)	4 (1)	3 (3)
Energy metabolism	132	19 (14)	17 (12)	12 (7)	7 (4)
Hypothetical	1076	169 (53)	133 (64)	92 (51)	70 (52)
Other categories	306	38 (22)	21 (9)	16 (7)	8 (3)
Photosynthesis and respiration	141	20 (13)	42 (37)	18 (14)	12 (11)
Purines, pyrimidines, nucleosides, and nucleotides	41	5 (2)	3 (1)	2 (1)	1 (1)
Regulatory functions	146	20 (8)	20 (11)	9 (4)	6 (4)
Transcription	30	8 (6)	6 (3)	2 (0)	2 (2)
Translation	168	23 (15)	17 (12)	8 (3)	4 (3)
Transport and binding proteins	196	36 (27)	28 (13)	28 (9)	16 (4)
Unknown	474	69 (21)	54 (31)	37 (31)	34 (22)
Total number	3165 ^b	467 (209)	413 (242)	279 (162)	188 (124)

^aGenes were considered differentially regulated when fold change >1.5 fold

^bTotal number of genes based on Kazusa annotation prior to May, 2002

^cNumber of genes with increased mRNA levels at pH 7.5 compared to pH 10.0 in a functional category

^dNumber of genes with increased mRNA levels in the mutant compared to wild type in a functional category

Note: WT = wild type and OU = Δ PsbO: Δ PsbU mutant

Table II. Selected genes^a showing altered transcript abundance on transition from pH 10.0 to pH 7.5 in *Synechocystis* sp. PCC 6803 wild type and a Δ PsbO: Δ PsbU strain

No.	Gene Name	H ₂ O ₂ ^b	WT ^c	OU ^c	WT/OU ^d		No.	Gene Name	H ₂ O ₂ ^b	WT ^c	OU ^c	WT/OU ^d	
					pH 10.0	pH 7.5						pH 10.0	pH 7.5
Biosynthesis of cofactors, prosthetic groups							<i>Phycobilisomes</i>						
slr0233	<i>trxQ</i>	I	-2.3	-1.6	1.2	-1.2	sll1577	<i>cpcB</i>	-	1.2	1.7	1.4	1.0
slr0600	<i>ntr</i>	-	-1.5	-1.6	-1.3	-1.3	sll1579	<i>cpcC2</i>	R	1.8	2.6	1.2	-1.1
slr1562	<i>grxX1</i>	-	-1.5	-1.4	1.0	1.1	sll1580	<i>cpcC1</i>	R	1.4	1.7	1.2	1.0
Cellular processes							slr0335	<i>apcE</i>	R	1.0	2.1	2.0	-1.1
sll0170	<i>dnaK2</i>	I	-2.1	-1.4	1.3	-1.1	ssr3383	<i>apcC</i>	R	1.8	3.2	1.6	-1.2
sll0430	<i>htpG</i>	I	-2.3	-2.2	1.0	1.0	slr1687	<i>nblB</i>	I	-2.3	-2.0	-1.4	-1.6
sll1514	<i>hspA</i>	I	-6.0	-1.7	1.6	-2.2	ssl0452	<i>nblA1</i>	I	-1.2	-1.5	-1.4	-1.1
sll1933	<i>dnaJ</i>	-	-1.6	-1.1	1.4	1.1	ssl0453	<i>nblA2</i>	I	-2.1	-2.4	-1.1	1.0
sll0093	<i>dnaJ</i>	I	-1.7	-1.2	1.0	-1.3	<i>Soluble electron carriers</i>						
sll0755	<i>tpx</i>	I	-1.3	-1.5	-1.2	1.0	sll1584	<i>fdl</i>	-	1.3	1.7	1.1	-1.3
Hypothetical							slr1828	<i>petF</i>	R	1.3	1.7	-1.4	-1.8
sll0939	<i>hypo</i>	I	-4.0	-2.9	1.0	-1.4	ssl0020	<i>petF</i>	-	1.1	2.1	1.6	-1.2
slr1128	<i>hypo</i>	-	-2.2	-1.5	1.0	-1.4	Regulatory functions						
slr1963	<i>ocp</i>	I	-1.7	-1.1	1.8	1.2	sll0797	<i>rppA</i>	-	1.5	1.5	-1.1	-1.1
Other							sll0798	<i>rppB</i>	-	1.6	2.2	1.1	-1.2
sll0550	<i>flv3</i>	I	-1.7	-1.3	1.2	-1.1	sll1392	<i>pfsR</i>	-	-3.9	-3.3	1.0	-1.1
sll1621	<i>ahpC</i>	I	-1.1	-1.9	-1.4	-1.3	slr0311	<i>hik29</i>	-	1.5	2.0	1.0	-1.3
ssl2542	<i>hliA</i>	I	-1.4	-1.5	-1.6	-1.5	slr0533	<i>hik10</i>	-	1.5	2.2	1.0	-1.3
ssr2595	<i>hliB</i>	I	-1.7	-4.1	-2.6	-1.1	slr0947	<i>rpaB</i>	I	-1.7	-2.3	-1.2	-1.1
Photosynthesis and Respiration							slr1285	<i>hik34</i>	I	-2.8	-1.7	1.2	-1.3
<i>Respiration</i>							slr1529	<i>ntrX</i>	-	1.5	1.9	-1.1	-1.4
slr1136	<i>ctaD1</i>	-	1.0	2.1	1.0	-2.0	Transcription						
slr1137	<i>ctaC1</i>	-	-1.1	1.5	1.2	-1.5	sll0184	<i>sigC</i>	I	-1.9	-1.2	1.1	-1.4
slr1138	<i>ctaE1</i>	-	1.0	1.6	1.0	-1.6	sll0306	<i>sigB</i>	I	-2.5	-1.7	1.0	-1.5
<i>NADH dehydrogenase</i>							sll1818	<i>rpoA</i>	-	2.9	1.4	-2.1	1.0
sll0026	<i>ndhF4</i>	R	1.8	2.0	1.1	1.0	slr1129	<i>rne</i>	-	2.7	1.5	-1.4	1.3
sll0027	<i>ndhD4</i>	R	2.1	1.8	1.2	1.4	Translation						
sll0223	<i>ndhB</i>	-	-1.3	1.6	1.6	-1.3	sll0020	<i>clpC</i>	I	-2.0	-1.2	1.1	-1.5
slr0331	<i>ndhD1</i>	R	-1.5	2.9	2.4	-1.8	slr0257	<i>ctpB</i>	-	-1.7	-1.2	1.3	-1.1
<i>Photosystem I</i>							slr1641	<i>clpB1</i>	I	-1.6	-1.2	1.1	-1.2
slr1655	<i>psaL</i>	R	1.0	1.9	1.8	-1.1	slr1751	<i>ctpC</i>	-	-3.1	-1.3	1.9	-1.2
ssr0390	<i>psaK1</i>	-	1.1	1.9	1.7	1.0	Gene clusters						
ssr2381	<i>psaE</i>	R	1.0	1.5	1.0	-1.6	sll0788	<i>hypo</i>	I	-4.0	-5.8	1.1	1.5
<i>Photosystem II</i>							sll0789	<i>rre34</i>	I	-3.5	-11.5	-2.1	1.6
sll0258	<i>psbV</i>	R	1.7	1.6	-1.1	1.0	sll0790	<i>hik31</i>	R	-1.3	-12.5	-6.2	1.5
sll1398	<i>psb28</i>	-	1.6	1.2	-1.2	1.1	slr0074	<i>ycf24</i>	I	-2.0	-2.3	-1.1	1.0
slr1739	<i>psb28-2</i>	I	-3.2	-2.4	1.1	-1.2	slr0075	<i>ycf16</i>	I	-1.5	-2.1	1.0	1.4

^aGenes were considered differentially regulated when fold change >1.5 fold

^bGenes previously shown to have altered mRNA levels following exposure to H₂O₂ (Li et al., 2004; Kanesaki et al., 2007).

R=repressed, I=induced

^cGenes with altered mRNA levels at pH 7.5 vs pH 10.0

^dGenes with altered mRNA levels in the WT vs OU

Note: WT = wild type; OU = Δ PsbO: Δ PsbU mutant

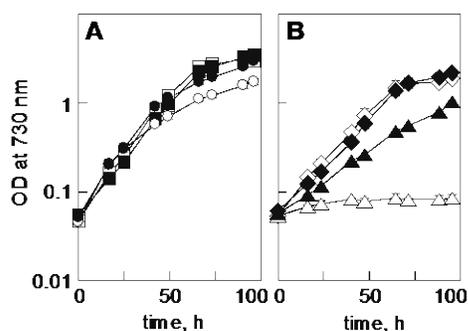


Figure 1. Growth of *Synechocystis* sp. PCC 6803 strains in BG-11 medium as measured by the optical density at 730 nm. A. Wild type: photoautotrophic growth in media buffered at pH 7.5 (open circles) and in media buffered at pH 10.0 (closed circles); photoheterotrophic growth in the presence of atrazine and glucose in media buffered at pH 7.5 (open squares) and media buffered at pH 10.0 (closed squares). B. Δ PsbO: Δ PsbU mutant: photoautotrophic growth in media buffered at pH 7.5 (open triangles) and in media buffered at pH 10.0 (closed triangles); photoheterotrophic growth in the presence of atrazine and glucose in media buffered at pH 7.5 (open diamonds) and media buffered at pH 10.0 (closed diamonds). In A and B the data are the average \pm the standard error of three independent experiments. Error bars not visible are smaller than the symbols.

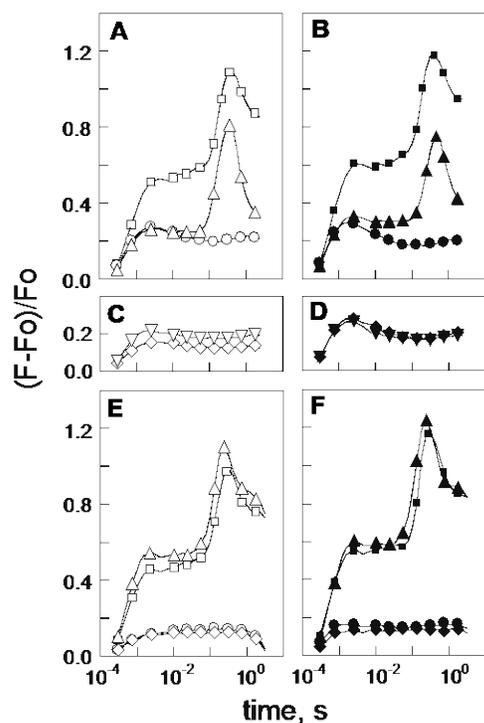


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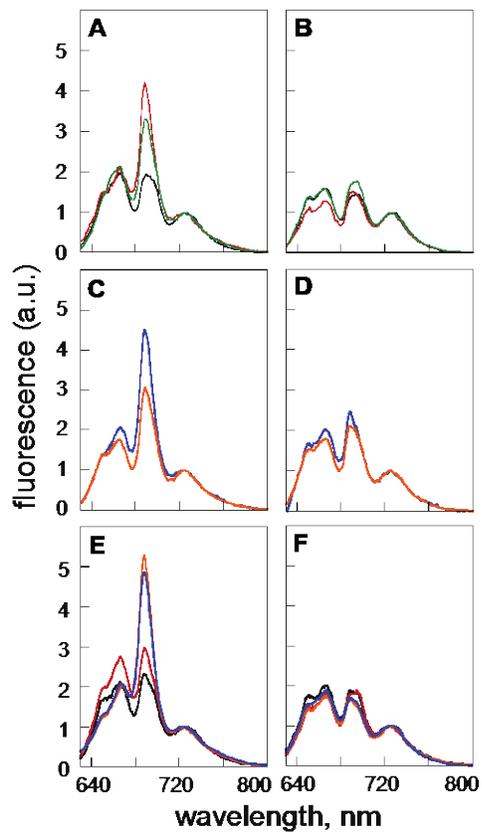


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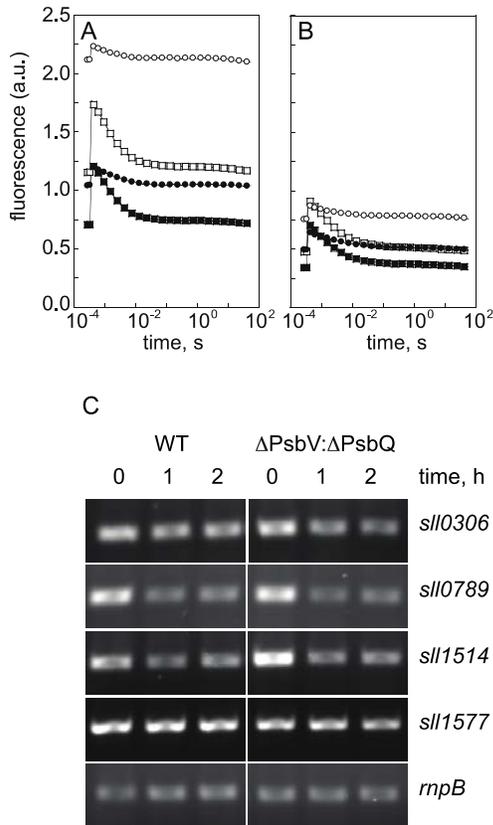


Figure 4. pH dependent effects on Photosystem II mutants lacking the PsbV and CyanoQ proteins. A. Comparison of using red (625 nm) and blue (455 nm) measuring flashes on the level of F_0 and the variable fluorescence decay following a single turnover actinic flash in wild type (squares) and the Δ PsbV strain (circles) at pH 7.5. Open symbols, 625 nm measuring flash; closed symbols, 455 nm measuring flash. The actinic flash (455 nm) was fired after the second measuring flash. The CyanoQ mutant was similar to the wild type and the Δ PsbV: Δ CyanoQ strain was similar to the Δ PsbV strain (data not shown). B. Same as Panel A but at pH 10.0. C. Wild type and Δ PsbV: Δ CyanoQ cells were grown in BG-11 at pH 10.0 and samples harvested at t_0 , then remaining cells were transferred to pH 7.5 and samples were harvested at 1 and 2 h following transfer, as indicated above the lane. Equal amounts of RNA were used for each time sample and for each transcript. Transcripts amplified were: *sll0306*, *sigB*; *sll0789*, *rre34*; *sll1514*, *hspA*; *sll1577*, *cpcB* and *rnpB*.

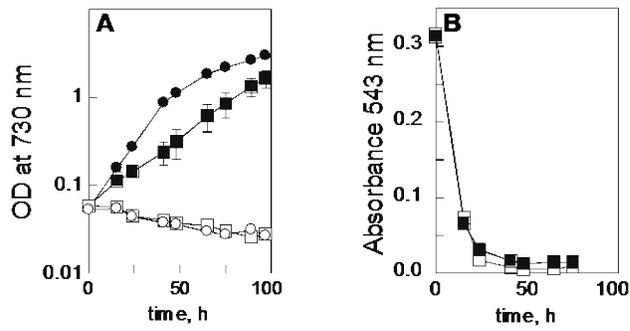


Figure 5. A. Photoautotrophic growth of the wild-type *Synechocystis* sp. PCC 6803 strains in BG-11 medium as measured by the optical density at 730 nm. Growth of the wild-type strain plus 5 μ M rose bengal (squares) and 2.5 μ M rose bengal (circles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). B. Absorbance at 543 nm of 5 μ M rose bengal in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols).

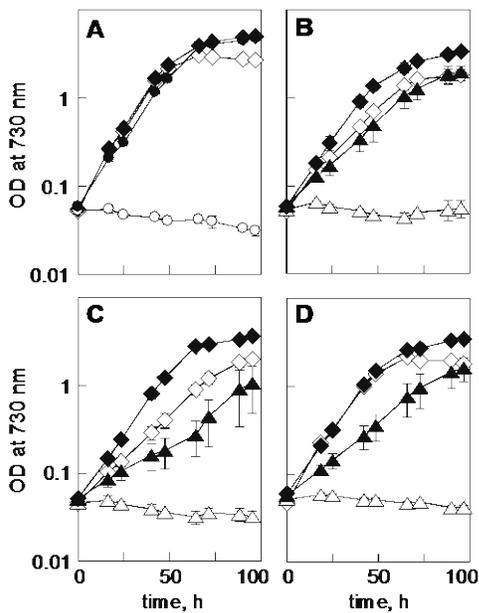


Figure 6. Photomixotrophic growth of the *Synechocystis* sp. PCC 6803 strains in BG-11 medium in the presence of 5 mM glucose as measured by the optical density at 730 nm. A. Growth of the wild-type strain (diamonds), plus 2.5 μ M rose bengal (circles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). B. Growth of the Δ PsbO: Δ PsbU strain (diamonds) and with the addition of 1 μ M rose bengal (triangles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). C. Growth of the Δ PsbV: Δ CyanoQ strain (diamonds) and with the addition of 1 μ M rose bengal (triangles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols). D. Growth of the Δ PsbO: Δ PsbU pseudorevertant strain (diamonds) and with the addition of 1 μ M rose bengal (triangles), in BG-11 medium buffered at pH 7.5 (open symbols) or pH 10.0 (closed symbols).

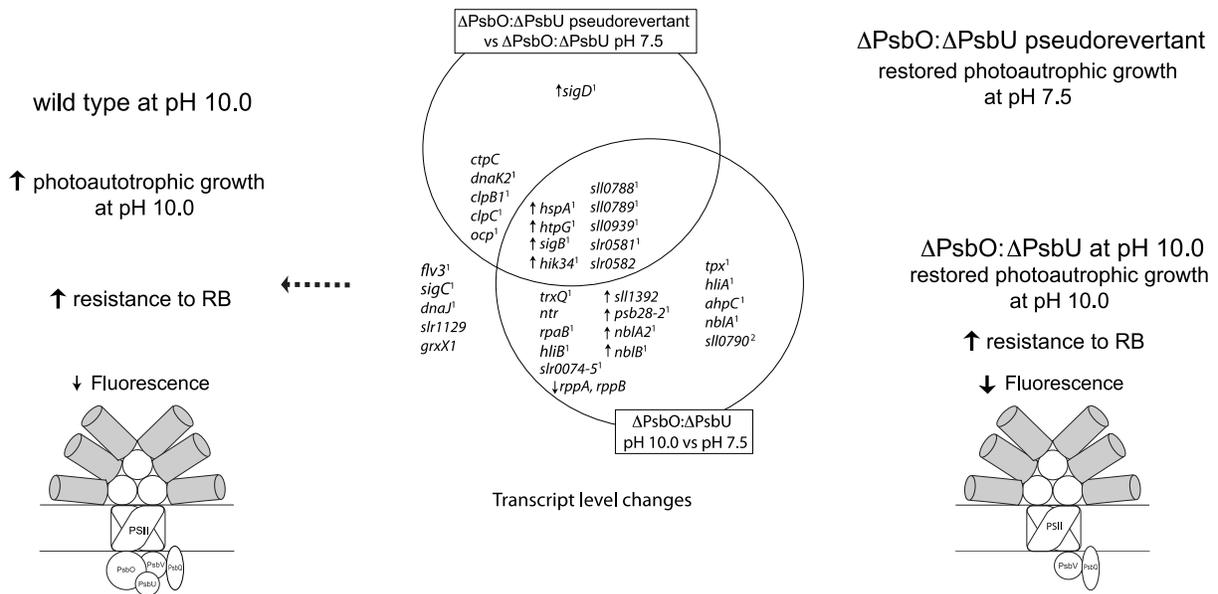


Figure 7. Model of the response to transition from pH 10.0 to pH 7.5 of *Synechocystis* sp. PCC 6803 wild type (WT) and a Δ PsbO: Δ PsbU mutant that is unable to grow photoautotrophically at pH 7.5, and comparison of a Δ PsbO: Δ PsbU mutant with a Δ PsbO: Δ PsbU pseudorevertant strain that is able to grow photoautotrophically at pH 7.5. Selected differentially abundant genes (1.5 fold change or greater) at pH 10.0 compared to pH 7.5 in the wild type and the Δ PsbO: Δ PsbU strain are listed. The results are presented in the form of a Venn diagram that highlights the overlap among oxidative stress induced genes and genes involved in scavenging reactive oxygen species that exhibit increased mRNA levels at pH 10.0. In addition, genes with increased transcript levels in the pseudorevertant compared to the Δ PsbO: Δ PsbU strain at pH 7.5 are shown (Summerfield et al., 2007). Genes designated as¹ were reported previously to exhibit elevated transcripts following exposure to oxidative stress and genes designated² exhibited decreased transcripts following exposure to oxidative stress (Li et al. 2004; Kanesaki et al. 2007). We suggest global stress-induced gene expression changes, are sufficient to account for restoration of photoautotrophic growth in the Δ PsbO: Δ PsbU strain and the increased resistance to rose bengal observed in all strains at pH 10.0 and directly or indirectly led to changes in light harvesting.