Investigating the role of Ycf48 in the biogenesis of Photosystem II

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Abstract

Photosystem II is a large, multi-subunit pigment-protein super-complex capable of splitting water to generate protons and electrons ultimately for the reduction of carbon dioxide into sugars: and as a by-product of this reaction molecular oxygen is produced. Photosystem II is composed of more than 20 protein subunits and in addition an increasing number of protein factors have been discovered that are required for biogenesis or assembly of the photosystem; however, these assembly factors are not present in the mature complex. Light-induced water-splitting by Photosystem II results in photodamage and therefore the photosystem continually undergoes a self repair process. In this project the roles of five hydrophilic protein assembly factors in Photosystem II biogenesis and repair have been investigated. The protein factors investigated were: Ycf48, PsbP, Psb27, Psb28, and Psb28-2. For this study a number of knockout mutants were utilised and the following mutants: ∆Ycf48, ∆PsbP, ∆PsbP:∆Ycf48, ∆Psb27, ∆Psb27:∆Ycf48, ∆Psb28, ∆Psb28:∆Ycf48, ∆Psb28-2, ∆Psb28-2:∆Ycf48, ∆Psb28:∆Psb28-2, and ∆Psb28:∆Psb28-2:∆Ycf48 have been compared to wild type. The ∆PsbP mutant was largely indistinguishable from wild type, with similar photoautotrophic growth and oxygen evolution rates. However, the ∆PsbP mutant did have lower levels of light-harvesting phycobilisomes and carotenoid pigments than wild type. The ∆PsbP:∆Ycf48 strain was found to have increased carotenoids and reduced chlorophyll a, suggesting an enhanced susceptibility to light stress in this strain. However, photoautotrophic growth, oxygen evolution and 77 K fluorescence emission with a 440 nm chlorophyll-specific excitation (a probe of the relative assembly levels of Photosystem II) all indicated the ∆PsbP:∆Ycf48 strain resembled the ∆Ycf48 single mutant. However, using a 580 nm excitation wavelength that excites the bilin pigments in the light-harvesting phycobilisomes 77 K fluorescence emission suggested an altered phycobilisome-Photosystem II interaction in the ∆PsbP:∆Ycf48 mutant. The ∆Psb27 strain had a similar doubling time to wild type. However, the ∆Psb27 mutant’s oxygen evolution rate and total photosystem level were both higher than wild type. An increased Photosystem II to Photosystem I ratio in the ∆Psb27 mutant appeared to be responsible for these differences. The ∆Psb27:∆Ycf48 strain was unable to grow photoautotrophically. The ∆Psb27:∆Ycf48 mutant had a reduced oxygen evolution rate, and reduced total levels of photosystems compared to wild type. The ∆Psb27:∆Ycf48 strain also had the increased Photosystem II to Photosystem I ratio found in the ∆Psb27 strain. Removal of either or both of the Psb28 proteins, Psb28 and Psb28-2, had minimal effects on physiological fitness. However, removal of either or both of the Psb28 proteins in combination with the removal of Ycf48 resulted in strains unable to grow photoautotrophically. Similar to the ∆Psb27:∆Ycf48 mutant, the ∆Psb28:∆Ycf48, ∆Psb28-2:∆Ycf48, and ∆Psb28:∆Psb28-2:∆Ycf48 mutants had decreased total photosystem levels, and an increased Photosystem II to Photosystem I ratio. These results suggest either an increased turnover or slow repair cycle is operating in these non-photoautotrophic strains. Results are discussed with regard to what is currently known about these assembly factors and the strains in which they were constructed.
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Abbreviations

Amp Ampicillin
APC allophycocyanin
BSA Bovine serum albumin
Chl a chlorophyll a
CpcG1 phycobilisome rod-core linker polypeptide 1
CpcG2 phycobilisome rod-core linker polypeptide 2
CtpA C-terminal processing A
Cyt b559 cytochrome b559
DCBQ 2,6-dichloro-p-benzoquinone
DCMU 3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DM n-Dodecyl-β-D-maltopyranoside
EDTA 2-(2-[Bis(carboxymethyl)amino]ethyl(carboxymethyl)amino)acetic acid
FRP fluorescence recovery protein
GT-O1 glucose tolerant Otago 1
GT-O2 glucose tolerant Otago 2
LRC rod-core linker
LB Lysogeny Broth
LED light emitting diode
LHC light-harvesting complex
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<tr>
<td>LMM</td>
<td>low molecular mass</td>
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<tr>
<td>NPQ</td>
<td>Non photochemical quenching</td>
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<td>OCP</td>
<td>Orange Carotenoid Protein</td>
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<td>OEC</td>
<td>oxygen-evolving centre</td>
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<td>$^3P_{680}$</td>
<td>triplet excited state $P_{680}$</td>
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</tr>
<tr>
<td>PETC</td>
<td>photosynthetic electron transport chain</td>
</tr>
<tr>
<td>Phe</td>
<td>Pheophytin</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonyl fluoride</td>
</tr>
<tr>
<td>POR</td>
<td>protochlorophyllide oxido-reductase</td>
</tr>
<tr>
<td>PPL-1</td>
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<td>PQ</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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Chapter 1

Introduction

1.1 Photosynthesis

Photosynthesis, the utilisation of sunlight to fix carbon dioxide into carbohydrates, is responsible for the vast majority of primary production globally. The photosynthesis of this project is the predominant form, oxygenic photosynthesis, which is utilised by cyanobacteria, algae, and higher plants. This process is also responsible for the high levels of molecular oxygen present in the planet’s atmosphere (reviewed by Hohmann-Marriott and Blankenship (2011)). Photosynthesis occurs in two groups of reactions known as the light and dark reactions.

\[ \text{H}_2\text{O} + \text{CO}_2 = (\text{CH}_2\text{O})_n \] (1.1)

Simplistically these reactions result in the generation of carbohydrates according to Equation (1.1). The light-dependent reaction, which takes as its input light and water, produces molecular oxygen and energy equivalents. This process, which is highly conserved between photoautotrophs, is reliant on electron transfer through a series of pigment-protein intermediates known as the photosynthetic electron transport chain (PETC).

1.2 Photosystem II

Photosystem II (PS II) is a multisubunit membrane protein complex, common to oxygenic photosynthetic organisms, with water-plastoquinone oxidoreductase activity (for
Figure 1.1: Structure of PS II from *Thermosynechococcus vulcanus*. Coloured with the monomer on the left showing extrinsic protein subunits: PsbO in pink, PsbU in olive, and PsbV in magenta, intrinsic protein subunits in light blue and the monomer on the right showing extrinsic protein subunits in yellow, D1 in red D2 in orange, CP47 in Green, and CP43 in blue. Image of 1D3ARC (Umema et al., 2011) from PDB (www.rcsb.org) (Berman et al., 2000) and made in PyMOL (Schrödinger, LLC, 2010).

recent reviews see Kern and Renger (2007), Pagliano et al. (2013), and Vinyard et al. (2013)). This activity is highly modulated by the interactions of PS II with a number of accessory complexes and cofactors, allowing for activity under a broad range of environmental conditions. In cyanobacteria, as in higher plants, assembled PS II is found in the thylakoid membrane, and in its physiologically active form is in a homodimeric complex with another PS II. The structure of PS II (Figure 1.1) has been determined several times, most recently by Umema and colleagues in the thermophilic cyanobacterium *Thermosynechococcus vulcanus* to a resolution of 1.8 Å (Ferreira et al., 2004; Guskov et al., 2009; Kamiya and Shen, 2003; Loll et al., 2005; Umema et al., 2011; Zouni et al., 2001). Individual PS II complexes are heterodimeric, with each being composed of approximately 20 protein subunits. The reaction centre (RC), i.e. the minimum requirement for charge separation, is made up of cytochrome *b*$_{559}$ (Cyt *b*$_{559}$) (comprised of PsbE and PsbF), D1, and D2. Whereas the centre required for oxidation of water is comprised of the chlorophyll *a* (Chl *a*)-binding D1, D2, CP43, and CP47 proteins, and Cyt *b*$_{559}$. The Mn$_4$CaO$_5$ cluster, the catalytic core of the oxygen-evolving centre
Photosystem II (OEC), assembles at the luminal face of the RC near D1 where it facilitates electron production via the splitting of water. The cluster is aided and protected by luminal extrinsic proteins present in mature PS II (reviewed by Bricker et al. (2012), Fagerlund and Eaton-Rye (2011), and Roose et al. (2007)). Around this central core are a number of comparatively low molecular mass (LMM) transmembrane protein subunits, whose composition varies amongst and between cyanobacteria and higher plants (for a review see Shi et al. (2012)).

Figure 1.2: Photosynthetic electron transport chain in *Synechocystis* sp. PCC 6803. Major components of the photosynthetic electron transport chain in the thylakoid membrane (TM), Lumen, and cytoplasm (C). Fd, ferredoxin; SDH, succinate dehydrogenase; Cyt b6, cytochrome b6 oxidase; Cyt C6, cytochrome C6; SUC, succinate; FUM, fumarate; COX, cytochrome c oxidase; FNR, ferredoxin:NADP\(^+\) oxidoreductase; PC, plastocyanin; NDH-1, type I NADPH dehydrogenase; and NDH-2, type II NADPH dehydrogenase. Light or excitation energy from phycobilisomes represented by yellow waves. Adapted from Nowaczyk et al. (2010).

The PETC, depicted in Figure 1.2, has three major components: PS II, Cyt b6/f complex, and Photosystem I (PS I). The PETC produces NADPH and ATP, coordinating and regulating their relative levels to minimise damage to components of the system resulting from depletion of reductants such as plastoquinone (PQ) as discussed below. Photosystem I and II are named for the order in which they were discovered not the order in which they appear in the PETC. The chain begins with PS II, which uses excitation energy to induce charge separation between the reaction centre chlorophylls that make up the P\(_{680}\) pigment complex of the PS II RC and pheophytin (Phe) (Nelson and Ben-Shem, 2004). Phe then transfers an electron to the primary plastoquinone Q\(_A\), which in turn passes it to Q\(_B\) via the non-heme iron. The positively charged P\(_{680}^+\) is rapidly neutralised by tyrosine 161 of D1 (Y\(_Z\)), which is neutralised in turn by the Mn\(_4\)CaO\(_5\) cluster (Figure 1.3).

The Mn\(_4\)CaO\(_5\) cluster is a vital component of the OEC, its ability to handle a broad range of redox states facilitating water-splitting via the ‘S-state mechanism’. The S-state mechanism, proposed by Kok et al. (1970) and depicted in Figure 1.4, is the
Chapter 1. Introduction

Figure 1.3: Photosynthetic electron transfer within Photosystem II. Major components of photosynthetic electron transfer within Photosystem II: Electron movement indicated by solid black lines; P680 composed of ChlD1, Chl P680D1, ChlD2, and Chl P680D2 shown in green with mg in yellow; Mn4CaO5 cluster with Mn2+ in purple and Ca2+ in yellow; quinones in purple; the non-heme iron (Fe) in red; pheophytin in light blue; and Cyt b559 in red. Image of ID:3ARC (Umena et al., 2011) from PDB (www.rcsb.org)(Berman et al., 2000) and made in PyMOL (Schrödinger, LLC, 2010).

sequential release of protons and light-driven oxidation (with electrons going to YZ) of the Mn4CaO5 cluster. Oxidation is required for each step: from S1, through S2 and S3 (centres which will decay to S1 in the absence of light) to S4. Following S4 molecular oxygen is released and S0 is reached, requiring a further proton release and oxidation event to return to S1. This is reliant on manganese’ stability at a range of oxidation states (Roelofs et al., 1996; Yano and Yachandra, 2007).

1.3 Light-harvesting complexes

Both cyanobacteria and higher plants possess light-harvesting complexes; large, mobile chromophore containing complexes able to increase the effective absorption cross section available to drive photosynthesis (Maksimov et al., 2011; Mullineaux, 2007).
1.3. Light-harvesting complexes

Figure 1.4: S-state cycle of the OEC. The OEC moves between five redox states (S1 to S0) driven by light-induced charge separation occurring in PS II, represented here by yellow waves. Molecular oxygen is produced between S4 and S0. Adapted from Kok et al. (1970).
Besides participating in light harvesting these complexes have also been found to have photoprotective roles (Bailey and Grossman, 2008; Horton and Ruban, 2005; Kana, 2013; Pascal et al., 2005; Tamary et al., 2012). In higher plants these light-harvesting complexes (LHCs) bind chlorophylls and are characterised as associating with either PS I or PS II (Barros and Kühbrandt, 2009; Melkozernov and Blankenship, 2005). Conversely, in cyanobacteria the predominant light-harvesting complexes are the phycobilisomes (PBS); large, thylakoid membrane-associated complexes with core and rod substructures that vary both between, and within, cyanobacteria. Although the LHCs of cyanobacteria and higher plants are functionally similar a number of differences exist between them. Notably, the LHCs of higher plants use chlorophylls as their chromophores whilst cyanobacteria use bilins. *Synechocystis* sp. PCC 6803 has two distinct PBS, differing by rod-core linker polypeptides (L_RC): phycobilisome rod-core linker polypeptide 1 (CpcG1) links rods to a core containing three cylinders composed of specific combinations of allophycocyanins (APC), with each core binding multiple rods; and phycobilisome rod-core linker polypeptide 2 (CpcG2), which links rods directly to photosystems (Kondo et al., 2005, 2009, 2007). Rods, constructed of phycocyanin (PC), phycoerythrin (PE), and phycoerythrocyanin (PEC), utilise chromophore-chromophore and chromophore-protein interactions to modify the absorption properties of these components (Scharnagl and Schneider, 1991). This allows them to absorb light over a broad range and pass the resulting energy down through increasingly long-wavelength-absorbing pigments toward the membrane, before being released through the terminal emitter (TE) (Holzwarth, 1991). Although CpcG1-PBS and CpcG2-PBS are able to associate with both photosystems CpcG1-PBS preferentially transfers energy to PS I whereas CpcG2-PBS associates with PS I or PS II depending on the energy requirements of the cell.

The state transition refers to changes favouring excitation of either PS II or PS I, with state one favouring energy transfer to PS II and state two favouring energy transfer to PS I. PBS are central to the state transition, modulating excitation of either photosystem and spillover from PS I to PS II (Joshua and Mullineaux, 2004; McConnell, 2002). Normal thylakoid membrane morphology, PS II/PS I ratio, and photosystem packing within the thylakoid membrane are all partially dependent on PBSs (Collins et al., 2012). They are also involved in photoprotection via a non-photochemical quenching (NPQ) mechanism involving orange carotenoid protein (OCP). This is a photocative protein which, as the name suggests, utilises a carotenoid as its chromophore. In its inactive, orange form (OCP^o) it does not bind the PBS. OCP^o is converted by light into its red form (OCP^r), altering its absorption and fluorescence properties (Berera
et al., 2013; Stadnichuk et al., 2009; Wilson et al., 2008). OCP\textsuperscript{r} is able to bind the PBS and reduce energy transfer to photosystems, instead dissipating this excess energy as heat (Kirilovsky and Kerfeld, 2013; Stadnichuk et al., 2013). The last component of this photoprotective mechanism is the fluorescence recovery protein (FRP). Active (dimeric) FRP acts to remove OCP from the PBS by converting it from OCP\textsuperscript{r} into OCP\textsuperscript{o}, allowing the PBS to continue funnelling excitation energy into PS II and PS I (Gwizdala et al., 2013; Sutter et al., 2013).

1.4 Synthesis, Damage, and Repair

The assembly of PS II is a highly coordinated and regulated process, and has been the subject of multiple recent reviews (Komenda et al., 2012b; Nickelsen and Rengstl, 2013; Nixon et al., 2010). Assembly occurs throughout the thylakoid and plasma membrane systems, with different steps occurring in different locations: the plasma membrane, which separates the cytosol from the Mn\textsuperscript{2+} enriched periplasmic space; the thylakoid membrane, the location for active photosystems; and the PratA defined membrane (PDM), specialised assembly centres with traits of both the plasma and thylakoid membrane. PS II assembly begins with Cyt \textit{b}\textsubscript{559} assembly. This precedes the attachment of D2 and then pD1 and PsbI to form the RC. These PS II assembly complexes, lacking core antennae proteins CP43 and CP47, are present in the plasma membrane (Keren et al., 2005; Zak et al., 2001). Interestingly PsbO, one of the three luminal extrinsic proteins that protect and stabilise the Mn\textsubscript{4}CaO\textsubscript{5} cluster in both cyanobacteria and higher plants, was also detected in the plasma membrane (Zak et al., 2001).

In both cyanobacteria and higher plants D1 is usually produced with a C-terminal extension, that must be removed, by a C-terminal processing protein A (CtpA), for active PS II to be generated (Anbudurai et al., 1994; Oelmüller et al., 1996; Shestakov et al., 1994). In \textit{Synechocystis} sp. PCC 6803 D1 is produced with a 16 residue C-terminal extension (pD1) that is removed by CtpA in two stages, CtpA cleaves eight residues producing intermediate D1 (iD1) and then eight more to produce mature D1. This C-terminal extension has been shown to be important, conferring a competitive advantage over a strain without the C-terminal extension; this strain was also found to be more sensitive to photoinhibition under high-light conditions (Diner, 2001; Ivleva et al., 2000; Kuviková et al., 2005). This may be, at least in part, due to the C-terminal extension’s role in protein-protein interactions. PratA binds directly to the
Chapter 1. Introduction

C-terminus of D1, both iD1 and mature D1, where it is thought to have roles in assisting D1 processing and loading Mn$^{2+}$ onto D1 (Klinkert, 2004; Schottkowski et al., 2009a,b; Stengel et al., 2012). Perhaps unsurprisingly, PratA is also required for the formation of the PDM. The next step in PS II assembly, the formation of RC47, is the attachment of the core antennae CP47 along with the PsbH, PsbL, and PsbT subunits. The CP43 antennae protein, along with the PsbK, PsbZ, and Psb30 subunits, are the last transmembrane proteins to be included. Assembly of the Mn$_4$CaO$_5$ cluster and the luminal side extrinsic proteins is the final step for the assembly of the PS II monomer (for recent review see Becker et al. (2011)).

An unavoidable consequence of using highly energetic light in photosynthesis is photodamage. Photoinhibition, the decrease in photosynthetic activity due to light, is primarily due to damage to PS II (Aro et al., 1993). The major mechanistic pathways of photodamage are the generation of reactive oxygen species (ROS) and damage of the catalytic Mn$_4$CaO$_5$ cluster. Both pathways are at least partially caused by both visible and ultraviolet (UV) light (reviewed in Vass (2011)). UV light leads to damage of the Mn$_4$CaO$_5$ cluster, Q$_A$, Q$_B$, tyrosine-D, and tyrosine-Z. Of these processes the damage to the Mn$_4$CaO$_5$ cluster contributes the most to photoinhibition (Hakala et al., 2005; Larkum et al., 2001).

Some forms of damage are exacerbated by lack of reductant, which results in relatively long lifetimes of redox species increasing the chance of recombination and thus ROS generation. Delayed electron transport through Q$_A$ and Q$_B$ facilitates the recombination of Phe$^-$ with P$^+_680$, which results in triplet excited state P$_{680}$ ($^3$P$_{680}$). This $^3$P$_{680}$ is able to react with O$_2$ to form highly damaging singlet oxygen, $^1$O$_2$. Alternatively, the less destructive, but still damaging, superoxide, O$_2^*$ can also be formed by acceptor side reductants, namely Q$_A^*$ (Pospíšil, 2009). Donor side damage also occurs, and is facilitated by damage to the Mn$_4$CaO$_5$ cluster and rapid electron removal by P$^+_680$. The loss of a functional OEC causes the production of long-lived P$^+_680$ and Tyr-Z$^*$ radicals that can damage their protein environment (Jegerschoeld et al., 1990). Alternately a dysfunctional Mn$_4$CaO$_5$ cluster may produce hydrogen peroxide and this can go on to form highly reactive, protein-damaging hydroxyl radicals, OH$^*$. Regardless of its cause, photodamage primarily affects the D1 subunit of PS II, which includes or coordinates most of the electron transport components of PS II. Having a preferentially damaged subunit seems to have facilitated the development of an efficient repair cycle. In this cycle PS II must only be partially disassembled, releasing CP43 (along with the PsbK, PsbZ, and Psb30 subunits) to leave RC47 containing damaged D1, allowing D1 to be removed/degraded, primarily by the thylakoid membrane.
localised FtsH proteases (Komenda et al., 2012b; Lindahl et al., 2000). Following the replacement of D1, and processing of this replacements to mD1, PsbO and Psb28 associate with the assembly complex, possibly to provide protection (Liu et al., 2011). It has been reported that CtpA is located exclusively in the cytoplasmic membrane (Zak et al., 2001). This raises questions regarding the processing and integration of D1 in the repair cycle: does it occur in the PDM and then move to TMs before joining RC47; or is it integrated into RC47 directly at the TM, and if so how and where is it processed? The tight coupling of D1 degradation and replacement, and the presence of pD1 in both PratA-defined and thylakoid membranes have been suggested to support direct integration of replacement D1 into damaged RC47 at the TM (Nickelsen and Rengstl, 2013).

1.5 Ycf48

Ycf48, homologous to HCF136 in Arabidopsis thaliana, is encoded by slr2034 in Synechocystis sp. PCC 6803. In Arabidopsis thaliana HCF136 is an essential component of PS II assembly, its removal resulting in a mutant unable to accumulate PS II (Meurer et al., 1998; Plücken et al., 2002). The structure of the Ycf48 homologue from Thermosynechococcus elongatus, shown in Figure 1.5, has been solved to a resolution of 1.5 Å (F. Michoux, K. Takasaka, P. Nixon, J. Murray, unpublished). This seven-bladed beta-propeller structure is likely anchored to the membrane through an N-terminal modification. On removal of Ycf48 from Synechocystis sp. PCC 6803 a less severe phenotype was observed by Komenda et al. (2008), with mutants displaying decreased levels of pD1, iT1, mature D1, and assembled PS II. Komenda and colleagues identified, by way of yeast-two hybrid studies, interactions between Ycf48 and both pD1 and iD1 but did not show an interaction between Ycf48 and mature D1. However, Ycf48 was found in the same assembly complexes in a strain that lacks the C-terminal extension on D1. This may indicate a transient interaction between Ycf48 and mature D1 or that it is not the only factor determining Ycf48 binding. These results were taken to suggest a role in protecting newly synthesized D1 and facilitating its incorporation into a D2-Cyt b559-containing pre-RC assembly complex. Rengstl and colleagues investigated the effects of removing Ycf48, either alone or in concert with sll0933, and found that removal of Ycf48 is accompanied by reduced levels of sll0933 and protochlorophyllide oxido-reductase (Rengstl et al., 2013, 2011). POR catalyses the conversion of protochlorophyllide a to chlorophyllide a and its removal severely reduces the accumulation of Chl a and the Chl a-containing photosystems (He et al., 1998). Furthermore,
POR has been observed in both the PDM and TM, suggesting a role not only in pigment synthesis, but also in pigment insertion (Nickelsen et al., 2011; Schottkowski et al., 2009b). Rengstl and colleagues report an increased rate of oxygen evolution in the ΔYcf48 strain and suggest this is due to their normalisation based on Chl a concentration.

1.6 Extrinsic Proteins

1.6.1 PsbP

PsbP (often called CyanoP to differentiate it from the PsbP of higher plants with which it shares moderate sequence homology) is a luminal side assembly factor of PS II. Cyanobacterial PsbP is more similar to PsbP-like 1 (PPL-1) in plants, with higher plant PsbP having a role distinct from that in cyanobacteria (reviewed by Bricker et al. (2013) and Ifuku et al. (2008)). PPL1 in plants is thought to have a role in the repair cycle with mutant plant PS II being more susceptible to high-light-induced photodamage, and slower to recover than wild-type Arabidopsis thaliana (Ishihara et al., 2007). Unlike in Arabidopsis thaliana, cyanobacterial PsbP is bound to the membrane by an N-terminal lipid moiety. Structurally, PsbP adopts an αβα sandwich
configuration in both cyanobacteria and higher plants, and contains cation binding sites, the importance of some of these being contentious in cyanobacteria (Jackson et al., 2012; Michoux et al., 2010). Expression of PsbP appears to be tightly associated with PS II, with PS II-less mutants having no detectable PsbP (Ishikawa et al., 2005). Previous studies into the function of cyanobacterial PsbP suggest a role in aiding the luminal side configuration of PS II, supporting optimal activity in Cl\(^-\) and Ca\(^{2+}\) limited conditions (Ishikawa et al., 2005; Summerfield et al., 2005; Sveshnikov et al., 2007; Thornton et al., 2004). PsbP was also found to contribute to *Synechocystis* sp. PCC 6803 thermal tolerance, although being less influential than PsbO, PsbU, or PsbV (Summerfield et al., 2005).

### 1.6.2 Psb27

In *Synechocystis* sp. PCC 6803 Psb27 is encoded by slr1645 and forms an 11 kDa protein. Psb27 is targeted to the thylakoid lumen and membrane-bound due to a modified N-terminus. Psb27 acts as an assembly factor of PS II, binding to inactive PS II on the luminal side and near the Mn\(_4\)CaO\(_5\) cluster assembly site and stopping the premature binding of luminal-side extrinsic subunits (Nowaczyk et al., 2006). Psb27 has been shown to bind to CP43-containing assembly complexes (Roose and Pakrasi, 2004). This binding is through an interaction between the E loop of CP43 and a number of highly conserved residues in Psb27 (Mabbitt et al., 2013). This interaction is thought to prevent the extrinsic subunits PsbV and PsbU (although not PsbO) from associating with the luminal face of PS II during assembly and repair cycles, and aid the assembly of the Mn\(_4\)CaO\(_5\) cluster (Roose and Pakrasi, 2007). CP43-Psb27 assembly complexes were found to accumulate in a ∆CtpA strain, i.e. when pD1 processing is arrested, and under cold stress, supporting a role in both *de novo* assembly and the repair of damaged PS II (Grasse et al., 2011; Roose and Pakrasi, 2004). Psb27 also protects CP43 from degradation in the absence of the extrinsic subunits, with a ∆CP47:∆Psb27 strain showing reduced levels of CP43 and protein fragments consistent with the enzymatic degradation of CP43 (Komenda et al., 2012a). Psb27 may also have a broader role in photosystem assembly, being able to bind PS I as well as PS II, and the reported role of an *Arabidopsis thaliana* Psb27 homologue acting in PS II-LHC supercomplex formation (Dietzel et al., 2011; Komenda et al., 2012a).
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1.6.3 Psb28 and Psb28-2

Psb28 and Psb28-2, encoded in *Synechocystis* sp. PCC 6803 by sll1398 and slr1739, respectively, are the only known extrinsic polypeptides of PS II that are found on the cytoplasmic side of the thylakoid membrane. The majority of investigations done to date suggest that Psb28 is the main functional protein with Psb28-2 acting in an accessory role or in response to some environmental stress (Dobakova et al., 2008; Sakata et al., 2013). Psb28 is involved in the assembly of damaged PS II, being present in sub-stoichiometric amounts and associated with PS II assembly complexes. There have been conflicting reports with regard to the importance and function of Psb28, particularly when grown under low light conditions. Dobakova et al. (2008) report ΔPsb28 to have significantly less Chl a than wild type, and a greater PS II/PS I ratio. They also report reduced levels of CP47, D1, and D2, suggesting that Psb28 acts in the synthesis of chlorophyll-binding proteins and/or has a role in Chl a synthesis. Conversely, Sakata et al. (2013) found only mildly less Chl a and wild type ratio of PS II/PS I. These differences may reflect different cell culturing methodologies or data acquisition. Psb28 was found to accumulate in mutants defective in lipid synthesis (Sakurai et al., 2007a,b). Psb28 has also been reported to have an interaction with the small CAB-like protein (SCP) ScpD, suggesting a contribution to CP47 ScpD interaction (Yao et al., 2007). Psb28 is primarily involved in protection of PS II during repair, its absence is only becoming physiologically significant when assembly is slowed, increasing the likelihood of damage occurring. Conflicting results have been found for the following: the removal of Psb28-2 either alone, or in conjunction with Psb28 (Moore, 2011; Sakata et al., 2013). Psb28-2 has been shown to associate with RC47 much like Psb28 (Boehm et al., 2012).

1.6.4 Aims of this study

This study aims to determine the means by which Ycf48 is involved in D1 processing and PS II biogenesis. A number of factors are involved in PS II assembly and repair pathways, some of which are likely as yet unknown. Previous studies show that in *Synechocystis* sp. PCC 6803 Ycf48 aids PS II assembly and repair by facilitating correct processing of the newly synthesized D1 subunit of the reaction centre. Whether this is an evolutionarily conserved role is not known; however, HCF136, the homologous protein in *Arabidopsis thaliana*, is indispensable for PS II assembly. Other cell components
involved in PS II assembly may act with or in parallel to Ycf48, with the recently determined structure of a Ycf48 homologue suggesting a capacity for multiple interactions. The specific details with regard to the transport of assembly complexes between the cytoplasmic, PratA-defined, and thylakoid membranes are still unknown. This study will characterise the effects of interrupting \textit{str2034}, the gene encoding Ycf48, in conjunction with other proteins seen to affect D1 and the assembly of PS II. \textit{ΔYcf48: ΔPsb27, ΔPsb28, ΔPsb28-2, ΔPsb28:ΔPsb28-2, and ΔPsbP} strains will be constructed and, along with their single and double mutant counterparts, characterised to determine the role of Ycf48 in concert with each.
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Chapter 2

Materials and Methods

2.1 General

2.1.1 Chemicals, reagents and kits

Analytical grade reagents were used throughout this study. Unless otherwise noted reagents were sourced from Ajax Finechem, Australia; AppliChem, Germany; BDH Laboratory Supplies, UK; Bio-Rad Laboratories, USA; ChemService, Inc., USA; Invitrogen, USA; Merck KGaA, Germany; Roche, Switzerland; Scharlab S.L., Spain; Sigma-Aldrich, USA; ThermoFisher Scientific, USA; or USB Corporation, USA.

2.1.2 Software

Protein structure figures were produced with PyMOL (Schrödinger, LLC, 2010). Data was processed and graphed using Microsoft Office Excel 2007. Figures were annotated and edited using the GNU Image Manipulation Program v2.8.3 (GIMP) (Wernicke et al., 2010).
2.2 *Synechocystis* sp. PCC 6803

2.2.1 *Synechocystis* sp. PCC 6803 strains

*Synechocystis* sp. Pasteur Culture Collection (PCC) 6803 is referred to as *Synechocystis* sp. PCC 6803. The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 is the wild type of this study (Williams, 1988). Other strains used in this study, shown in Table 2.1 were produced by transformation of this wild type.

Table 2.1: *Synechocystis* sp. PCC 6803 strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transformation</th>
<th>Reference</th>
<th>Background^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>-</td>
<td>Williams (1988)</td>
<td>GT-O2</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>wild type transformed with pAD-2034::specR</td>
<td>This study</td>
<td>GT-O1</td>
</tr>
<tr>
<td>ΔPsb27</td>
<td>wild type transformed with pΔPsb27</td>
<td>Mabbitt (2013)</td>
<td>GT-O2</td>
</tr>
<tr>
<td>ΔPsb27:ΔYcf48</td>
<td>ΔPsb27 transformed with pAD-2034::specR</td>
<td>This study</td>
<td>GT-O2</td>
</tr>
<tr>
<td>ΔPsbP</td>
<td>wild type transformed with pΔPsbP</td>
<td>Summerfield et al. (2005)</td>
<td>GT-O1</td>
</tr>
<tr>
<td>ΔPsbP:ΔYcf48</td>
<td>ΔPsbP transformed with pAD-2034::specR</td>
<td>This study</td>
<td>GT-O1</td>
</tr>
<tr>
<td>ΔPsb28</td>
<td>wild type transformed with pΔPsb28</td>
<td>Moore (2011)</td>
<td>GT-O2</td>
</tr>
<tr>
<td>ΔPsb28:ΔYcf48</td>
<td>ΔPsb28 transformed with pAD-2034::specR</td>
<td>This study</td>
<td>GT-O2</td>
</tr>
<tr>
<td>ΔPsb28-2</td>
<td>wild type transformed with pΔPsb28-2</td>
<td>Moore (2011)</td>
<td>GT-O2</td>
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<tr>
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<td>This study</td>
<td>GT-O2</td>
</tr>
</tbody>
</table>

^a Refers to the background the strain was eventually shown to have been derived from. Discussed in Section 4.6. See also Table 4.1, and Appendix D.

2.2.2 *Synechocystis* sp. PCC 6803 growth conditions

2.2.2.1 Solid media

Strains were grown on solid agar BG-11 (1.5% agar weight to volume (w/v)) plates containing 5 mM glucose, 20 µM atrazine, 0.3% sodium thiosulfate, 10 mM 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES)/NaOH (pH 8.2),
and appropriate antibiotics. When included, antibiotics were at the following concentrations: kanamycin, 25 µg mL\(^{-1}\); spectinomycin, 25 µg mL\(^{-1}\); chloramphenicol, 15 µg mL\(^{-1}\); and atrazine, 20 µM. Cells on plates were grown at 30 °C under constant light (50 µE m\(^{-2}\) s\(^{-1}\)).

### 2.2.2 Liquid media

Strains were grown in 300 mL or 150 mL of BG-11 in specially modified Erlenmeyer flasks (Eaton-Rye, 2004) at 30 °C under constant light (50 µE m\(^{-2}\) s\(^{-1}\)). Continuous aeration was provided by aquarium pumps. Air was passed through sterile ddH\(_2\)O to minimize evaporation and was filtered with Millex-FG filters (Millipore, USA). Unless otherwise noted cells were grown in the presence of 5 mM glucose.

### 2.2.3 Determination of chlorophyll \(a\) concentration

Cell-containing liquid culture was thoroughly mixed with 100% methanol and spun at 13400 \(g\) for 2 min to pellet cell material. The supernatant was measured at OD\(_{600\ nm}\) and Chl \(a\) concentration calculated using Equation (2.1). For determining the Chl \(a\) concentration of isolated thylakoid samples 80% acetone was used instead of 100% methanol.

### 2.2.4 Oxygen Evolution

Cells were grown under mixotrophic conditions as previously described to an OD\(_{730\ nm}\) of approximately 0.8. Cells were concentrated by centrifugation at 2760 \(g\) and washed twice in pH 7.5 BG-11 containing 25 mM HEPES/NaOH before being diluted to a Chl \(a\) concentration of 5 µg mL\(^{-1}\). Cells were incubated at 30 °C under constant light (50 µE m\(^{-2}\) s\(^{-1}\)) and shaking for at least 30 min before being assayed. One and a half millilitres of cells were assayed in a Clark-type electrode (Hansatech, UK) also at 30 °C and stirred by an electronic flea. Either 15 mM sodium bicarbonate or 1 M potassium ferricyanide \((K_3Fe(CN)_6)\) and 200 µM 2,6-dichloro-p-benzoquinone (DCBQ) were added to assays. Oxygen concentration was measured for 5 min; 1 min with ambient light, 3 min with saturating light, followed by another min in ambient light. Saturating light was produced by an FLS1 light source (Hansatech, UK) fitted with a 580\(_{\text{nm}}\) bandpass filter and a 0.3 OD neutral density filter (both from Meller Griot, USA) at an intensity of 5 000 µE m\(^{2}\) s\(^{-1}\). Oxygen evolution rates were calculated from the voltage change (\(\Delta V\)) between 60 and 90 s using Equation (2.2)
2.2.5 Photoautotrophic growth curves

Liquid cultures were grown to ~OD$_{730}$ nm. Cells were spun down at 2760 g and washed twice in BG-11 to remove extracellular glucose. These cells were used to inoculate growth curve cultures at a starting OD$_{730}$ nm of 0.05. Growth curve liquid cultures were grown at 30 °C and 50 µE m$^{-2}$ s$^{-1}$ with OD$_{730}$ nm measured every 12 h. Doubling times were calculated by fitting a line to the logarithmic growth stage.

2.2.6 77 K fluorescence emission spectroscopy

Liquid cultures were grown to an OD$_{730}$ nm of ~1.0. Cells were spun down at 2760 g, washed twice in pH 7.5 BG-11 containing 25 mM HEPES/NaOH and resuspended at a Chl a concentration of 5 µg mL$^{-1}$. Five hundred microlitres of cells were pipetted into a thin glass tube (internal diameter: 4 mm, external diameter: 6 mm) and frozen in liquid nitrogen. Measurements were made in a MPF-3L fluorescence spectrophotometer (Perkin-Elmer, USA) custom modified for 77 K Chl a fluorescence emission measurements. Excitation slit widths were 12 nm and 8 nm for excitation at 440 nm and 580 nm, respectively. The emission slit width was 2 nm and scanning speed was set to 4 nm s$^{-1}$ for both 440 nm and 580 nm.

2.2.7 Whole cell absorption spectra

Liquid cultures were grown to an OD$_{730}$ nm of ~1.0. Cells were spun down at 2760 g, washed twice in pH 7.5 BG-11 containing 25 mM HEPES/NaOH and resuspended at an OD$_{800}$ nm of 0.3. Absorption was measured from 400 nm to 800 nm at a scan speed of 200 nm min$^{-1}$. Cellotape was placed on both sides of cuvettes to account for light scattering by whole cells.

2.2.8 Fluorescence induction and $Q_A^-$ reoxidation

For room temperature fluorescence experiments cells were grown mixotrophically as previously described (Section 2.2.2.2) spun down at 2760 g, washed twice in pH 7.5 BG-11 containing 25 mM HEPES/NaOH, resuspended at a Chl a concentration of
5 μg mL$^{-1}$, and incubated in 50 μE m$^{-2}$ s$^{-1}$ at 30 °C shaking in 25 mL Erlenmeyer flasks for at least 30 min. Cells were diluted with pH 7.5 BG-11 containing 25 mM HEPES/NaOH to a Chl a concentration of 1.5 μg mL$^{-1}$ and 2.5 μg mL$^{-1}$ for fluorescence induction and $Q_{A^-}$ reoxidation experiments, respectively, and dark adapted for at least 20 min. For dark adaptation onward, experiments were carried out in a dark room. An FL 3300 fluorometer (Photon Systems Instruments, Czech Republic) running 455 nm actinic and measuring light emitting diodes (LEDs) was used for all room temperature Chl a variable fluorescence yield measurements.

### 2.2.8.1 Fluorescence induction

Two millilitres of cells either without addition or containing 40 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in a plastic cuvette were exposed to continuous actinic light and Chl a variable fluorescence induction measured. Detailed protocols can be found in Appendix A.

### 2.2.8.2 $Q_{A^-}$ reoxidation

Two millilitres of cells either without addition or containing 40 μM DCMU in a plastic cuvette were exposed to a single saturating actinic flash and fluorescence decay measured by a series of measuring flashes. Detailed protocols can be found in Appendix A.

### 2.3 Molecular Biology

#### 2.3.1 Oligonucleotides

Oligonucleotide primers for PCR and sequencing were obtained from Sigma-Aldrich, USA.

#### 2.3.2 PCR

PCR amplification of DNA was done using the Touchdown PCR system (Don et al., 1991), either Platinum Taq or Phusion Hot Start II High Fidelity polymerase, and appropriate reaction buffer. PCR reactions were generally 10 or 50 μL in volume and used different cycles (Table 2.3) and reaction mix compositions as detailed in Section 2.3.2.1.
### Table 2.2: List of primers used in this study

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Table 2.3: PCR cycles

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<td>Time</td>
<td>Temp (°C)</td>
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<td>30 s</td>
<td>94</td>
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<td>Extension</td>
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<td>68</td>
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<td>Cycle two (x10 a)</td>
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<td>Extension</td>
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<td>1 min/kbp</td>
<td>68</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>1 min/kbp</td>
<td>68</td>
</tr>
</tbody>
</table>

a Cycle two was done x15 for colony PCR that used *Synechocystis* sp. PCC 6803 as template.

b Annealing temperatures started at the higher value and were reduced by 1 °C/cycle.

2.3.2.1 PCR reaction mix compositions

**PCR**: 50 µL reactions with reaction mix containing 1× reaction buffer, 1.5 mM MgCl₂, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 µM dNTPs, 10 U/mL Platinum Taq polymerase, and 2 µg mL⁻¹ template DNA.

**Colony PCR**: 10 µL reactions with reaction mix made the same as that used for basic PCR but with 50 U/mL Platinum Taq polymerase and using 1 µL of cell water in place of template DNA. Cell water generated by resuspending a colony in 5-10 µL water. Positive controls used generic PCR reaction mix.

**SLIC PCR**: 50 µL reactions with reaction mix containing 1× reaction buffer, 0.4 µM forward primer, 0.4 µM reverse primer, 0.2 µM dNTPs, 10 U/mL Phusion Hot Start II High Fidelity polymerase, and 2 µg mL⁻¹ template DNA.
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2.3.3 Agarose gel electrophoresis

Gels were 0.8% (w/v) agarose, made with and run in 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris)/borate/2-(2-[bis(carboxymethyl)amino]ethyl(carboxymethyl)amino) acetic acid (EDTA) (TBE) buffer. Samples were made up 10% volume to volume (v/v) with loading dye.

$5 \times \text{TBE (1 L): } 54.0 \text{ g trizma base; 27.5 g boric acid (anhydrous); 20.0 mL 0.5 M EDTA (pH 8.0).}$

$\text{Loading dye (20 mL): 0.05 g bromophenol blue (0.25%); 0.05 g xylene cyanol FF (0.25%); 6.0 mL glycerol (30%); made up to 20 mL with ddH}_2\text{O.}$

2.3.4 Plasmid construction

The sequence and ligation independent cloning (SLIC) method was used for plasmid construction, with primers designed to produce fragments that overlap 20 bp at both the 5' and 3' ends (Li and Elledge, 2007). SLIC fragments were made with PCR (Section 2.3.2), cleaned using a Purelink Quick PCR Purification Kit, and quantified according to Section 2.3.7. Cleaned PCR products were then digested for 30 min at 37 °C with T4 DNA polymerase at 0.5 U/µg DNA and 1× reaction buffer, which exhibits 3'→5' exonuclease activity in the absence of dNTPs. The digestion reaction was stopped by adding 1/10th volume 10 mM dCTP. SLIC fragments were then mixed in sequential order with the 5' most fragment first and the plasmid backbone last. The volume of each fragment was calculated according to Equation (2.3) where the amount of backbone fragment is 150 ng. One tenth volume T4 10× ligase buffer was then added and the solution was incubated at room temperature for 30 min before being used to transform DH5α.

2.3.5 Plasmid isolation

For plasmid amplification 5 mL of Lysogeny Broth (LB) media containing appropriate antibiotics was inoculated with E. coli using a sterile wooden toothpick and grown overnight at 30 °C. Four and a half millilitres of this was then subjected to alkaline lysis plasmid isolation. Cells were pelleted by centrifugation at 21000 g at room temperature, resuspended in 200 µL Solution 1 (50 mM glucose, 25 mM Tris pH 8, 10 mM
2.3.6 Restriction enzyme digests of plasmids

Restriction enzymes were sourced from Roche (Switzerland) and New England Biolabs (USA) and digests were carried out according to manufacturer’s instructions. Typical reactions were 10 µL total volume with 2 µL of plasmid (corresponding to 1-4 µg of DNA), 1 x appropriate reaction buffer, and 1 µL of 1 U µL⁻¹ restriction enzyme digested for 60-90 min at the temperature instructed by the manufacturer.

2.3.7 DNA quantification

A NanoDrop (Thermo Fisher Scientific, USA) spectrophotometer was used to quantify DNA, by absorption spectroscopy, according to the manufacturer’s instructions.

2.3.8 DNA sequencing

DNA sequencing was performed by Genetic Analysis Services using an ABI 3730xl DNA Analyser to perform capillary electrophoresis.
2.4 *Escherichia coli*

*E. coli* was grown at 37 °C in either liquid or agar plates containing: 1% bactotryptone; 0.5% yeast extract; 1% NaCl; 1.5% bacteriological agar (for agar plates only). When antibiotics were added they were to the following final concentrations: ampicillin, 50 µg mL⁻¹; kanamycin, 50 µg mL⁻¹; spectinomycin, 50 µg mL⁻¹; chloramphenicol, 30 µg mL⁻¹. Long term storage stocks were made in LB media containing 15% (v/v) glycerol and kept at -80 °C.

2.4.1 *E. coli* strain

The *E. coli* used in this study was the DH5α strain (Invitrogen, USA).

2.4.2 *E. coli* transformation

2.4.2.1 Preparation of competent *E. coli*

Competent *E. coli* were generated using the RbCl₂ method (Hanahan, 1985). Briefly, *E. coli* were grown in ψB media shaking at 37 °C to OD₆₀₀ mm 0.3-0.4. Then, working on ice and with chilled equipment from here on, cells were chilled for 5 min on ice and centrifuged for 10 min at 2760 g. Supernatant was discarded and the pellet resuspended in 15 mL TfBI and again centrifuged for 10 min at 2760 g. Supernatant was discarded and the pellet gently resuspended in 2 mL TfBII. Finally 200 µL aliquots were snap frozen, using ethanol and dry ice, in sterile microfruge tubes and stored immediately at -80 °C.

ψB media (1 L): 20 g Tryptone, 5 g Yeast Extract, 5 g MgSO₄, adjust pH to 7.6 with POH, autoclave

TfBI (500 mL): 1.47 g CH₃CO₂K, 0.61 g Rubidium Chloride, 0.74 g Calcium Chloride, 4.95 g Manganese Chloride, add sterile glycerol to 15% (v/v), adjust pH to 5.8 with 0.2M acetic acid and filter sterilize.

TfBII (50 mL): 10mM MOPS, 0.15 g PIPES, 0.55 g Calcium Chloride, 0.06g Rubidium Chloride, 7.5 mL Glycerol and filter sterilize.
2.4.2.2 Heat-shock transformation method

For transformation DNA (1 µL of plasmid or 20 µL of ligation mix) was added to chilled, competent *E. coli* followed by a 30 min incubation on ice. The cell plasmid mix was then heat shocked by placing in a 37 °C water bath for 2 min followed by 3 min in ice cold water. Then 1 mL of LB media was added and cells were incubated on a rotating platform for 1 h at 37 °C, centrifuged at 15000 g for 30 s and the supernatant removed. Cells were then resuspended in 100 µL LB media and spread over LB agar plates containing appropriate antibiotics.

2.5 Transformation of *Synechocystis* sp. PCC 6803

Strains to be transformed were grown in liquid media as previously described, concentrated by centrifugation at 2760 g. Cells were resuspended in BG-11 to 0.5 mL at OD_{730} of 10 in a glass test tube and 10 µL plasmid DNA (pDNA) was added. Cells were then incubated at 30 °C under 30 µE m^{-2} s^{-1} light for 5-6 h. Half way through this incubation cells were shaken to resuspend them and reoxygenate the BG-11. After 5-6 h cells were plated directly onto BG-11 plates containing glucose, half-strength antibiotics to select for integration, and appropriate antibiotics for strains other than wild type.

2.6 Protein Methods

2.6.1 Thylakoid Isolation

Large volumes of cells were grown in liquid cultures as described, centrifuged at 6000 g for 15 min, resuspended in 100 mL of Medium A (50 mM HEPES-NaOH pH 7.5, 30 mM CaCl$_2$), centrifuged again at 6000 g for 15 min and resuspended in 100 mL of Medium B (50 mM HEPES-NaOH pH 7.5, 30 mM CaCl$_2$, 800 mM sorbitol, 1 mM 6-amino-γ-caproic acid, 1 M gycine betaine, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 mM benzamidine). Then working at 4 °C in the dark, samples were mixed with an equal volume of 0.1 mm diameter glass beads and blended with a bead beater (Biospec Products, USA) fifteen times for 15 s with 2 min rests on ice between blends.
Chapter 2. Materials and Methods

Homogenate was washed from beads with Medium B and separated from unbroken cells by centrifugation at 1000 g for 2 min. Pooled homogenate was centrifuged at 8000 g for further purification, supernatants were centrifuged at 25000 g for 1 h, pellets resuspended in Medium B and again centrifuged at 25000 g for 1 h resuspended in Medium B and stored at 77 K.

2.6.2 Blue-Native PAGE

Blue Native polyacrylamide gel electrophoresis (PAGE) was performed according to Kügler et al. (1997) with the changes of Rokka et al. (2005) for loading solubilised thylakoids. Thylakoids were thawed on ice and transferred into pre-chilled microcentrifuge tubes. Twice, thylakoids were topped up with Washing Buffer (0.33 M sorbitol, 0.5 mM Bis-tris HCl pH 7.0, 0.25 mg mL\(^{-1}\) Pefabloc) and spun at 13400 g for 15 min at 4 °C. Thylakoids were then resuspended in 200 µL Buffer A (25 mM Bis-tris HCl pH 7.0, 20% glycerol, 0.25 mg mL\(^{-1}\) Pefabloc) with an equal volume of Buffer A containing 2% (w/v) n-dodecyl-\(\beta\)-D-maltopyranoside (DM) and incubated on ice for 15 min to solubilise. Insoluble material was removed by another spin at 13400 g for 15 min at 4 °C and supernatant was transferred to fresh pre-chilled microcentrifuge tubes. One tenth volume of Sample Buffer (0.5 M amino-caproic acid, 100 mM Bis-tris HCl pH 7.0, 30% sucrose (w/v), 50 mg mL\(^{-1}\) Serva Blue G) was added to samples. Samples were run using a Protean II cell (Bio-Rad Laboratories, USA) at 4 °C through a 4% loading gel onto a 5-12% gradient gel at 5 mA.

2.6.3 Western blot

BN-PAGE gels were western blotted onto Polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot cell (Bio-Rad, USA) according to the manufacturer’s instructions running at 4 °C and 40 V for 1 h.

2.6.4 Enchanced chemiluminescence

Polyvinylidene fluoride (PVDF) membranes were briefly destained with destaining solution (20% (v/v) methanol, 7% (v/v) acetic acid), rinsed with water, and blocked with
Blot-O (4% bovine serum albumen (BSA), 3 µM NaN₃, made in Tris-Buffered Saline (TBS) pH 7.5) for 1 h rocking at room temperature. Membranes were then rinsed thoroughly with water and incubated overnight at 4 °C with constant rolling in 250 mL Nalgene centrifuge bottles containing 1° antibody. Membranes were then washed thrice with TBS + 0.1% Tween-20 at room temperature and water rinses between and after washes before being incubated with 2° antibody for 2 h at room temperature. Four washes in TBS + 0.1% Tween-20 with water rinses between and following followed. Finally the membrane was submerged in equal volumes of ECL Reagent A (100 mM Tris-HCl, 2.5 mM luminol, 132 µM p-coumaric acid) and ECL Reagent B, (100 mM Tris-HCl, 0.025% hydrogen peroxide (v/v)) which had been freshly mixed, and imaged with a FujiFilm PS-3000 imager (Fujifilm, Japan).

### 2.6.4.1 Stripping and reprobing

To strip PVDF membrane for reprobing the following protocol was used. After ECL and imaging (see Section 2.6.4) membranes were washed in TBS + 0.1% Tween-20 twice. Membranes were then incubated at 50 °C rocking in Stripping Solution (63 mM, 2% SDS (w/v), 0.781% β-mercaptoethanol(v/v)) for 30 min. Finally membranes were washed three times in TBS + 0.1% Tween-20.

### 2.7 Equations

#### 2.7.0.1 Chlorophyll determination

\[
[\text{Chl } a](\mu \text{g mL}^{-1}) = A_{663} [\text{nm}] \times 1000 \times \text{dilution factor} \div 82
\]  

(2.1)

#### 2.7.0.2 Oxygen evolution rate

\[
\text{Rate (µmoles O}_2 \text{ mg}^{-1} \text{Chl } a^{-1} \text{ h}^{-1}) = \frac{0.235 \ \mu \text{moles O}_2 \text{ mL}^{-1} \times \Delta V \times 2 \times 60 \ \text{sec}}{\text{Baseline } V \times [\text{Chl } a](mg \text{ mL}^{-1})}
\]  

(2.2)
2.7.0.3 SLIC fragment volume determination

\[ V_{frag} = \frac{L_{frag} \times N_{plas}}{L_{plas} \times C_{frag}} \] (2.3)

Where \( V_{frag} \) is the volume of a specific fragment in µL, \( L_{frag} \) is the length of the fragment in kbp, \( L_{plasmid} \) is the length of the desired plasmid in kbp, \( N_{plas} \) is the amount of plasmid backbone used (150 ng), and \( C_{frag} \) is concentration of the fragment in µg mL\(^{-1}\).
Chapter 3

Results

3.1 Cloning

To investigate the role of Ycf48 in *Synechocystis* sp. PCC 6803, a strain lacking *slr2034*, the gene responsible for this protein’s production, was made. The SLIC method was employed (see Section 2.3.4) to generate a pUC19 plasmid containing *slr2034* interrupted with a spectinomycin-resistance cassette. Construction of the pAD-2034::specR required four SLIC fragments: 2034 pUC19, the entirety of pUC19 allowing for selection of transformed *E. coli* with ampicillin (Amp); 2034 Left Flank, comprising nucleotides 1-515 of *slr2034*; 2034 2034 Right Flank, comprising nucleotides 516-1029 of *slr2034*; and 2034 SpecR, a spectinomycin-resistance cassette containing the gene *aadA*. 2034 Left Flank, 2034 Right Flank, 2034 SpecR, and 2034 pUC19 were amplified by PCR (according to Section 2.3.2) using primers: LF-slr2034-F1 and LF-slr2034-R1; RF-slr2034-F1 and RF-slr2034-R1; SpecR-slr2034-F1 and SpecR-slr2034-R1, and pUC19-slr2034-F1 and pUC19-slr2034-R1, respectively. These fragments were used to construct the pAD-2034::specR as described in Section 2.3.4.

The plasmid was isolated (according to Section 2.3.5) from *E. coli* and digested to confirm the correct construction (Figure 3.2). The plasmid constructed contained 2034 Left Flank, 2034 Right Flank, and 2034 SpecR fragments joined together correctly. They were inverted relative to the pUC19 vector fragment (see Figure 3.1); however, still contained the left and right flanks of *slr2034* separated by the spectinomycin-resistance cassette and thus could be used to generate a ΔYcf48 strain of *Synechocystis* sp. PCC 6803. The pAD-2034::specR plasmid was sequenced to confirm that no mutations had been introduced during cloning (Appendix B).
Figure 3.1: Map of pAD-2034::specR. Plasmid map of pAD-2034::specR used for generation of ΔYcf48 strains of *Synechocystis* sp. PCC 6803. The ampicillin resistance conferred by APr in the pUC19 backbone allows for selection of transformed *E. coli*. 2034 Left Flank and 2034 Right Flank, the left and right halves of *slr2034*, allow for homologous recombination resulting in interruption of *slr2034* whilst SpecR allows selection for this interruption.

*Synechocystis* sp. PCC 6803 was transformed with pAD-2034::specR according to Section 2.5. Due to the presence of multiple copies of the chromosome in *Synechocystis* sp. PCC 6803 it was necessary to ensure complete segregation of the inactivated gene or genes. This integration and segregation was confirmed for all novel single, double, and triple mutants used in this study (Figure 3.3). Upon segregation all novel strains were also added to the lab collection, with stocks in BG-11 containing 15% glycerol (v/v) stored at -80 °C.
3.1. Cloning

Figure 3.2: Restriction digest of pAD-2034::specR. (A) Lane 1, 1 Kb DNA marker (Invitrogen, Carlsbad, CA); lane 2, pAD-2034::specR digested with PvuII. (B) Lane 1, as in Panel A; lane 2, pAD-2034::specR digested with PstI. (C) Lane 1, as in Panel A; lane 2, pAD-2034::specR digested with DraIII. Sizes on the left side of each panel indicate ladder bands in kbp and expected band sizes based on virtual digests in the SnapGene program (from GSL Biotech; available at snapgene.com) are indicated on the right side of each panel in bp.
Figure 3.3: Segregation of mutants lacking the Ycf48 protein and one or more of the PsbP, Psb27, Psb28-1, and Psb28-2 proteins. (A) Diagram of the SLIC-assembled construct showing the position of the 1.4 kbp spectinomycin-resistance cassette (SpecR) in the slr2034 ORF. Arrows indicating the position of primers located upstream and downstream of slr2034 used to confirm segregation of the transformants in Panel B and C are shown. (B) Segregation of the interrupted slr2034 gene introduced into the ∆PsbP, ∆Psb27, and ∆Psb28:∆Psb28-2 strains. Colony PCR was performed using wild-type or transformed strain cells and the primers shown in (A) as in Table 2.3. Lane 1, 1 Kb DNA marker (Invitrogen, Carlsbad, CA); lane 2, the ∆Psb27:ΔYcf48 mutant; lane 3, the ∆PsbP:ΔYcf48 mutant; lane 4, the ∆Psb28:∆Psb28-2:ΔYcf48 mutant; lane 5, the ΔYcf48 mutant; lane 6, wild type. (C) Segregation of the interrupted slr2034 gene introduced into the ∆Psb28, ∆Psb28-2, and ∆Psb28:∆Psb28-2 strains. Lane 1, 1 Kb DNA marker (Invitrogen, Carlsbad, CA); lane 2, the wild-type strain; lane 3, the ΔYcf48 mutant; lane 4, the ∆Psb28:ΔYcf48 mutant; lane 5, the ∆Psb28-2:ΔYcf48 mutant; lane 6, the ∆Psb28:∆Psb28-2:ΔYcf48 mutant.
3.2 PsbP

3.2.1 ∆PsbP Photoautotrophic growth

For photoautotrophic growth experiments cultures were grown as described in Section 2.2.2.2 but without the addition of glucose. Cultures grown photoautotrophically, i.e. with light as their only energy source, are only able to survive and multiply if they have functional photosystems. As such, decreases in photoautotrophic growth capability may indicate reduced levels of functional PS II.

Figure 3.4: Photoautotrophic Growth Curve. Cell density determined by measuring OD$_{730}$ nm of the following strains: Wild type (closed squares); ∆PsbP (open circles); ∆Ycf48 (closed triangles), and ∆PsbP:∆Ycf48 (open diamonds). Error bars are the standard error of three or more independent experiments. Error bars that are not visible are smaller than their accompanying symbols.
Table 3.1: Doubling times of the ΔPsbP strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (h) ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.6 ± 2.0</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>31.5 ± 2.4</td>
</tr>
<tr>
<td>ΔPsbP</td>
<td>13.5 ± 2.0</td>
</tr>
<tr>
<td>ΔPsbP:ΔYcf48</td>
<td>34.2 ± 3.6</td>
</tr>
</tbody>
</table>

Photoautotrophically the ΔPsbP mutant was seen to grow as well as wild type (Section 3.2.1) with doubling times of 13.5 h (s.d. 2.0 h) and 10.6 h (s.d. 2.0 h), respectively. Removal of Ycf48 was found to retard growth in either the wild-type or ΔPsbP background. However, there is no significant difference between the growth rates of the ΔYcf48 and ΔPsbP:ΔYcf48 strains for which doubling times were 31.5 h (s.d. 2.4 h) and 34.2 h (s.d. 3.6 h), respectively.

3.2.2 ΔPsbP Whole cell absorption spectra

*Synechocystis* sp. PCC 6803 has a number of pigments, most of which are constitutively or transiently associated with PS II and/or PS I. These pigments have important roles in light harvesting, photoprotection, and energy transfer (Grossman et al., 1993; Kirilovsky and Kerfeld, 2012; MacColl, 1998; Wilson et al., 2006). Measuring whole cell absorption is an easy way to assay the relative levels of these pigments and thus the complexes they are associated with. Absorption at 435 nm and 680 nm comes from Chl a-containing PS I and PS II, at 475-550 nm from carotenoids, and at 625 nm comes from PBS. The OCP is a central form of NPQ photoprotection in cyanobacteria. The OCP has a broad and dynamic contribution to absorption: in its inactive form OCP<sub>o</sub>, it has absorption maxima at 467 nm and 496 nm; whereas in its active form OCP<sub>r</sub>, it has an absorption maximum at roughly 505 nm (Wilson et al., 2008).

Pigment composition analysed by room temperature absorption measurement showed ΔPsbP to have lower absorption in the 450-525 nm range and a reduced peak at 625 nm relative to wild type (Figure 3.5). Removal of Ycf48 in the wild-type background did not alter absorption compared to wild type. Conversely, removal of Ycf48 in the ΔPsbP background resulted in appreciable changes in absorption; increases in the 475-550 nm range

34
3.2.3 ΔPsbP Oxygen evolution

Oxygen evolution assays (Section 2.2.4) were used to measure PS II activity. These were carried out in the presence of K₃Fe(CN)₆ and DCBQ, which support PS II-specific oxygen evolution, or sodium bicarbonate, which keeps the concentration of carbon dioxide high facilitating whole-chain photosynthesis. These assays probe the capacity of mutant strains to carry out PS II-specific electron transport and total photosynthetic electron transport, respectively.
Chapter 3. Results

Figure 3.6: Oxygen evolution. (A) Rates of oxygen evolution supported by 15 mM sodium bicarbonate (blue) or 1 M K$_3$Fe(CN)$_6$ and 200 µM DCBQ (red). (B) Rates of oxygen evolution supported by 15 mM sodium bicarbonate or 1 M K$_3$Fe(CN)$_6$ and 200 µM DCBQ as a percentage of wild type rate under same conditions. Errors bars are s.d. and data are from at least three separate experiments. Traces of oxygen evolution in the presence of 15 mM sodium bicarbonate (C) or 1 M K$_3$Fe(CN)$_6$ and 200 µM DCBQ (D): wild type (black); ΔPsbP (red); ΔYcf48 (blue), and ΔPsbP:ΔYcf48 (purple). Representative traces are shown.

Assaying whole cells for oxygen evolution as described (in Section 2.2.4), all four strains showed similar rates in the presence of DCBQ. If anything wild type had a lower rate of oxygen evolution on a Chl a basis. With the addition of bicarbonate, however, the two strains that lack Ycf48, the ΔYcf48 and ΔPsbP:ΔYcf48 mutants, show reduced rates of oxygen evolution (Figure 3.6). Removal of Ycf48 in the ΔPsbP background did not reveal an additive effect in reducing oxygen-evolving capacity.
3.2.4 ∆PsbP Fluorescence induction and Q\textsubscript{A}$^-\textsuperscript{reoxidation}$

Fluorescence induction, also known as the Kautsky or O-J-I-P transient, is the multiphasic progression of Chl \textit{a} fluorescence from a photosynthetic system in response to continuous light (Reviewed by Papageorgiou and Govindjee (2011) and Papageorgiou et al. (2007)). This fluorescence is dependent on a number of dynamic, interconnected variables making interpretation difficult (Govindjee, 1995). Excitation at 445 nm specifically targets Chl \textit{a}, allowing for more direct measurement of changes in PS II fluorescence. The term O-J-I-P comes from the notation for four common features present in a fluorescence induction trace plotted on a logarithmic timescale. \textit{O}, with intensity $F_0$, is the minimal fluorescence of dark adapted cells where it is assumed that the RCII primary charge separation rate and $Q_A$ oxidation are both maximal. Using a 445 nm LED \textit{O} comes from PS II, PS I, and to a lesser extent from PBS. $F_V$ is dependent solely on PS II (Byrdin et al., 2000; Schlodder et al., 2005)

![Figure 3.7: Room temperature fluorescence induction. Measured in the absence (A) and presence (B) of DCMU. Wild type (black), ∆PsbP (red), ∆Ycf48 (blue), and ∆PsbP:∆Ycf48 (purple). Traces are the average of at least three independent experiments.](image)

Fluorescence induction was similar between the ∆PsbP and wild-type cells in the presence or absence of DCMU. Removal of Ycf48 in either the wild type or ∆PsbP background resulted in reduced variable fluorescence, more so in the ∆PsbP:∆Ycf48 than ∆Ycf48 strains. The ∆Ycf48 mutant had a greatly reduced I inflection and a reduced P peak. This is enhanced in the ∆PsbP:∆Ycf48 mutant, which had no I inflection and a much reduced P peak (Figure 3.7).

Chl \textit{a} fluorescence relaxation following excitation occurs by a number of mechanisms, characterised by their relative rates. In the absence of DCMU, $Q_A$ reoxidation
occurs via three mechanisms: primarily, $Q_A^-$ to $Q_B$; secondarily, $Q_A^-$ to $Q_B$ where the $Q_B$ site is originally unoccupied and must first become occupied; and thirdly, $Q_A^-$ recombining with the donor side (Renger et al., 1995; Robinson and Crofts, 1983; Vass et al., 1999). In the presence of the herbicide DCMU, which binds the $Q_B$ site and blocks forward electron transfer, only recombination with the donor side occurs. This recombination occurs at two different rates due to sub-populations of PS II (Fufezan et al., 2007).

In the absence of DCMU fluorescence relaxation following a single actinic flash was similar between wild type, the $\Delta Ycf48$, and $\Delta PsbP:Ycf48$ strains. In the presence of DCMU relaxation was similar between the wild-type, $\Delta PsbP$, and $\Delta Ycf48$ cells but consistently initially faster in the $\Delta PsbP:Ycf48$ mutant (Figure 3.8). $F_v$ following a single actinic flash was similar between wild type and the $\Delta PsbP$ strain, being reduced in the $\Delta Ycf48$ mutant and even more so in the $\Delta PsbP:Ycf48$ mutant (Figure 3.9).

### 3.2.5 $\Delta PsbP$ 77 K fluorescence emission

Using low temperature (77 K) fluorescence measurements it is possible to distinguish between individual complexes. A 440 nm excitation beam excites Chl $a$, resulting in fluorescence from those complexes containing Chl $a$: PS I, with a maxima of ~725 nm; CP43 of PS II, with a maxima of ~685 nm; and CP47 of PS II, with a maxima
of ∼695 nm. Whereas a 580 nm excitation beam excites, and produces fluorescence resulting from the excitation of, the bilin pigments of the PBS: PC, with a maxima of ∼650 nm; APC, with a maxima of ∼665 nm; TE and CP43 of PS II, with a maxima of 685 nm; and CP47 of PS II, with a maxima of 695 nm.

The 77 K fluorescence emission spectra at an excitation wavelength of 440 nm showed a very slight reduction in PS II compared to PS I for strains lacking Ycf48 (Figure 3.10). Conversely, the removal of PsbP did cause an appreciable change in fluorescence at this excitation wavelength in the wild type or in the ∆Ycf48 mutant background. When a 580 nm wavelength excitation beam was used a number of differences were observed. Compared to wild type, both the ∆Ycf48 and the ∆PsbP:∆Ycf48 cells had moderate increases in the emission maxima at 680 nm. ∆Ycf48 and ∆PsbP cells had small decreases in the 665 nm emission maxima. The ∆PsbP mutant had a large decrease in 680 nm emission maxima, yet removal of Ycf48 in this background resulted in a peak above the 680 nm emission from wild-type cells.
### Table 3.2: Variable fluorescence of the ∆PsbP strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>$F_V$ $^a$ ±$^a$ (-DCMU)</th>
<th>$F_V$ $^a$ ±$^a$ (+DCMU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100.0 ±14.0</td>
<td>100.0 ±11.6</td>
</tr>
<tr>
<td>∆Ycf48</td>
<td>64.9 ±13.7</td>
<td>67.0 ±16.3</td>
</tr>
<tr>
<td>∆PsbP</td>
<td>91.9 ±16.5</td>
<td>89.8 ±17.2</td>
</tr>
<tr>
<td>∆PsbP:∆Ycf48</td>
<td>50.3 ±9.3</td>
<td>51.2 ±13.5</td>
</tr>
</tbody>
</table>

$^a$ $F_V$ values are given as % of WT for given treatment.

#### 3.2.6 ∆PsbP BN-PAGE

PS II assembly is a complicated process involving the sequential addition, facilitated by numerous assembly factors, of subunits, followed by dimerisation to the biologically functional complex. Blue Native gels utilizes mild conditions to allow separation of intact, enzymatically-active complexes (in a manner at least partially determined by size) (Schägger et al., 1994; Schägger and von Jagow, 1991). Thus BN-PAGE can be used to determine the presence and relative abundance of major complexes and intermediates in PS II assembly (Aro et al., 2005; Rokka et al., 2005). Specific antibodies allow for the determination of those complexes and assembly intermediates known to contain or lack certain protein subunits.

Figure 3.11 shows the separation of thylakoid membrane complexes, via BN-PAGE, visualised by immunodetection of the CP47, CP43, and D1 proteins. There were multiple bands present across the strains and using all the antibodies, which have been assigned as PS II dimers, PS II monomers, CP43 lacking PS II monomers, and lower molecular weight assembly sub-complexes. Bands consistent with PS II dimers were present in all lanes, under all three immunodetection methods, supporting that all strains were able to assemble dimeric PS II. However, the ratio of dimeric to monomeric PS II appears to be lower in ∆PsbP:∆Ycf48 cells. When probes against CP43 or D1 were used, both strains lacking PsbP had less CP47 compared to either wild type or the ∆Ycf48 mutant. However, when anti-CP47 antibodies were used there was no difference between ∆Ycf48 cells and wild type, or the ∆PsbP:∆Ycf48 and ∆PsbP mutants. Strains that lack Ycf48 (the ∆Ycf48 and ∆PsbP:∆Ycf48 strains), imaged
for D1 or CP43, had reduced levels of assembly sub-complexes compared to wild type and the ΔPsbP strain.
Figure 3.11: Immunodetection of PS II core complexes in the ΔPsbP strains. Following BN-PAGE, carried out according to Section 2.6.2, protein was transferred to PVDF (see Section 2.6.3) and probed with (A) CP47, (B) CP43, and (C) D1 antibodies and imaged according to Section 2.6.4. Complexes indicated by arrows are: PS II (2), dimeric PS II; PS II (1), monomer PS II; PS II (1)*, assembly intermediate PS II monomer lacking CP43; AC, PS II pre-assembly complex; AC*, PS II pre-assembly complex lacking CP47. Samples were diluted such that each lane contains 1.5 µg Chl a.
3.3 Psb27

3.3.1 ΔPsb27 Photoautotrophic growth

Figure 3.12: Photoautotrophic Growth Curve. Cell density determined by measuring OD_{730 nm} of the following strains: Wild type (closed squares), ΔPsb27 (open circles), ΔYcf48 (closed triangles), and ΔPsb27:ΔYcf48 (open diamonds). Error bars are the standard error of three independent experiments. Error bars that are not visible are smaller than their accompanying symbols.

Wild type and the ΔPsb27 strain displayed similar growth characteristics under the conditions used (Section 3.3.1). Doubling times for these strains were calculated to be 13.2 h (s.d. 1.6 h) and 10.6 h (s.d. 2.0 h), respectively. Removal of Ycf48 was found to retard growth in both the wild-type or ΔPsb27 mutant background. Additionally there was a significant difference between the growth rates of ΔYcf48 and ΔPsb27:ΔYcf48 for which doubling times were 31.5 h (s.d. 2.4 h) and 85.1 h (s.d. 23.1 h), respectively.
Table 3.3: Doubling times of the ΔPsb27 strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (h)</th>
<th>±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.6</td>
<td>2.0</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>31.5</td>
<td>2.4</td>
</tr>
<tr>
<td>ΔPsb27</td>
<td>13.2</td>
<td>1.6</td>
</tr>
<tr>
<td>ΔPsb27:ΔYcf48</td>
<td>85.1</td>
<td>23.1</td>
</tr>
</tbody>
</table>

3.3.2 ΔPsb27 Whole cell absorption spectra

Pigment composition was analysed by room temperature fluorescence of whole cells (Section 2.2.7). The ΔPsb27 strain showed a reduced maximum absorption peak at 680 nm compared to wild type (Figure 3.13). When both Ycf48 and Psb27 were removed, more substantial changes were observed. The ΔPsb27:ΔYcf48 mutant had increased absorption in the 475-500 nm range and a severely reduced maximum absorption peak at 680 nm. Again in both strains lacking Ycf48, the ΔYcf48 and ΔPsb27:ΔYcf48 mutants, there is a shoulder in the area of decreasing absorption between 500 nm and 570 nm, suggesting increased OCP$^\text{r}$ relative to wild type and the ΔPsb27 cells.

3.3.3 ΔPsb27 Oxygen evolution

All four strains showed similar rates of oxygen evolution in the presence of DCBQ, if anything wild-type and ΔPsb27:ΔYcf48 cells having slightly lower rates than the ΔYcf48 or ΔPsb27 cells. Supported by bicarbonate, the two strains that lack Ycf48, the ΔYcf48 and ΔPsb27:ΔYcf48 strains, showed reduced rates of oxygen evolution (Figure 3.14). Removal of Ycf48 in the ΔPsb27 background did not reveal a significant additive effect in reducing oxygen evolving capacity.

3.3.4 ΔPsb27 Fluorescence induction and $Q_A^-$ reoxidation

Kautsky induction fluorescence was similar between the ΔPsb27 strain and wild type in the presence or absence of DCMU. Removal of Ycf48 in either the wild type or
3.3.5 ∆Psb27 77 K fluorescence emission

Figure 3.13: Whole cell absorption spectra. Absorption spectra of strains: Wild type (black); ∆Psb27 (orange); ∆Ycf48 (blue), and ∆Psb27:∆Ycf48 (dark red) normalised to the Soret band at 435 nm. Traces are representative of three or more separate experiments.

In the absence of DCMU fluorescence relaxation following a single actinic flash was indistinguishable between wild type and the ∆Ycf48, ∆Psb27, and ∆Psb27:∆Ycf48 mutants. In the presence of DCMU relaxation was similar between wild type and ∆Ycf48 cells but consistently initially faster in both the ∆Ycf48 and ∆Psb27:∆Ycf48 strains, with the ∆Psb27:∆Ycf48 strain being faster still than the ∆Ycf48 strain (Figure 3.17).

3.3.5 ∆Psb27 77 K fluorescence emission

The 77 K fluorescence emission spectrum at an excitation wavelength of 440 nm showed a very slight reduction in PS II compared to PS I for ∆Ycf48 (Figure 3.10). Conversely,
Chapter 3. Results

Figure 3.14: Oxygen evolution. (A) Rates of oxygen evolution supported by 15 mM sodium bicarbonate (blue) or 1 M K₃Fe(CN)₆ and 200 µM DCBQ (red). (B) Rates of oxygen evolution supported by 15 mM sodium bicarbonate or 1 M K₃Fe(CN)₆ and 200 µM DCBQ as a percentage of wild-type rate under the same conditions. Errors bars are s.d. and data is from at least three separate experiments. Traces of oxygen evolution supported by (C) 15 mM sodium bicarbonate or (D) 1 M K₃Fe(CN)₆ and 200 µM DCBQ: wild type (black); ∆Psb27 (orange); ∆Ycf48 (blue), and ∆Psb27:∆Ycf48 (dark red). Representative traces are shown.

the removal of Psb27 caused an appreciable change in fluorescence at this excitation wavelength in wild type or the ∆Ycf48 backgrounds, with the ∆Psb27:∆Ycf48 mutant higher still than the ∆Psb27 mutant. The ∆Psb27:∆Ycf48 cells also showed either an ablation of the 695 nm peak or its masking beneath a large 685 nm peak. In both the ∆Ycf48 and the ∆Psb27:∆Ycf48 mutants there was also an increase in 683 nm fluorescence emission, which originates from partially assembled PS II complexes and IsiA protein (Burnap et al., 1993; Keren et al., 2005; Seibert et al., 1988) When
3.3.5 $\Delta$Psb27 77 K fluorescence emission

Figure 3.15: Room temperature fluorescence induction. Measured in the absence (A) and presence (B) of DCMU. Wild type (black), $\Delta$Psb27 (orange), $\Delta$Ycf48 (blue), and $\Delta$Psb27:$\Delta$Ycf48 (dark red). Representative traces are shown.

A 580 nm wavelength excitation beam was used, differences were observed. Strains lacking Psb27, the $\Delta$Psb27 and $\Delta$Psb27:$\Delta$Ycf48 mutants, showed moderately increased 665 nm peaks compared to wild type. Compared to wild type both the $\Delta$Ycf48 and the $\Delta$Psb27 strains had moderate increases in the emission maxima at 680 nm. The $\Delta$Psb27:$\Delta$Ycf48 cells had a large increase in emission at this peak, the change being more than the combined effect of removing Ycf48 or Psb27 individually. Additionally, the $\Delta$Ycf48 cells had a small decrease in the 665 nm emission maximum.
Figure 3.16: Variable fluorescence decay. Wild type (black), ΔPsb27 (orange), ΔYcf48 (blue), and ΔPsb27:ΔYcf48 (dark red) in the presence (~) or absence (*) of DCMU.

Table 3.4: Variable fluorescence of the ΔPsb27 strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>F_V ±</th>
<th>F_V ±</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-DCMU)</td>
<td>(+DCMU)</td>
</tr>
<tr>
<td>Wild type</td>
<td>100.0</td>
<td>14.0</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>64.9</td>
<td>13.7</td>
</tr>
<tr>
<td>ΔPsb27</td>
<td>115.6</td>
<td>10.0</td>
</tr>
<tr>
<td>ΔPsb27:ΔYcf48</td>
<td>21.4</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*F_V values are given as % of WT for given treatment.
Figure 3.17: Chl \( a \) fluorescence decay following a single actinic flash. Measured in the absence (A) and presence (B) of DCMU. Wild type (black), \( \Delta Psb27 \) (orange), \( \Delta Ycf48 \) (blue), and \( \Delta Psb27:\Delta Ycf48 \) (dark red). Representative traces are shown.

Figure 3.18: 77 K fluorescence emission. 77 K fluorescence emission spectra at (A) 440 nm excitation, and (B) 580 nm excitation. Wild type (black), \( \Delta Psb27 \) (orange), \( \Delta Ycf48 \) (blue), and \( \Delta Psb27:\Delta Ycf48 \) (dark red). Traces are the average of at least three independent experiments and are normalised to the PS I emission maxima at 725 nm.
3.4 Psb28 and Psb28-2

3.4.1 ΔPsb28 and ΔPsb28-2 Photoautotrophic growth

Figure 3.19: Photoautotrophic Growth. Cell density determined by measuring OD\textsubscript{730 nm} of the following strains: Wild type (open squares); ΔPsb28 (open circles); ΔPsb28-2 (open diamonds); ΔPsb28:ΔPsb28-2 (open triangles); ΔYcf48 (closed squares); ΔPsb28:ΔYcf48 (closed circles); ΔPsb28-2:ΔYcf48 (closed diamonds), and ΔPsb28:ΔPsb28-2:ΔYcf48 (closed triangles). Error bars are the standard error of three independent experiments. Error bars that are not visible are smaller than their accompanying symbols.

Photoautotrophic growth was perturbed by removal of Psb28 or, to a lesser degree, Psb28-2. Removal of both Psb28 and Psb28-2 hindered photoautotrophic growth more than removal of either individually (Section 3.4.1). Removal of Ycf48 in any background increased doubling time (Table 3.5). Interestingly there is a comparable reduction of photoautotrophic growth capability between the ΔYcf48 strains lacking either Psb28 or Psb28-2, and that lacking both Psb28 and Psb28-2.
Table 3.5: Doubling times of the ΔPsb28 strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (h)</th>
<th>±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.6</td>
<td>2.0</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>31.5</td>
<td>2.4</td>
</tr>
<tr>
<td>ΔPsb28</td>
<td>19.2</td>
<td>2.0</td>
</tr>
<tr>
<td>ΔPsb28:ΔYcf48</td>
<td>85.7</td>
<td>38.1</td>
</tr>
<tr>
<td>ΔPsb28-2</td>
<td>16.7</td>
<td>0.2</td>
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<tr>
<td>ΔPsb28-2:ΔYcf48</td>
<td>104.9</td>
<td>47.0</td>
</tr>
<tr>
<td>ΔPsb28:ΔPsb28-2</td>
<td>20.7</td>
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</tr>
<tr>
<td>ΔPsb28:ΔPsb28-2:ΔYcf48</td>
<td>155.2</td>
<td>25.3</td>
</tr>
</tbody>
</table>

3.4.2 ΔPsb28 and ΔPsb28-2 Whole cell absorption spectra

Pigment composition analysed by room temperature absorption measurement revealed similar effects with the removal of Ycf48 in the ΔPsb28, ΔPsb28-2, and ΔPsb28:ΔPsb28-2 background strains. Removal of one or both of Psb28 and Psb28-2 resulted in the suppression of the 625 nm and 680 nm peaks and increased absorption in the 475-550 nm range relative to wild type. Removal of Ycf48 in the ΔPsb28, ΔPsb28-2, or ΔPsb28:ΔPsb28-2 strains exacerbates both the suppression of the 625 nm peak and the increased absorption of the 475-550 nm range relative to wild type (Section 3.4.2). In all strains, that lacked Ycf48, a shoulder was displayed in the decreasing absorption between 500 nm and 570 nm, perhaps reflecting an increase in the amount OCP$^r$ relative to wild-type, ΔPsb28, and ΔPsb28:ΔPsb28-2 cells.
Figure 3.20: Whole cell absorption spectra of \( \Delta \text{Psb28}, \Delta \text{Psb28-2}, \) and \( \Delta \text{Psb28:}\Delta \text{Psb28-2} \) strains. (A) Wild type (black), \( \Delta \text{Ycf48} \) (dotted black), \( \Delta \text{Psb28} \) (blue), and \( \Delta \text{Psb28:}\Delta \text{Psb28-2} \) (dotted blue). (B) Wild type (black), \( \Delta \text{Ycf48} \) (dotted black), \( \Delta \text{Psb28-2} \) (red), and \( \Delta \text{Psb28-2:}\Delta \text{Ycf48} \) (dotted red). (C) Wild type (black), \( \Delta \text{Ycf48} \) (dotted black), \( \Delta \text{Psb28:}\Delta \text{Psb28-2} \) (purple), and \( \Delta \text{Psb28:}\Delta \text{Psb28-2:}\Delta \text{Ycf48} \) (dotted purple). All traces are normalised to the Soret band at 435 nm. Representative traces are shown.

3.4.3 \( \Delta \text{Psb28} \) and \( \Delta \text{Psb28-2} \) Oxygen evolution

In the presence of bicarbonate, rates of oxygen evolution were similar between wild type, the \( \Delta \text{Ycf48} \) and the \( \Delta \text{Psb28} \) cells. Confoundingly, under these conditions the \( \Delta \text{Psb28-2} \) and \( \Delta \text{Psb28:}\Delta \text{Psb28-2} \) mutants were seen to evolve oxygen at a higher rate than wild type on a Chl \( \alpha \) basis. Conversely, strains lacking Ycf48 and either or both of Psb28 and Psb28-2 displayed reduced rates of oxygen evolution (Section 3.4.3). In the presence of DCBQ all strains able to synthesise Ycf48 had similar rates of oxygen evolution, including the \( \Delta \text{Psb28-2} \) and \( \Delta \text{Psb28:}\Delta \text{Psb28-2} \) mutants. The removal of Ycf48 from wild type, and the \( \Delta \text{Psb28}, \Delta \text{Psb28-2}, \) or \( \Delta \text{Psb28:}\Delta \text{Psb28-2} \) strains reduced oxygen evolution capability. No additional reduction in oxygen evolution capability was
3.4.3 $\Delta$Psb28 and $\Delta$Psb28-2 Oxygen evolution

![Figure 3.21: Oxygen evolution of the $\Delta$Psb28, $\Delta$Psb28-2, and $\Delta$Psb28:$\Delta$Psb28-2 strains.](image)

(A) Rates of oxygen evolution supported by 15 mM sodium bicarbonate (blue) or 1 M $K_3Fe(CN)_6$ and 200 $\mu$M DCBQ (red). (B) Rates of oxygen evolution supported by 15 mM sodium bicarbonate or 1 M $K_3Fe(CN)_6$ and 200 $\mu$M DCBQ as a percentage of wild type rate under same conditions. Errors bars are s.d. and data is from at least three independent experiments.

seen when both Psb28 and Psb28-2 were removed in concert with Ycf48, compared to either Psb28 or Psb28-2 and Ycf48.
Figure 3.22: Traces of oxygen evolution of ∆Psb28, ∆Psb28-2, and ∆Psb28:∆Psb28-2 strains. (A) Supported by sodium bicarbonate: wild type (black); ∆Ycf48 (dotted black); ∆Psb28 (blue), and ∆Psb28:∆Ycf48 (dotted blue). (B) Supported by DCBQ and K₃Fe(CN)₆, colouring as in (A). (C) Supported by sodium bicarbonate: wild type (black); ∆Ycf48 (dotted black); ∆Psb28-2 (red), and ∆Psb28-2:∆Ycf48 (dotted red). (D) Supported by DCBQ and K₃Fe(CN)₆, colouring as in (C). (E) Supported by sodium bicarbonate: wild type (black); ∆Ycf48 (dotted black); ∆Psb28:∆Psb28-2 (purple), and ∆Psb28:∆Psb28-2:∆Ycf48 (dotted purple). (F) Supported by DCBQ and K₃Fe(CN)₆, colouring as in (E). Black arrows indicate when the light was turned on and then, 180 s later, off again. Representative traces are shown.
3.4.4 $\Delta$Psb28 and $\Delta$Psb28-2 Fluorescence induction and $Q_A^-$ reoxidation

Figure 3.23: Fluorescence induction of $\Delta$Psb28, $\Delta$Psb28-2, and $\Delta$Psb28:∆Psb28-2 strains. (A) Without addition: wild type (black); $\Delta$Psb28 (blue); $\Delta$Psb28-2 (red), and $\Delta$Psb28:∆Psb28-2 (purple). (B) In the presence of DCMU, colouring as in (A). (C) Without addition: $\Delta$Ycf48 (spotted black); $\Delta$Psb28:∆Ycf48 (dotted blue); $\Delta$Psb28-2:∆Ycf48 (dotted red), and $\Delta$Psb28:∆Psb28-2:∆Ycf48 (dotted purple). (D) In the presence of DCMU, colouring as in (C). Traces are the average of at least three independent experiments.

Measured by Kautsky induction fluorescence. Variable fluorescence was found to be reduced, compared to wild type, in the $\Delta$Psb28 and $\Delta$Psb28:∆Ycf48 strains. Strains lacking Psb28-2, however, had $F_V$ values similar to wild type (Figure 3.23). Removal of Ycf48 again reduced variable fluorescence, and an additive effect was observed when either or both of Psb28 and Psb28-2 were removed, with the $\Delta$Psb28, $\Delta$Psb28-2, and $\Delta$Psb28:∆Psb28-2 mutants having reduced fluorescence compared to the $\Delta$Psb28:∆Ycf48, $\Delta$Psb28-2:∆Ycf48, and $\Delta$Psb28:∆Psb28-2:∆Ycf48 mutants, respectively. Removing Ycf48 in the $\Delta$Psb28-2 background resulted in a reduction of variable fluorescence.
Table 3.6: Variable fluorescence of the ΔPsb28, ΔPsb28-2, and ΔPsb28:ΔPsb28-2 strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>FV  a ± a (-DCMU)</th>
<th>FV  a ± a (+DCMU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>100.0 14.0</td>
<td>100.0 11.6</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>64.9 13.7</td>
<td>67.0 16.3</td>
</tr>
<tr>
<td>ΔPsb28</td>
<td>88.0 4.7</td>
<td>85.2 5.9</td>
</tr>
<tr>
<td>ΔPsb28:ΔYcf48</td>
<td>25.4 2.7</td>
<td>25.3 3.0</td>
</tr>
<tr>
<td>ΔPsb28-2</td>
<td>93.0 5.7</td>
<td>93.9 7.6</td>
</tr>
<tr>
<td>ΔPsb28-2:ΔYcf48</td>
<td>28.7 2.8</td>
<td>27.1 4.1</td>
</tr>
<tr>
<td>ΔPsb28:ΔPsb28-2</td>
<td>79.8 11.3</td>
<td>81.8 15.4</td>
</tr>
<tr>
<td>ΔPsb28:ΔPsb28-2:ΔYcf48</td>
<td>29.8 3.1</td>
<td>28.9 3.1</td>
</tr>
</tbody>
</table>

a values are given as % of WT with same treatment.

as severe as that seen for the ΔPsb28:ΔYcf48 and ΔPsb28:ΔPsb28-2:ΔYcf48 strains, which is interesting given there was no observable reduction when removing Psb28-2 alone.

Fluorescence decay following a single actinic flash, which is predominantly dependent on the reoxidation of Q_−_A, was similar between all strains. However, when cells were pre-treated with DCMU initial fluorescence decay was consistently faster in the ΔPsb28:ΔYcf48, ΔPsb28-2:ΔYcf48, ΔPsb28:ΔPsb28-2:ΔYcf48 strains, and, again to a lesser extent, the ΔYcf48 strain.
3.4.4 $\Delta$Ps28 and $\Delta$Ps28-2 Fluorescence induction and $Q_A^-$ reoxidation

Figure 3.24: Variable fluorescence decay. (A) In the presence of DCMU: wild type (solid grey); $\Delta$Ycf48 (dotted grey); $\Delta$Ps28 (solid light blue), and $\Delta$Ps28:$\Delta$Ycf48 (dotted light blue). In the absence of DCMU: wild type (solid black); $\Delta$Ycf48 (dotted black); $\Delta$Ps28 (solid blue), and $\Delta$Ps28:$\Delta$Ycf48 (dotted blue). (B) In the presence of DCMU: wild type (solid grey); $\Delta$Ycf48 (dotted grey); $\Delta$Ps28-2 (solid light red), and $\Delta$Ps28-2:$\Delta$Ycf48 (dotted light red). In the absence of DCMU: wild type (solid black); $\Delta$Ycf48 (dotted black); $\Delta$Ps28-2 (solid red), and $\Delta$Ps28-2:$\Delta$Ycf48 (dotted red). (C) In the presence of DCMU: wild type (solid grey); $\Delta$Ycf48 (dotted grey); $\Delta$Ps28:$\Delta$Ps28-2 (solid light purple), and $\Delta$Ps28:$\Delta$Ps28-2:$\Delta$Ycf48 (dotted light purple). In the absence of DCMU: wild type (solid black); $\Delta$Ycf48 (dotted black); $\Delta$Ps28:$\Delta$Ps28-2 (solid purple), and $\Delta$Ps28:$\Delta$Ps28-2:$\Delta$Ycf48 (dotted purple).
Figure 3.25: Chl a fluorescence decay following a single actinic flash in the absence of DCMU. (A) Wild type (black), ΔYcf48 (dotted black), ΔPsb28 (blue), and ΔPsb28:ΔYcf48 (dotted blue). (B) Wild type (black), ΔYcf48 (dotted black), ΔPsb28-2 (red), and ΔPsb28-2:ΔYcf48 (dotted red). (C) Wild type (black), ΔYcf48 (dotted black), ΔPsb28:ΔPsb28-2 (purple), and ΔPsb28:ΔPsb28-2:ΔYcf48 (dotted purple). Traces are the average of at least three independent experiments.
3.4.4 ΔPsb28 and ΔPsb28-2 Fluorescence induction and Q₄⁻ reoxidation

Figure 3.26: Chl a fluorescence decay following a single actinic flash in the presence of DCMU. (A) Wild type (black), ΔYcf48 (dotted black), ΔPsb28 (blue), and ΔPsb28:ΔYcf48 (dotted blue). (B) Wild type (black), ΔYcf48 (dotted black), ΔPsb28-2 (red), and ΔPsb28-2:ΔYcf48 (dotted red). (C) Wild type (black), ΔYcf48 (dotted black), ΔPsb28:ΔPsb28-2 (purple), and ΔPsb28:ΔPsb28-2:ΔYcf48 (dotted purple). Traces are the average of at least three independent experiments.
3.4.5 ΔPsb28 and ΔPsb28-2 77 K fluorescence emission

Figure 3.27: 77 K fluorescence emission spectra at 440 nm excitation. (A) Wild type (black), ΔYcf48 (dotted black), ΔPsb28 (blue), and ΔPsb28:ΔYcf48 (dotted blue). (B) Wild type (black), ΔYcf48 (dotted black), ΔPsb28-2 (red), and ΔPsb28-2:ΔYcf48 (dotted red). (C) Wild type (black), ΔYcf48 (dotted black), ΔPsb28:ΔPsb28-2 (purple), and ΔPsb28:ΔPsb28-2:ΔYcf48 (dotted purple). Traces are the average of at least three independent experiments and are normalised to the PS I emission maxima at 725 nm.

The removal of either Psb28 or Psb28-2 alone does not result in appreciable differences in 77 K fluorescence emission with a 440 nm excitation wavelength (Figure 3.27). However, the ΔPsb28:ΔPsb28-2 strain had increased fluorescence emission in the 685 nm peak, coming from CP43, compared to the wild-type, ΔPsb28, or ΔPsb28-2 strains. Again the emission spectrum showed a slight reduction in the PS II/PS I ratio for the ΔYcf48 mutant. When Ycf48 and one or both Psb28 and Psb28-2 were removed more
drastic changes appeared. The ΔPsb28:ΔYcf48, ΔPsb28-2:ΔYcf48, and ΔPsb28:ΔPsb28-2:ΔYcf48 cells all showed altered emission spectra with either an ablation of the 695 nm peak or its masking beneath a large 685 nm peak.

Figure 3.28: 77 K fluorescence emission spectra at 580 nm excitation. (A) Wild type (black), ΔYcf48 (dotted black), ΔPsb28 (blue), and ΔPsb28:ΔYcf48 (dotted blue). (B) Wild type (black), ΔYcf48 (dotted black), ΔPsb28-2 (red), and ΔPsb28-2:ΔYcf48 (dotted red). (C) Wild type (black), ΔYcf48 (dotted black), ΔPsb28:ΔPsb28-2 (purple), and ΔPsb28:ΔPsb28-2:ΔYcf48 (dotted purple). Traces are the average of at least three independent experiments and are normalised to the PS I emission maxima at 725 nm.

77 K fluorescence emission with a 580 nm excitation wavelength showed negligible difference resulting from the removal of either of Psb28 and Psb28-2 alone or together; the ΔPsb28, ΔPsb28-2, and ΔPsb28:ΔPsb28-2 mutants having similar emission spectra to wild type (Figure 3.28). The ΔYcf48 strain had an increased peak in the 685-695 nm range i.e. fluorescence emission coming from PS II via PBS. Removing Ycf48 in
the ΔPsb28, ΔPsb28-2, and ΔPsb28:ΔPsb28-2 background strains had further effects. The 685-695 nm peak was similarly amplified by the removal of Ycf48 in strains lacking either Psb28 or Psb28-2. Removing Psb28, Psb28-2, and Ycf48 in unison resulted in an even greater increase in the 685-695 nm peak.
Chapter 4

Discussion

4.1 Introduction

The Ycf48 protein is a 37-kDa hydrophilic polypeptide that has been shown to be involved in the assembly of PS II and the repair cycle of the photosystem (Komenda et al., 2008). The function of Ycf48 appears to be stabilisation of early assembly complexes containing the D1 and D2 heterodimer and the processing of the C-terminal extension on the pD1 protein (Nickelsen and Rengstl, 2013). This thesis has presented new evidence that suggests Ycf48 may interact with, or work together with, other protein assembly factors during the early stages of assembly. Mutants have been created and studied that combine the removal of Ycf48 with the removal of the PsbP, Psb27, Psb28, and Psb28-2 proteins.

4.2 ΔYcf48

Consistent with previous reports, removal of Ycf48 adversely affected photoautotrophic growth (Komenda et al., 2008; Rengstl et al., 2013). Whole cell absorption spectra (Figure 3.5) indicate a similar pigment composition between wild-type and ΔYcf48 cells; however, it has also been reported that ΔYcf48 cells have increased absorption at 540 nm and decreased absorption at 680 nm, indicating comparatively more carotenoids and fewer Chl a-containing photosystems, respectively (Rengstl et al., 2013). Interestingly, it was observed that the rate of electron transport supported by bicarbonate was...
similar between the wild type and the ΔYcf48 strain. This is in agreement with the findings of Komenda et al. (2008), who reported an oxygen evolution rate 89% that of wild type for their ΔYcf48 strain. This contrasts with the situation when electron transport is supported by the PS II-specific electron acceptors DCBQ and K₃Fe(CN)₆. Supported by DCBQ and K₃Fe(CN)₆, we found the ΔYcf48 strain to have an oxygen evolution rate roughly 40% of wild type. This is similar to the 65% reported by Komenda et al. (2008), and at odds with the 140% of wild-type rate reported by Rengstl et al. (2013). Rengstl et al. (2013) attribute this to the lower Chl a levels of their ΔYcf48 strain.

Using $F_v$ to estimate the level of functional, assembled PS II, the ΔYcf48 mutant has fewer than wild type. The $F_v$ of the ΔYcf48 cells was found to be 67% that of wild type (Section 3.2.4), consistent with the 71% $F_v$ reported by Komenda et al. (2008). In the presence of K₃Fe(CN)₆ and the artificial electron acceptor, DCBQ, i.e. PS II-specific electron acceptors, the ΔYcf48 strain has severely reduced oxygen evolution capability compared to wild type. This would suggest either an altered ability to utilise DCBQ as an electron acceptor, or changes to the donor side reactions of PS II in the ΔYcf48 mutant. The latter seems unlikely given the currently known functions of Ycf48 and isn’t supported by results of this investigation. The shape of the fluorescence induction curve was similar, although the variable fluorescence was smaller, between the ΔYcf48 strain and wild type (Section 3.2.4). The reduced amplitude of the variable fluorescence found for the ΔYcf48 mutant, compared to wild type, in both fluorescence induction and fluorescence decay experiments, most likely reflects a reduced number of functional centres, due to less efficient assembly and repair in the absence of Ycf48.

### 4.3 ΔPsbP strains

Removal of PsbP did not render *Synechocystis* sp. PCC 6803 incapable of photoautotrophic growth in either a wild-type or ΔYcf48 background, with doubling times being similar between the ΔPsbP and wild-type, and the ΔPsbP:ΔYcf48 and ΔYcf48 strains (Table 3.1). This is in agreement with earlier reports, although Sveshnikov et al. (2007) found that their ΔPsbP mutant had a disadvantage in competitive growth experiments with wild type. Similarly, the absence of PsbP had no effect on oxygen evolution, with the ΔPsbP and ΔPsbP:ΔYcf48 mutants having a similar, if not higher, rate of oxygen evolution than wild type and the ΔYcf48 strain, respectively.
4.4 \( \Delta \text{Psb27} \) strains

(Figure 3.6). This was equally true for oxygen evolution in the presence of bicarbonate or \( \text{K}_3\text{Fe(CN)}_6 \) and DCBQ. Previous investigations also report no change in oxygen evolution in \( \Delta \text{PsbP} \) strains (Ishikawa et al., 2005; Summerfield et al., 2005; Thornton et al., 2004). The \( \Delta \text{PsbP} \) mutant appeared to have a lower level of PBS and carotenoids, compared to wild type (Figure 3.5). The \( \Delta \text{PsbP}:\Delta \text{Ycf48} \) cells, however, showed increased carotenoids and reduced Chl \( \alpha \), supporting an increased light-stress response in this strain, and a shoulder in the 500 nm to 550 nm region of the absorption spectrum (Figure 3.5). This shoulder may reflect an increase in OCP\(^r\) relative to wild type, concomitant with either or both increased light stress in these strains or reduced PS I and PS II, where the absence of PS II and PS I has previously been shown to increase OCP\(^r\)-mediated NPQ (Rakhimberdieva et al., 2011). In terms of photoautotrophic growth capability, whole cell pigment composition, oxygen evolution and 77 K fluorescence emission with 440 nm excitation, the phenotype of the \( \Delta \text{PsbP}:\Delta \text{Ycf48} \) cell was largely dominated by the effect of removing Ycf48.

The \( \Delta \text{PsbP} \) mutant was also found to have reduced fluorescence from PS II-associated PBS compared to wild type (Figure 3.10). This may, in part, be due to this strain’s lower PBS levels, but could also reflect tighter coupling between PS II and PBS in a strain lacking PsbP. Conversely in the \( \Delta \text{PsbP}:\Delta \text{Ycf48} \) mutant similarly high peaks were observed, despite the \( \Delta \text{PsbP}:\Delta \text{Ycf48} \) strain having lower levels of PBS than the \( \Delta \text{Ycf48} \) strain. These findings may suggest an as-yet uncharacterised ability of PsbP to modulate the interaction between PS II and PBS. The \( \Delta \text{PsbP} \) strain has previously been reported to display lower thermal tolerance (Summerfield et al., 2005). Given that PBS detachment is partially dependent on temperature, it is tempting to speculate that this thermal-tolerance phenotype results from an altered connectivity between PBS and PS II in the absence of PsbP (Sarcina et al., 2001; Stoitchkova et al., 2007). Given that it is targeted to the lumen, PsbP may act via the OEC which has been shown to affect PS II-PBS interaction (Hwang et al., 2008).

4.4 \( \Delta \text{Psb27} \) strains

There was a negligible difference in photoautotrophic growth capability between wild-type and \( \Delta \text{Psb27} \) cells (Table 3.3). The \( \Delta \text{Psb27} \) strain was found to evolve oxygen faster than wild type when measured using both bicarbonate to support whole chain electron transport and when using \( \text{K}_3\text{Fe(CN)}_6 \) and DCBQ to support PS II-specific
electron transport (Figure 3.14). The ΔPsb27 mutant also displayed greater variable fluorescence in both fluorescence induction and fluorescence decay measurements following a single actinic flash (Figure 3.15, Figure 3.17). These observations are consistent with the large increase in PS II to PS I ratio that 77 K fluorescence emission spectra using a 440 nm excitation wavelength revealed for this mutant (Figure 3.18). These results are, however, at odds with previously reported phenotypes resulting from the removal of Psb27 from *Synechocystis* sp. PCC 6803 (Grasse et al., 2011; Nowaczyk et al., 2006; Roose and Pakrasi, 2004, 2007). However, Psb27 may also have a role in PS I assembly and in supercomplexes between PS II and the PBS in a similar manner to a role for Psb27 in interactions between PS II and LHCs as reported in higher plants (Dietzel et al., 2011; Komenda et al., 2012a).

On removal of Ycf48 from the ΔPsb27 strain there is a significant reduction in physiological fitness, more-so than the effects of removal of either Ycf48 or Psb27 alone. Removal of Ycf48 from the ΔPsb27 background decreases Chl *a*-containing complexes and increases absorption associated with carotenoids, and absorption in the 500 nm to 550 nm shoulder (Figure 3.13), possibly reflecting increased OCP <sup>r</sup> levels, all of which is indicative of light-induced stress. The ΔPsb27:ΔYcf48 strain is incapable of photoautotrophic growth, evolves oxygen at a lower rate than wild-type, ΔYcf48, or ΔPsb27 mutant (Figure 3.14), has a large reduction in F<sub>V</sub> (Section 3.3.4), and has the askew PS II to PS I ratio of the ΔPsb27 mutant (Figure 3.18). At a 440 nm excitation wavelength, 77 K fluorescence emission spectra also revealed a higher ratio of CP43 to CP47-associated fluorescence emission in ΔPsb27:ΔYcf48 cells. At a 580 nm excitation wavelength, 77 K fluorescence emission spectra of the ΔPsb27:ΔYcf48 strain had a large 685 nm peak, suggesting major dissociation of PBS (Figure 3.18). Interestingly, assembled PS II display altered photochemical characteristics in the ΔPsb27:ΔYcf48 and ΔYcf48 strains, with faster Q<sub>A</sub><sup>-</sup> to donor side recombination than wild type or ΔPsb27 cells and an O-J-I-P transient with no I or P inflections. Without Ycf48, when PS II assembly and repair is hindered, cells may be more vulnerable to damage during the repair cycle, making them more reliant on Psb27, due to its putative role in the protection of the Mn<sub>4</sub>CaO<sub>5</sub> cluster. Similarly, in the absence of Ycf48, the level of D1, which is required for Mn<sub>4</sub>CaO<sub>5</sub> cluster assembly, is reduced. The absence of a Mn<sub>4</sub>CaO<sub>5</sub> cluster may also be responsible for the faster Chl *a* fluorescence decay observed in DCMU-treated cells (Johnson et al., 1995; Krieger-Liszkay et al., 2008).
4.5 \( \Delta \text{Psb28}, \Delta \text{Psb28-2}, \text{and } \Delta \text{Psb28}:\Delta \text{Psb28-2} \) strains

There was minimal reduction in photoautotrophic growth capability on removal of Psb28, Psb28-2, or both Psb28 and Psb28-2, although the strain lacking both fared worse than strains lacking either, and Psb28 removal had a larger effect than Psb28-2 removal (Table 3.5). This pattern was also present with regards to the decrease in PBS and Chl \( \alpha \)-containing complexes, as determined by whole cell spectroscopy (Section 3.4.2). The peaks associated with PBS and Chl \( \alpha \)-containing complexes were reduced most, on the removal of both Psb28 isoforms, and least on removal of Psb28-2 alone. Strains lacking Psb28-2: \( \Delta \text{Psb28-2}, \Delta \text{Psb28-2}:\Delta \text{Ycf48}, \Delta \text{Psb28}:\Delta \text{Psb28-2}, \) and \( \Delta \text{Psb28}:\Delta \text{Psb28-2}:\Delta \text{Ycf48}, \) displayed greater oxygen evolution than similar strains lacking Psb28. Yet, this was only the case in the presence of bicarbonate i.e. when whole chain electron transport is occurring. Between strains with and without Psb28, rates were indistinguishable, at least between those strains with, or those without, Ycf48. However, as with the \( \Delta \text{Psb27} \) mutant, removal of Ycf48 from strains already \( \Delta \text{Psb28}, \Delta \text{Psb28-2}, \) or \( \Delta \text{Psb28}:\Delta \text{Psb28-2} \) resulted in an inability to grow photoautotrophically (Table 3.5). Removal of Ycf48 from a strain lacking either or both of Psb28 and Psb28-2 resulted in: a large reduction in oxygen-evolving capability in the presence of either bicarbonate, or \( \text{K}_3\text{Fe(CN)}_6 \) and DCBQ (Section 3.4.3); largely reduced \( \text{F}_V \) (Figure 3.24), and removal of the I and P inflections in fluorescence induction traces (Figure 3.23). The smaller amplitude of variable fluorescence found for strains lacking one or both of the Psb28 isoforms and Ycf48, in both fluorescence induction and fluorescence decay experiments, again reflected a reduced number of functional centres due to less efficient assembly and repair in these strains. These experiments revealed a phenotype similar to that observed with the \( \Delta \text{Psb27}:\Delta \text{Ycf48} \) mutant in the \( \Delta \text{Psb28}:\Delta \text{Ycf48}, \Delta \text{Psb28-2}:\Delta \text{Ycf48}, \) and \( \Delta \text{Psb28}:\Delta \text{Psb28-2}:\Delta \text{Ycf48} \) strains. Low temperature fluorescence emission spectra revealed both an increase in the PS II to PS I ratio, especially in strains that are \( \Delta \text{Ycf48} \) and \( \Delta \text{Psb28}, \) and a large accumulation of CP43 complexes (Figure 3.27). This supports an increased turnover and delayed processing of the D1 subunit and delayed incorporation into PS II in these strains (Komenda et al., 2008). A similar Chl \( \alpha \) fluorescence decay phenotype to that measured in the \( \Delta \text{Psb27}:\Delta \text{Ycf48} \) mutant was observed (Figure 3.26, Figure 3.25), again suggestive of comparatively fewer photoactivated centres.

Collectively these experiments have provided the first evidence to suggest that in the \( \Delta \text{Ycf48} \) strains both Psb28 isoforms have vital roles, such that in their absence the other isoform cannot suffice. This is understandable with Psb28, whose proposed
function includes the protection of PS II assembly complexes, which likely accumulate in ∆Ycf48 cells.

4.6 Background Strains

Recently it was discovered that there are two strains of *Synechocystis* sp. PCC 6803 in the lab that are used as wild type (Morris et al., 2014). These are: glucose tolerant Otago 1 (GT-O1), closely related to the Kasuza strain originally sequenced by Kaneko et al. (1996) given the corrections of Tajima et al. (2011); and glucose tolerant Otago 2 (GT-O2), containing a number of additional single nucleotide polymorphisms resulting in mutations with unknown effects on physiology (see Table 4.1).

The wild-type background for the strains used in this study were therefore checked by PCR and sequencing to confirm if the genetic background belonged to either GT-O1 or GT-O2 (Dr. Simon Jackson, personal communication). These results are summarised in Table D.1. The majority of the strains used in this study, including the wild type intended to act as the control, are in the GT-O2 background.

The phenotype of the ∆Ycf48 strain is largely in agreement with that previously reported (Komenda et al., 2008) and this strain is in the GT-O1 background. The phenotype of Ycf48 was also similar to the GT-O2 wild type: especially in terms of whole cells absorption and oxygen evolution in the presence of bicarbonate. This suggests that one or more of the additional mutations in the GT-O2 strain may in fact give rise to a phenotype that is similar to the removal of Ycf48. One likely possibility is that the G195E mutation found in the ChlH subunit of magnesium-protoporphyrin IX chelatase may give rise to the observed phenotype of GT-O2. This is supported by the fact that the Ycf48 mutant was reported to have a reduced level of POR (Rengstl et al., 2013) and therefore both the ∆Ycf48 and the GT-O2 strains may have impaired Chl a biosynthesis. In addition, ChlH has previously been shown to act as an anti-sigma factor to SigE, whose regulons are involved in positive regulation of sugar catabolism (Osanai et al., 2009). It seems likely that the practice of maintaining strains in the presence of glucose has selected for this G195E mutation, facilitating constitutive expression of glucose catabolism pathway components.

The ∆PsbP strain is in the GT-O1 background and displays a phenotype largely indistinguishable from wild type, consistent with previous reports that found little or
no effect on removal of PsbP (Ishikawa et al., 2005; Summerfield et al., 2005). Differences between the wild-type and ΔPsbP strains were observed in whole cell absorption spectra and 77 K fluorescence emission spectra using a 580 nm excitation wavelength, both of which are normalised to Chl a-containing peaks. The GT-O2 wild type may have a reduced ability to produce Chl a. It is therefore possible that the phenotype of the ΔPsbP mutant arises from the comparison with the GT-O2 wild type and that in fact the ΔPsbP strain exhibits a similar absorption spectrum to GT-O1.

Likewise, the phenotype of the ΔPsb27 and ΔPsb27:ΔYcf48 mutants may originate, at least partially, from one or more of the mutations present in the GT-O2 strain in which they were constructed. That said, there is still some interplay of missing factors, that is revealed when Psb27 is absent that results in a large change in the PS II to PS I ratio. Similarly, the ΔPsb27:ΔYcf48 strain has a severe phenotype, it just remains to be established if another mutation or mutations, present in the GT-O2 strain, are required for, or the cause of, this phenotype.

The ΔPsb28, ΔPsb28-2, and ΔPsb28:ΔPsb28-2 strains are all in the GT-O2 strain. The severe phenotypes observed on the removal of Ycf48 from the ΔPsb28, ΔPsb28-2, and ΔPsb28:ΔPsb28-2 background strains, but not on removal of Ycf48 from a GT-O1 strain, argues that one or more of the mutations in the GT-O2 strain are required for this phenotype. The mutation to ChlH likely reduces the availability of Chl a. This, along with the reduced stability of D1 during processing in the absence of Ycf48, may be the cause of the phenotypes observed in the ΔPsb28:ΔYcf48, ΔPsb28-2:ΔYcf48, and ΔPsb28:ΔPsb28-2:ΔYcf48 strains.

### 4.7 Conclusions and future directions

As the GT-O2 strain has a number of mutations with potentially detrimental effects on physiology it would be informative to recreate the ΔPsb27:ΔYcf48, ΔPsb28:ΔYcf48, ΔPsb28-2:ΔYcf48, and ΔPsb28:ΔPsb28-2:ΔYcf48 strains with GT-O1 background strains. Given that all the plasmids required for such transformations are available (Table 2.1) this should be a relatively simple task. The observation that strains lacking either or both of the Psb28 and Psb28-2 assembly factors are equally impaired by the removal of Ycf48 makes it tempting to conclude that these phenotypes are due to one or more of the mutations present in the GT-O2 strain and being made ΔYcf48. As such, strains
ΔPsb28:ΔYcf48, ΔPsb28-2:ΔYcf48, and ΔPsb28:ΔPsb28-2:ΔYcf48 in a GT-O1 background would be expected to resemble the ΔYcf48 strain of this study.

Unlike the results of ΔPsb28 and ΔPsb28-2 series, the ΔPsb27 series revealed distinct phenotypes between the wild-type ΔPsb27, and ΔPsb27:ΔYcf48 strains, all of which are in the GT-O2 background (as well as between these strain and the GT-O1 ΔYcf48 strain). Removal of either Ycf48 or Psb27 resulted in strains capable of photoautotrophic growth. Whereas the ΔPsb27:ΔYcf48 strain is an obligate heterotroph. Recently, these differences were also found to be present in GT-O1 background variants of these mutants, representing a novel requirement for Ycf48 in ΔPsb27 cells, and Psb27 in ΔYcf48 cells (Jackson et al., 2014).

A number of the mutations present in the GT-O2 strain could be expected to influence photosynthetic processes. The generation of GT-O1 strains homozygous for individual mutations could be used to investigate such influences. In particular, it would be interesting to engineer a ChlH mutant line homozygous for the G195E mutation in the GT-O1 background as this may create a genetic background with reduced Chl a availability. Recently it has been reported that Chl a processing is coupled to photosystem assembly at the point of nascent protein membrane insertion, and that this coupling is reliant on a complex or series of complexes containing ScpE, ChlG, and sll0933 (Chidgey et al., 2014; Knoppova et al., 2014). PS II assembly is suggested to stall during D1 processing awaiting this Chl a incorporation. As such a ChlH mutant line homozygous for the G195E mutation may emphasise or reveal roles for the Ycf48, Psb27, Psb28, and Psb28-2 assembly factors.
Table 4.1: Mutations in background strains used in this study

<table>
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<th>No.</th>
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<th>Type</th>
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<th>GT-O2</th>
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<th>Annotation</th>
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<td>-</td>
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<td>—</td>
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<td>G</td>
<td>N37S</td>
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<td>Chr:Heterozygous</td>
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<td>T</td>
<td>C</td>
<td>C</td>
<td>None</td>
<td>Hypothetical protein - predicted to have transferase activity</td>
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<td>R641Q</td>
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<td>G195E</td>
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<td>A</td>
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<td>G</td>
<td>G</td>
<td>A</td>
<td>R65C</td>
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<td>T</td>
<td>T</td>
<td>A422T</td>
<td>flv3 - flavoprotein</td>
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† GT-K (GT-Kazusa) in GenBank database under accession number AP012205.


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Appendix A

Fluorescence Induction Protocol
$\mathcal{Q}_A^{-}$ Protocol
Figure A.2: Q\textsuperscript{-}A Protocol

Timing of Hashes

Setting Parameters
Appendix B
Figure B.1: M-13 Forward primer sequencing of the pAD-2034::specR plasmid.
Figure B.2: M-13 Reverse primer sequencing of the pAD-2034::specR plasmid.
Figure C.1: slr1055 sequencing for determination of background. Nucleotide at position 509098 used to differentiate between GT-O1 (G) and GT-O2 (A) strains. Difference from GT-K highlighted in red. The ΔYcf48 and ΔPsbP strains are normal with respect to GT-K. Wild type, the ΔPsb27, ΔPsb28, ΔPsb28-2, and the ΔPsb28:ΔPsb28-2 strains show mutations indicative of GT-O2.
Figure C.2: sll1428 sequencing for determination of background. Nucleotide at position 1581467 used to differentiate between GT-O1 (A) and pre-GT-O1 (G) strains. Difference from GT-K highlighted in red. None of the wild-type, ∆Ycf48, ∆Psb27, ∆Psb28, ∆Psb28-2, and ∆Psb28:∆Psb28-2 strains are shown to be pre-GT-O1.
Figure C.3: slr0750 sequencing for determination of background. Nucleotide at position 2422495 used to differentiate between GT-O1 (G) and GT-O2 (A) strains. Difference from GT-K highlighted in red. The ∆Ycf48 and ∆PsbP strains are normal with respect to GT-K. The wild-type, ∆Psb27, ∆Psb28, ∆Psb28-2, and ∆Psb28:∆Psb28-2 strains show mutations indicative of GT-O2.
Appendix D

Background of strains used in this study

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<th>Strain</th>
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<td>slr1055 (ChlH)</td>
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<td>G195E</td>
<td>Q115L</td>
</tr>
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<td>∆Ycf48&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Q115L</td>
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<td>∆Psb27</td>
<td>G195E</td>
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<td>G195E</td>
<td>Q115L</td>
</tr>
</tbody>
</table>

<sup>a</sup> The ∆Ycf48 strain was constructed in a GT-O1 background, whereas the wild type of this study is GT-O2.

<sup>b</sup> These strains were not checked by PCR and sequencing but their genotypes were inferred from the parent strain used to make these mutants.

<sup>c</sup> With change referring to difference from the GT-Kazusa strain in GenBank database under accession number AP012205. No change is indicated by ‘–’.

Table D.1: Backgrounds of strains used in this study
Appendix E
Removal of both Ycf48 and Psb27 in Synechocystis sp. PCC 6803 disrupts Photosystem II assembly and alters QA oxidation in the mature complex

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Synechocystis sp. PCC 6803
Ycf48

Abstract

The Photosystem II (PS II) assembly factors Psb27 and Ycf48 are transiently associated with PS II during its biogenesis and repair pathways. We investigated the function of these proteins by constructing knockout mutants in Synechocystis sp. PCC 6803. In ΔYcf48 cells, PS II electron transfer and stable oxygen evolution were perturbed. Additionally, Psb27 was required for photautotrophic growth of cells lacking Ycf48 and assembly beyond the RC47 assembly complex in ΔYcf48:ΔPsb27 cells was impaired. Our results suggest the RC47 complex formed in ΔYcf48 cells is defective and that this deficiency is exacerbated if CP43 binds in the absence of Psb27.

1. Introduction

Photosystem II (PS II) is a large multi-subunit pigment-protein complex which catalyzes the light-driven oxidation of water and reduction of plastoquinone to plastoquinol [1]. The PS II holoenzyme, located in the thylakoid membrane of oxygenic photoautotrophs, consists of at least 20 polypeptides incorporating a core complex containing the reaction center (RC) D1 and D2 proteins and the chlorophyll α-binding proximal antenna proteins CP43 and CP47 [2]. The heterodimeric RC formed by the D1 and D2 proteins binds the majority of the redox active co-factors including the catalytic Mn4CaO5 cluster of the oxygen-evolving complex. As a consequence of PS II activity the RC complex is subject to light-induced photodamage resulting in PS II undergoing a constant repair cycle to achieve sustained photosynthetic water splitting [3,4]. Both biogenesis and repair processes of PS II involve the coordinated assembly of many components and pre-assembly complexes [5]. Assembly is assisted by a complement of transiently-associated protein factors that are not found in the mature photosystem [6]. These additional proteins participate in stabilization of pre-assembly complexes and processing of precursor and intermediate forms of the D1 protein (pD1 and iD1, respectively). Two assembly proteins whose functions are not yet understood are Ycf48 and Psb27 [7].

The Arabidopsis thaliana homologue of Ycf48, HCF136, is indispensable for PS II assembly [8]. By comparison, a Synechocystis sp. PCC 6803 (hereafter Synechocystis 6803) Ycf48 knockout mutant displayed reduced PS II assembly and increased sensitivity to photoinhibition because the turnover and replacement of damaged D1 was impaired, suggesting a role for Ycf48 in repair processes [9]. The Ycf48 protein has been identified in isolated preparations of precursor PS II assembly complexes containing pD1 and iD1 up to the CP43-less assembly pre-complex (RC47) in both the so-called PratA-defined membrane (PDM) and thylakoid membrane systems [9,10]. Ycf48 is thought to interact directly with the C-terminal extension of D1 but can still bind in

Abbreviations: Bis–Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; BSA, bovine serum albumin; BME, blue measuring flashes; BN-PAGE, blue-native polyacrylamide gel electrophoresis; CP43, 43-kDa chlorophyll a-binding protein of the core antenna; CP47, 47-kDa chlorophyll a-binding protein of the core antenna; D1, Photosystem II reaction center protein subunit; D2, Photosystem II reaction center protein subunit; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ECL, enhanced chemiluminescence; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol

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the absence of the extension, albeit at reduced levels, suggesting this is not the only factor determining Ycf48 binding [9].

In contrast to Ycf48, there is no evidence for association of Psb27 with the PDM, instead it appears predominantly localized to the thylakoid lumen [10]. Removal of Psb27 from two cyanobacteria, *Thermoanaerobacterium thermosulfuricans* and *Synechocystis* 6803, resulted in mutants that exhibited reduced recovery from high-light-induced photodamage, a consequence of impairment of their PS II repair cycle [11–13]. The role of Psb27 in repair is supported by observations in an *A. thaliana* mutant [14].

Current evidence suggests that Psb27 interacts directly with the CP43 protein and may have a role in stabilizing this subunit as part of a CP43-containing pre-assembly complex prior to its amalgamation with the RC47 complex [13,15–18]. After binding of CP43 to the RC47 complex, but before formation of active PS II monomers or dimers, Psb27 is released allowing subsequent assembly of the MnaCaO2 cluster and binding of the extrinsic subunits [19–21]. In addition, the Psb27 protein is found associated with dimeric CP43-containing complexes specific to the PS II repair cycle that also lack the extrinsic subunits [12].

Given that both Ycf48 and Psb27 proteins bind pre-assembly or repair intermediates of PS II we hypothesized there might be a coordinated role for these subunits; particularly at the point of CP43 integration with RC47. To investigate this possibility we have studied the additive effect of removal of both Ycf48 and Psb27 in *Synechocystis* 6803, allowing us to evaluate their roles in formation and stabilization of PS II.

2. Materials and methods

2.1. Plasmid construction

To generate a construct for inactivation of the *ycf48* ORF ([slr02034] sequence and ligation independent cloning (SLIC) was used [22,23]. Overlapping PCR products corresponding to the upstream genomic region, a spectinomycin-resistance cassette (SpecR) [24], downstream genomic region and the pUC19 vector backbone were obtained. The primer pairs: (i) Ycf48_US_fwd and Ycf48_US_rev; (ii) Ycf48_SpecR_fwd and Ycf48_SpecR_rev; (i) Ycf48_DS_fwd and Ycf48_DS_rev, and (iv) Ycf48_pUC_fwd and Ycf48.pUC_rev, were used to generate these fragments, respectively ([Supplementary Table 1]). The fragments were subsequently combined in a single SLIC reaction to produce the plasmid pAYcf48::SpecR.

2.2. Growth of cyanobacterial strains

A sub-cultural derivative of the Williams glucose-tolerant strain of *Synechocystis* 6803, designated GT-01, is referred to throughout as wild type [25,26]. All strains were maintained on solid BG-11 plates (1.5% agar) supplemented with 5 mM glucose, 20 μM atrazine, 10 mM TES-NaOH (pH 8.2), 0.3% sodium thiosulfate and antibiotics (as below). Plates were stored at 30 °C under metal halide lamps at a light intensity of 10 μE m⁻² s⁻¹. Liquid cell cultures were grown mixotrophically in BG-11 containing 5 mM glucose at an illumination level of 50 μE m⁻² s⁻¹ with constant aeration in modified Erlenmeyer flasks according to Eaton-Rye [27]. Where appropriate, growth media contained spectinomycin at 25 μg mL⁻¹ and chloramphenicol at 15 μg mL⁻¹.

2.3. General pre-treatment of cells for physiological measurements

For all physiological characterizations the cells were grown mixotrophically in 300 mL of BG-11 in 500 mL flasks. For each given experiment, 50–100 mL of cells grown to an optical density at 730 nm (OD730) of between 0.8 and 1.2 (measured with a Jasco V-550 UV/Vis spectrophotometer; Jasco, International) were harvested by centrifugation at 2760 × g for 8 min. Cells were resuspended in BG-11 with 25 mM HEPES–NaOH (pH 7.5) to a chlorophyll a concentration of 5 μg mL⁻¹ (determined as in MacKinney [28]). Twenty milliliters of cell suspension was incubated in a 50 mL conical flask on an orbital shaker at 30 °C at a light intensity of 30 μE m⁻² s⁻¹. After a period of 30 min aliquots of cells were removed and used for the various physiological assays described. Where concentrations of chlorophyll a of less than 5 μg mL⁻¹ were specified the cells were diluted immediately prior to the measurements using additional BG-11 HEPES–NaOH (pH 7.5).

2.4. Whole cell absorption spectra

Whole cell absorption spectra were collected using a Jasco V-550 spectrophotometer at a cell density corresponding to an apparent OD at 800 nm of 0.3 with Scotch tape affixed in both the sample and reference optical paths. An absorption scan, against a reference sample of BG-11, was performed using a slit width of 1 nm.

2.5. Oxygen evolution assays

Oxygen evolution measurements were performed in 1 mL volumes using a Clarke-type electrode (Hansatech, UK) maintained at 30 °C by a recirculating water bath. Samples were measured at a chlorophyll a concentration of 5 μg mL⁻¹ in the presence of 0.2 mM 2,6-dichloro-1,4-benzoquinone (DCBQ) and 1 mM potassium ferricyanide (K₃[Fe(CN)₆]₂). Illumination of the samples was provided by an FL51 light source (Hansatech, UK) equipped with a 580 nm long-pass filter (Melles Griot, USA) and fiber optic cable assembly. The photon flux output at the chamber end of the cable was 8000 μE m⁻² s⁻¹. Data presented are for a minimum of five total replicates, gathered from at least three independent experiments. Rates were determined on the basis of the initial slope up to 30 s after the illumination began.

2.6. Low temperature (77 K) fluorescence emission spectra

For measurement of fluorescence emission at 77 K a modified MFP-3L fluorescence spectrometer (Perkin Elmer, USA) equipped with a custom made liquid nitrogen Dewar was used. Five hundred microliter samples of light-adapted cells at a chlorophyll a concentration of 2.5 μg mL⁻¹ were snap frozen, using liquid nitrogen, in glass tubes (6 mm outer diameter, 4 mm inner diameter). For data collected at an excitation peak of 440 nm the excitation and emission slit widths were set at 12 and 2 nm, respectively. At an excitation peak of 580 nm, slit widths of 8 and 2 nm were used. Emission spectra were collected at a scan rate of approximately 100 nm min⁻¹. For each strain 3–4 independent experiments, each consisting of two technical replicates were performed and data averaged. Normalization of the spectra were performed on the basis of baseline subtraction and normalization to the maxima of PS I emission at 725 nm.

2.7. Room temperature variable chlorophyll a fluorescence measurements

All room temperature variable chlorophyll a fluorescence measurements were made using a FL-3300 fluorometer (PSI instruments, Czech Republic) equipped with blue light emitting diodes (455 nm peak wavelength) for actinic illumination. Probe flashes employed either a blue measuring flash (BF, 455 nm) or red measuring flash (RFM, 625 nm), each with a 3 μs duration. Samples consisted of 2 mL of cells at a chlorophyll a concentration of 2 μg mL⁻¹.

and were dark adapted for 5 min prior to measurement. Where indicated, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added 2 min after commencement of dark adaptation at a final concentration of 50 μM. For the fluorescence induction protocol four BMF or RMF pulses spaced at 200 ms intervals were used to determine the Fs level before the blue actinic light (set at 50% intensity) was commenced. A logarithmic series of BMF or RMF was used to probe fluorescence induction in the 68 μs to 10 s timescale. For the fluorescence relaxation experiments a series of four BMF spaced at 200 ms intervals were used to determine the Fs level. This was followed 200 ms later by a 30 μs saturating actinic flash, and then a logarithmic series of BMF. The first data point used for analysis was 100 μs after the end of the actinic flash. For kinetic analyses of fluorescence relaxation in the absence of DCMU data up to 50 s were used, while in its presence the data sets were truncated to 10 s. Kinetic analyses were performed according to models previously described [29]. A correction was applied to account for the non-linear relationship between fluorescence and the QA oxidation state [30]. In the absence of DCMU, a correction was also applied for the oxidation of QA prior to the first data point measured.

2.8. Preparation of thylakoid membranes

Cells were grown as described for physiological experiments, harvested and re-suspended in isolation buffer (50 mM HEPES-NaOH (pH 7.2), 10 mM MgCl2, 5 mM CaCl2 and 1 M sucrose, 1 mM e-caproic acid, 1 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine). Cells were incubated on ice for 30 min in the dark before disruption using a Mini-Beadbeater (BioSpec Products, Inc., USA). Five cycles of bead beating (20 s each with 5 min rests on ice) were applied using a half volume of 0.1 mm diameter zirconia beads. Beads and unbroken cells were removed by centrifugation at 7500 g for 15 min. Samples containing 2 μg chlorophyll a equivalent of solubilized material were loaded on to precast 3–12% Bis-Tris gradient gels (Life Technologies, USA) and run at 4 °C. For western blotting and immunodetection the proteins were transferred to polyvinylidene difluoride membrane via electroblotting at 15 V for 3 h at 4 °C in buffer containing 25 mM Tris, 192 mM glycine, 10% v/v methanol and 0.05% w/v sodium dodecyl sulfate. Membranes were destained in methanol then blocked using 4% BSA. The primary antibodies for the PsbA (D1), PsbB (CP47) and PsbC (CP43) subunits were obtained from Agrisera, Sweden. The secondary antibody (Sigma, USA) was conjugated to peroxidase for detection using enhanced chemiluminescence (ECL). Detection was performed using a CCD detector system (Fuji imager PS3000) by incubating the membrane in freshly prepared ECL reagent (Abcam, UK).

3. Results

3.1. Construction and verification of mutants

The Synechocystis 6803 genome encodes one copy of a ycf48 homologue (slr2034). Transcript analysis suggests the slr2034 ORF is part of an operon with an upstream ORF encoding for a rubredoxin protein (RubA) [31,32]. In Synechocystis 6803 the tandem rubA-ycf48 genes are located immediately upstream of the psbEFLJ operon, a gene arrangement also found in many other cyanobacteria (Supplementary Table 2). To inactivate the ycf48 ORF a construct was designed to introduce a spectinomycin-resistance cassette at the equivalent of solubilized material were loaded on to precast 3–12% Bis–Tris gradient gels (Life Technologies, USA) and run at 4 °C. For western blotting and immunodetection the proteins were transferred to polyvinylidene difluoride membrane via electroblotting at 15 V for 3 h at 4 °C in buffer containing 25 mM Tris, 192 mM glycine, 10% v/v methanol and 0.05% w/v sodium dodecyl sulfate. Membranes were destained in methanol then blocked using 4% BSA. The primary antibodies for the PsbA (D1), PsbB (CP47) and PsbC (CP43) subunits were obtained from Agrisera, Sweden. The secondary antibody (Sigma, USA) was conjugated to peroxidase for detection using enhanced chemiluminescence (ECL). Detection was performed using a CCD detector system (Fuji imager PS3000) by incubating the membrane in freshly prepared ECL reagent (Abcam, UK).

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2.9. Blue-native polyacrylamide gel electrophoresis and Western blotting

Thylakoid membranes at a concentration of 0.5 mg chlorophyll a per mL were solubilized by incremental addition of 0.5 volumes of solubilization buffer containing 3% β-dodecyl maltoside (Anatrace, USA) (1/5 volume every 2 min) and incubated on ice for a further 10 min. Insoluble material was removed by centrifugation at 15000 × g for 15 min. Samples containing 2 μg chlorophyll a equivalent of solubilized material were loaded on to precast 3–12% Bis–Tris gradient gels (Life Technologies, USA) and run at 4 °C. For western blotting and immunodetection the proteins were transferred to polyvinylidene difluoride membrane via electroblotting at 15 V for 3 h at 4 °C in buffer containing 25 mM Tris, 192 mM glycine, 10% v/v methanol and 0.05% w/v sodium dodecyl sulfate. Membranes were destained in methanol then blocked using 4% BSA. The primary antibodies for the PsbA (D1), PsbB (CP47) and PsbC (CP43) subunits were obtained from Agrisera, Sweden. The secondary antibody (Sigma, USA) was conjugated to peroxidase for detection using enhanced chemiluminescence (ECL). Detection was performed using a CCD detector system (Fuji imager PS3000) by incubating the membrane in freshly prepared ECL reagent (Abcam, UK).

Fig. 1. Operon structure and segregation analysis. (A) Arrangement of the rubA and ycf48 genes and the psbEFLJ operon in Synechocystis 6803. (B) Confirmation of segregation by PCR for the inactivation of ycf48 by insertion of a 1.3-kb spectinomycin-resistance cassette (SpecR). (C) Confirmation of segregation by PCR for inactivation of psb27 by insertion of a 2.1-kb chloramphenicol-resistance cassette (CamR). Lanes in B and C are molecular weight marker (M), wild type (1), Δycf48 (2), ΔPsb27 (3) and Δycf48:ΔPsb27 (4).
an intragenic ClaI site using a plasmid developed by Bentley et al. [33]. This strategy allowed for the construction of both single ΔYcf48 and ΔPsb27 mutants and a ΔYcf48:ΔPsb27 double mutant. Colony PCR was performed to check integration and segregation of the constructs (Fig. 1B and C).

3.2. Photoautotrophic growth and oxygen evolution are blocked in the ΔYcf48:ΔPsb27 strain

Under the conditions tested, inactivation of the ycf48 ORF impaired photoautotrophic growth, increasing the doubling time from 11.5 h (wild type) to 21.7 h (ΔYcf48) (Fig. 2A). Removal of Psb27 only slightly reduced the photoautotrophic growth rate (13.8 h) compared to wild type. However, the removal of Psb27 from the ΔYcf48 strain resulted in severe loss of photoautotrophic growth capacity (exhibiting an initial doubling time of 35.5 h).

Removal of Ycf48 in the wild-type background reduced the saturated rate of steady-state oxygen evolution, measured in the presence of DCBQ and K3[Fe(CN)6], by approximately 52% (281 versus 592 μmol O2 per mg chlorophyll a per h for wild type) (Table 1). Removal of Psb27 in a ΔYcf48 strain retards photoautotrophic growth and oxygen evolution and modifies the whole-cell absorption spectrum. (A) Photoautotrophic growth curve determined by light scattering at 730 nm: wild type (solid squares), ΔYcf48 (open squares), ΔPsb27 (solid circles) and ΔYcf48:ΔPsb27 (open circles). Error bars represent the standard error from at least 3 independent experiments. (B) Oxygen evolution traces: wild type (i), ΔYcf48 (ii), ΔPsb27 (iii) and ΔYcf48:ΔPsb27 (iv). (C) Whole cell absorption spectra: wild type (solid line) and ΔYcf48 (dotted line). (D) Whole cell absorption spectra: ΔPsb27 (solid line) and ΔYcf48:ΔPsb27 (dotted line). In C and D, spectra are the average of three independent experiments and are normalized to the maxima at 435 nm.

<table>
<thead>
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<th>Table 1 Oxygen evolution rates. a</th>
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<tr>
<td>Wild type</td>
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<tr>
<td>ΔYcf48</td>
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<tr>
<td>ΔPsb27</td>
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<td>ΔYcf48:ΔPsb27</td>
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a Oxygen evolution was measured in the presence of DCBQ and K3[Fe(CN)6] as described in Section 2.

The ΔPsb27 strain was able to evolve oxygen at a comparable level to wild type, giving a rate of 585 μmol O2 per mg chlorophyll a per h. The depressed oxygen evolution capacity observed in the ΔYcf48 strain was compounded by removal of Psb27, resulting in a 72% reduction compared to wild type. Additionally, inactivation of ycf48 resulted in an apparent susceptibility to photoinactivation during the time course of the assay, again exacerbated by removal of the Psb27 subunit but not evident for the wild type or the Psb27 knockout strain (Fig. 2B).
During the growth curve measurements we noted a difference in the coloration of the strains lacking Ycf48 relative to wild type. To examine this further we analyzed the cellular pigment composition for each of the strains by room temperature absorption measurements. On a whole-cell basis the ΔYcf48 strain exhibited a small increase in the absorption region associated with carotenoids (475–520 nm) and a decrease in the absorption maxima at 625 and 680 nm (Fig. 2C). Aside from a minor decrease in the 625 nm peak there was no notable alteration in pigment content for the ΔPsb27 strain relative to wild type (Fig. 2C and D). However, in the absence of Ycf48 the effect of Psb27 removal resulted in large increases in the carotenoid absorption region and suppression of the 680 nm peak that originates from absorption by chlorophyll a (Fig. 2D).

3.3. Low temperature (77 K) fluorescence emission spectra

A reduction in the oxygen evolution capacity, referenced on a chlorophyll a basis, might be attributable to a change in the assembly levels of PS II. We therefore evaluated the ratio between PS II and PS I in each of the strains by performing low temperature (77 K) fluorescence emission experiments using an excitation beam with a peak wavelength of 440 nm that specifically targets chlorophyll a (Fig. 3A and B). When the spectra are normalized to the PS I maxima at 725 nm, inactivation of the ycf48 gene in the wild-type background resulted in a reduction of emission at both 685 and 695 nm and a small increase in the amplitude of the 685 nm peak relative to the 695 nm peak (Fig. 3A). These peaks at 685 and 695 nm are typically assigned to the CP43 and CP47 primary antenna subunits of PS II, respectively, but several other complexes might be responsible for the 685 nm emission peak: including, but not limited to, IsiA or unassembled CP43- or CP47-containing complexes [34,35]. For the ΔPsb27 strain the emission spectra showed a reduction in the amplitudes of both the 685 and 695 nm peaks, this time with the 685 nm peak more reduced compared to the 695 maxima (Fig. 3B). Inactivation of the psb27 gene in the Ycf48 knockout background induced substantial changes to the 77 K fluorescence emission spectra, including a large increase in the 685 nm peak and a concomitant reduction in the 695 nm shoulder (Fig. 3B).

Examination of 77 K fluorescence emission spectra collected at an excitation wavelength targeting phycobilisome pigments (580 nm) revealed differences in phycobilisome-coupled energy distribution for each of the mutant strains (Fig. 3C and D). Removal of Ycf48 in the wild-type background resulted in an increase in the spectral maxima observed at 680 nm, along with small reductions in the 649 and 664 nm peaks (Fig. 3C). For the ΔPsb27 strain the 680 nm peak was distinctly reduced compared to that in wild type, both in respect to its amplitude and relative level compared to the 649 and 664 nm peaks (Fig. 3C and D). For the ΔYcf48:ΔPsb27 strain the opposite effect was observed, whereby the 680 nm peak

![Fig. 3. 77 K fluorescence emission spectra.](image-url)
displayed a much higher emission than wild type or the parent ΔYcf48 strain.

3.4. Variable chlorophyll a fluorescence measurements

The charge separation and recombination characteristics of PS II centers in each strain were investigated with a series of experiments examining fluorescence induction and decay. Inactivation of ycf48 induced a significant reduction in variable fluorescence relative to wild type when measured in the presence or absence of DCMU with either BMF or RMF (Fig. 4A–D). The BMF examine the QA oxidation state by direct probing of chlorophyll fluorescence, while RMF provide phycobilisome-coupled fluorescence emission. Comparison of the two provides information on energy partitioning to complement the low temperature steady-state fluorescence data. It should be noted that the amplitudes of fluorescence presented are arbitrary and specific to the optical configuration of the fluorometer, in addition the relative amplitudes of data obtained with BMF versus RMF are not directly comparable as these are dependent on the flash intensity and gain settings.

For the ΔYcf48 strain the maximum amplitude of variable fluorescence ($F_v$, defined as $F – F_o$) was reduced to less than 50% of wild

**Fig. 4.** Room temperature fluorescence induction and relaxation of fluorescence following single-turnover actinic flashes. (A–D) Room temperature fluorescence induction observed upon illumination of dark-adapted cells with a constant actinic light. Strains are: wild type (solid), ΔYcf48 (dots), ΔPsb27 (dashes) and ΔYcf48:ΔPsb27 (dash-dots) in the absence (panels A and C) and presence (panels B and D) of DCMU. In all instances constant blue (455 nm) illumination was used with either blue (panels A and B) or red (625 nm) measuring flashes. The time scale is relative to the commencement of illumination. For clarity the x-axis in panels C and D is displayed equivalent to minus 0.2 on the y-axis, the actual zero $F – F_o$ level is shown with a dotted horizontal line. (E) Relaxation of fluorescence following single-turnover actinic flashes in the absence of DCMU. (F) Relaxation of fluorescence following single-turnover actinic flashes in the presence of DCMU. In panels E and F the strains are: wild type (solid), ΔYcf48 (dots), ΔPsb27 (dashes) and ΔYcf48:ΔPsb27 (dash-dots). Data displayed are for the average of at least 3 independent experiments.
type when accessed using both BMF and RMF. The ΔPsb27 strain exhibited similar fluorescence induction characteristics to wild type for all conditions examined but inactivation of the psb27 gene in the absence of Ycf48 exacerbated the depression of variable fluorescence observed for the ΔYcf48 strain. In addition, the characteristic J–P rise [38], observed in the absence of DCMU, was lost in the ΔYcf48:ΔPsb27 strain. The amplitude of the fluorescence in the P region was observed to change with addition of DCMU and interestingly for wild type as well as the ΔPsb27 strain, the fluorescence yield was similar or reduced in the presence of DCMU while in the ΔYcf48 and ΔYcf48:ΔPsb27 strains the yield was increased by addition of DCMU.

In all strains the fluorescence observed using RMF decreased after the P peak was reached but for the ΔYcf48:ΔPsb27 strain the reduction was accelerated, with the fluorescence dropping below the initial $F_o$ level; equivalent to zero on the $y$-axis for the $F$–$F_o$ data displayed. The $F_o$ data cannot be displayed on these figures as they occur prior to the start of the time-scale (x) axis (referred to the activation of actinic illumination) but are presented in Table 2. No appreciable difference between the $F_o$ for all strains was observed using BMF but when using RMF the $F_o$ level was decreased in ΔPsb27 and considerably elevated in the ΔYcf48: ΔPsb27 strain. An increase in $F_o$ would reduce the $F_o$/$F_m$ parameter that is classically quoted to illustrate PS II functionality but the underlying cause of the $F_o$ increase in the double mutant might be independent of PS II. Therefore we have presented $F$–$F_o$ during the time course of the assay as a more accurate representation of PS II activity, particularly given the observation that toward the end of the measurements $F$ drops below the initial $F_o$ in our data. This effect has previously been linked to alterations in PBS coupling during the fluorescence induction assay [37].

The decay of variable fluorescence following a single turnover saturating flash (455 nm) was measured using a BMF in the absence and presence of DCMU (Fig. 4E and F). For each of the strains the decay kinetics corresponding to the reoxidation of $Q_X$ were analyzed and these are summarized in Table 3. In the absence of DCMU the ΔYcf48 strain exhibited a similar fast phase to wild type but showed a decreased rate for the middle phase and increased rate and amplitude for the slow phase. For the ΔPsb27 strain the kinetics of $Q_X$ oxidation observed were similar to wild type. The ΔYcf48:ΔPsb27 strain showed a large increase in the rate of the fast phase but with slightly reduced amplitude compared to wild type. The decrease in the rate of the middle phase observed in ΔYcf48 was reversed by removal of Psb27, resulting in a faster rate than wild type but the increase in the rate and amplitude of the slow phase was retained.

In the presence of DCMU the oxidation of $Q_X$ for wild type was dominated by a slow phase (modeled as a hyperbolic component) with a rate constant of ~1.4 s$^{-1}$, with an additional exponential component (fast phase) present at negligible levels (Table 2). For the ΔPsb27 strain the slow component also dominated, with only a minor increase in the fast phase compared to wild type. For the ΔYcf48 strain the amplitude of the fast component increased to 7.4 ± 1.0%, apparent as a small increase in the initial decay of the fluorescence relaxation curve (Fig. 4F). The ΔYcf48:ΔPsb27 strain exhibited a large increase in the initial relaxation of fluorescence, corresponding with an approximately fourfold increase in the rate, and fivefold increase in the amplitude, of the fast kinetic component of $Q_X$ oxidation compared to wild type.

### Table 2

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<th>BMF (a.u.)</th>
<th>RMF (a.u.)</th>
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<tr>
<td>Wild type</td>
<td>0.501 ± 0.027</td>
<td>0.734 ± 0.050</td>
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<tr>
<td>ΔYcf48</td>
<td>0.495 ± 0.017</td>
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<tr>
<td>ΔPsb27</td>
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<td>0.509 ± 0.021</td>
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<td>ΔYcf48:ΔPsb27</td>
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*a The amplitudes between BMF and RMF are not directly comparable. Data shown were collected in the absence of DCMU. No significant difference was observed in its presence.*

### 3.5. Assembly of PS II complexes

Assembly of PS II complexes was analyzed by blue-native polyacrylamide gel electrophoresis (BN-PAGE) followed by western blotting (Fig. 5). The removal of Ycf48 did not preclude assembly of the three predominant macromolecular PS II complexes found in wild type (dimers, monomers and CP43-less monomers) but the level of assembly, particularly of the dimer complex, was reduced. In contrast, removal of the Psb27 subunit did not appear to impair assembly relative to wild type. However, the double mutant lacking both Ycf48 and Psb27 was largely unable to assemble beyond the RC47 (CP43-less monomer) stage. Furthermore, there was an enhanced accumulation of low-molecular-weight complexes which showed reactivity with the α-CP43 antibody in the ΔYcf48:ΔPsb27 strain.

### 4. Discussion

#### 4.1. A requirement for Psb27 in the absence of Ycf48

Inactivation of either ycf48 or psb27 in Synechocystis 6803 does not prevent photoautotrophic growth and for the wild-type and ΔPsb27 strains similar phenotypes are found when physiological measurements are performed on whole cells grown in BG-11 medium [9,13,19,33]. However, the combination of high light and low temperature has been shown to prevent photoautotrophic growth of a T. elongatus ΔPsb27 mutant [12].

Bentley et al. [33] reported that a ΔPsbM:ΔPsb27 double mutant was an obligate phototroph; however, we have recently sequenced the entire genome of this strain and identified a spontaneous mutation in the second transmembrane helix of

### Table 3

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<tr>
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<th>Fast phase ($t_{1/2}$/amplitude)</th>
<th>Middle phase ($t_{1/2}$/amplitude)</th>
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<td>No addition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>6.7 ms (±0.2)/26% (±1.2)</td>
<td>7.7 ms (±0.5)/27% (±0.6)</td>
<td>5.2 ms (±0.5)/26% (±1.1)</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>7.5 ms (±0.3)/33% (±2.7)</td>
<td>8.3 s (±1.9)/13% (±0.8)</td>
<td>8.5 s (±1.9)/13% (±0.8)</td>
</tr>
<tr>
<td>ΔPsb27</td>
<td>8.4 s (±1.9)/13% (±0.5)</td>
<td>9.2 s (±1.9)/13% (±0.5)</td>
<td>9.2 s (±1.9)/13% (±0.5)</td>
</tr>
<tr>
<td>ΔYcf48:ΔPsb27</td>
<td>9.2 s (±1.9)/13% (±0.5)</td>
<td>9.2 s (±1.9)/13% (±0.5)</td>
<td>9.2 s (±1.9)/13% (±0.5)</td>
</tr>
<tr>
<td>with DCMU</td>
<td>1.36 s (±0.10)/97.4% (±0.2)</td>
<td>1.55 s (±0.08)/92.6% (±1.0)</td>
<td>1.55 s (±0.08)/92.6% (±1.0)</td>
</tr>
<tr>
<td>Wild type</td>
<td>6.7 ms (±0.8)/33% (±2.7)</td>
<td>1.27 s (±0.06)/96.1% (±0.2)</td>
<td>1.70 s (±0.10)/85.1% (±1.5)</td>
</tr>
<tr>
<td>ΔYcf48</td>
<td>6.7 ms (±0.8)/33% (±2.7)</td>
<td>1.27 s (±0.06)/96.1% (±0.2)</td>
<td>1.70 s (±0.10)/85.1% (±1.5)</td>
</tr>
<tr>
<td>ΔPsb27</td>
<td>6.7 ms (±0.8)/33% (±2.7)</td>
<td>1.27 s (±0.06)/96.1% (±0.2)</td>
<td>1.70 s (±0.10)/85.1% (±1.5)</td>
</tr>
<tr>
<td>ΔYcf48:ΔPsb27</td>
<td>6.7 ms (±0.8)/33% (±2.7)</td>
<td>1.27 s (±0.06)/96.1% (±0.2)</td>
<td>1.70 s (±0.10)/85.1% (±1.5)</td>
</tr>
</tbody>
</table>

*a Kinetic analysis was performed on corrected fluorescence relaxation curves following a saturating single turnover flash in the absence or presence of DCMU as described in Section 2.*
CP43 (Gly116 to Asp substitution) in the original ΔPsbM:ΔPsb27 mutant; a new ΔPsbM:ΔPsb27 strain created without this mutation is capable of photoautotrophic growth (data not shown). Hence the requirement of Psb27 in the ΔYcf48 strain in Fig. 2 presents a novel observation where the Psb27 protein is essential to support sustained photoautotrophic growth and oxygen evolution in Synechocystis 6803 under standard growth conditions.

4.2. Is there an assembly bottleneck in the ΔYcf48:ΔPsb27 mutant?

The impaired growth of the ΔYcf48 strain correlated with a reduced level of PS II assembly as seen by the increased PS I/PS II ratio in Fig. 3A, a reduced PS II specific variable fluorescence (Fv/Fm) in Fig. 4A and B and the diminished levels of PS II monomers and dimers detected in Fig. 5. Likewise PS II assembly is severely restricted in the ΔYcf48:ΔPsb27 double mutant. In addition, the reduced levels of assembled PS II in both strains is accompanied by these mutants accumulating sub-complexes containing CP43 and CP47 (Fig. 5C and D). Isolated early assembly sub-complexes containing CP43 or CP47 have previously been shown to both exhibit 77 K fluorescence emission at a peak of 685 nm which might explain the increase in this peak observed in the ΔYcf48:ΔPsb27 cells [35].

Ycf48 has been shown to be associated with both an early assembly complex containing pD1 and a D1/D2 assembly intermediate (RCII) complex which goes on to form RC47 by combining with the CP47 pre-complex [9,38,39]. At this stage the Ycf48 protein is believed to have dissociated and a CP43 pre-assembly complex containing Psb27 binds to give an inactive monomeric PS II Ycf48 double mutant. A previously reported double mutant lacking both the Ycf48 and sll0933 assembly factors displayed phenotypes dominant by the absence of Ycf48; if the impairment of CP43 incorporation into RC47 is currently thought to be after the action and dissociation of Ycf48; if the impairment of CP43 incorporation into RC47 resulted wholly from the absence of Psb27 then the ΔPsb27 strain would show reduced PS II assembly. As such, our observations imply a direct interaction between complexes containing Ycf48 with those containing Psb27.

In this study cells were grown under a continuous light regime and characterized during the logarithmic phase of growth. Therefore the PS II pool will be a mixed population with a portion representing de novo biogenesis and the remainder in constant flux between mature functioning centers and those undergoing repair following photodamage. In the PS II repair cycle, CP47, along with several other subunits, remains associated with the core D2-cytochrome b559 component of RCII while newly synthesized D1 is incorporated (reviewed in Mabbitt et al. [7]). Hence the Psb27-containing CP43 subunit is incorporated directly after D1 addition without the intermediate step of CP47 binding. This process, along with D1 processing, may be partially coordinated by the Ycf48 and Psb27 assembly factors.

4.3. Centers formed in the ΔYcf48 and ΔYcf48:ΔPsb27 mutants are functionally distinct

The ΔYcf48 and ΔYcf48:ΔPsb27 strains remained capable of evolving oxygen, albeit at reduced rates, indicating that assembly can proceed beyond the ”bottleneck” in the biogenesis and repair pathways. Even in the double mutant the initial rate of PS II-specific oxygen evolution supported by DCMO was ~28% of the rates observed in wild type or the ΔPsb27 strain; however, the activity rapidly decreased during the assay suggesting an enhanced sensitivity to photoactivation. One possible explanation is that the PS II centers are receiving too much light as a result of an increased antenna to PS II center ratio. We explored this possibility by investigating phycobilisome connectivity in the 580 nm fluorescence emission spectra (Fig. 3D). The increased emission from PBS specific fluorophores suggests that many phycobilisomes are not coupled to photosystem centers (PS I or PS II) in the ΔYcf48:ΔPsb27 strain, thereby contradicting this hypothesis.

To investigate if the susceptibility to photoactivation arose from functional impairment of PS II centers we examined the effect of a single turnover flash on PS II electron transfer. In ΔYcf48 cells there was an increase in the millisecond component suggesting an altered Qa site [42]. Moreover, in the ΔYcf48:ΔPsb27 strain an enhanced millisecond component associated with Qa oxidation was observed in the presence of DCMO that appeared to “compete” with the slowed millisecond component in the single ΔYcf48 strain detected in the absence of DCMO (Table 3). This may indicate that side-path electron transfer through cytochrome b559, potentially involving β-carotene and chlorophyll Z on D2, is active in assembled PS II centers formed in the absence of Ycf48 and Psb27 [43]. Alternatively, there may be enhanced recombination with P680”. These observations suggest that although PS II assembly can proceed beyond the assembly bottleneck in these mutants the resulting centers are in fact compromised and might possibly be more susceptible to photodamage. This increased light sensitivity has previously been demonstrated for Ycf48 knockout strains and linked to photoinactivation [9,41]. Hence the inability of the double mutant to sustain photoautotrophic growth may arise from both an increased susceptibility to photodamage and a retarded assembly pathway during both biogenesis and repair.

In support of this theory is the accumulation of pigments absorbing in the carotenoid region of the whole cell absorption
spectra (Fig. 2C and D). Carotenoids, which play an important role in photoprotection of PS II, are found not only in mature centers but also in early assembly complexes of CP43 and CP47 [35]. Interestingly the orange carotenoid protein (OCP), a carotenoid-containing protein complex involved in photoprotection, absorbs in the same region with a maximal absorbance peak at 510 nm for the active form [44]. Furthermore, OCP is known to associate with phycobilisomes, pointing to coordination between phycobilisome coupling, regulation of photoprotection and energy distribution during the early stages of PS II biogenesis and repair.

4.4. Concluding remarks

While removal of Psb27 alone does not readily impair PS II performance in Synechocystis 6803, a clear role for Psb27 is observed in the absence of Ycf48. This requirement for Psb27 may be heightened in vivo if environmental factors adversely affect the production of D1 during recovery from photodamage. Although Ycf48 is not absolutely required for PS II biogenesis the centers formed in the absence of Ycf48 are compromised and the extent of this disruption appears to be partly compensated for by the presence of Psb27.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.08.024.

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