Investigation of the role of His-469 in the chlorophyll-binding protein, CP47 of Photosystem II

Shiny Sara Varghese

A thesis submitted for the degree of
Master of Science
at the University of Otago, Dunedin,
New Zealand
February, 2015
Abstract

CP47 is a chlorophyll-binding protein of the proximal antenna system of Photosystem II containing 16 chlorophyll molecules. Twelve histidine residues found within the transmembrane α-helices I-VI of CP47 serve as axial ligands for the magnesium ion of these chlorophyll molecules and a role for histidyl ligation of chlorophyll has been supported by various site-directed mutagenesis studies. Successful substitution of these histidine residues with either Asn or Gln within the transmembrane helices I-VI has been observed but replacement of His-469 with a glutamine residue within the helix VI of the CP47 protein resulted in a major reduction in the amount of assembled PS II reaction centres and photoautotrophic growth was retarded. A pseudorevertant for the strain carrying the His to Gln substitution (H469Qrev) was able to grow photoautotrophically at a rate similar to wild type but the secondary mutation was not present in the psbB gene that encodes CP47. Moreover, the introduction of Ala, Lys, Pro and Tyr at the 469 position resulted in obligate heterotrophic strains with reduced photoautotrophic growth and unassembled PS II centres. The current study was carried out to understand the role of the His-469 residue in CP47 and also to identify the gene responsible for the secondary mutation in the H469Qrev pseudorevertant. All strains carrying substitutions for His-469 (H469A, H469Arev, H469K, H469Krev, H469P, H469Prev, H469Q, H469Qrev, H469Y and H469Yrev) were studied using a range of physiological and biochemical measurements. Mutants created in the wild-type background exhibited loss of photoautotrophic growth with concomitant reduction in the oxygen evolution rates and reduced number of assembled PS II centres. However, physiological characterisation of the strains in the genetic background of the H469Qrev pseudorevertant resulted in a range of phenotypes showing varied levels of recovery when compared to their corresponding mutants in the wild-type background. Furthermore, the search for secondary mutations in the H469Qrev pseudorevertant in turn revealed seven unique gene mutations. Introduction of these mutations into the very impaired H469Y cells did not transform this strain but during these studies, a new pseudorevertant was isolated (H469Yrev+6). The new pseudorevertant did not contain any of the mutations identified in the H469Qrev strain but exhibited increased photoautotrophic growth and oxygen evolution rates at a comparable level to the wild-type strain. Additionally, the new suppressor present in the H469Yrev+6 strain was able to restore PS II activity to H469A, H469K and H469P cells but not the H469Q and H469Y strains.
Acknowledgements

This study would not have been possible without the support of many people. I take this opportunity to express my heartfelt gratitude to my supervisor, Prof. Julian Eaton-Rye, for giving me his time and excellent guidance as well as the encouragement throughout the study. I would like to take this instance to extend my sincere thanks to Jackie Daniels, for her guidance and mentoring.

A big thank you to all the members of the Lab 308 for their support and assistance in my studies. A special mention to Dr. Simon Jackson and Tim Crawford for their continuous technical assistance and also all the members of Department of Biochemistry.

Last, but not least, I would like to thank my husband and daughter for their endless support and patience throughout my research.
Contents

Abstract iii
Acknowledgements v
Contents vii
List of Tables xi
List of Figures xiii
Abbreviations xvii

1 Introduction 1
  1.1 Photosynthesis 1
  1.2 Photosystem II 2
    1.2.1 Structure, composition and function 2
    1.2.2 Biogenesis of PS II 6
    1.2.3 PS II Repair 7
  1.3 Chlorophyll a-binding protein, CP47 8
    1.3.1 Histidine residues in CP47 8
    1.3.2 Substitution of Histidine residues within helix VI of CP47 9
      by Glutamine
  1.4 Experimental Model: *Synechocystis* sp. PCC 6803 10
  1.5 Objectives 11
    1.5.1 Physiological characterisation of strains carrying substitutions 11
      for His-469
    1.5.2 Identification of secondary mutations in pseudorevertants 11
      retaining the introduced mutations at position 469

2 Experimental Methods 13
  2.1 Methods 13
    2.1.1 General 13
    2.1.2 *Synechocystis* sp. PCC 6803 strains 13
    2.1.3 Media and Growth Settings: *Synechocystis* sp. PCC 6803 13
    2.1.4 Genomic DNA Extraction 15
<table>
<thead>
<tr>
<th>Section Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.5 Polymerase Chain Reaction (PCR)</td>
<td>16</td>
</tr>
<tr>
<td>2.1.6 Gel Electrophoresis</td>
<td>18</td>
</tr>
<tr>
<td>2.1.7 DNA Sequencing</td>
<td>18</td>
</tr>
<tr>
<td>2.2 Physiological Studies</td>
<td>18</td>
</tr>
<tr>
<td>2.2.1 Photoautotrophic Growth Curves</td>
<td>18</td>
</tr>
<tr>
<td>2.2.2 Chlorophyll Analysis</td>
<td>19</td>
</tr>
<tr>
<td>2.2.3 Oxygen Evolution</td>
<td>19</td>
</tr>
<tr>
<td>2.2.4 $77$ K Fluorescence Emission Studies</td>
<td>20</td>
</tr>
<tr>
<td>2.2.5 Fluorescence Induction</td>
<td>20</td>
</tr>
<tr>
<td>2.2.6 Thylakoid Extraction and Solubilisation</td>
<td>21</td>
</tr>
<tr>
<td>2.2.7 Blue-Native PAGE</td>
<td>22</td>
</tr>
<tr>
<td>2.2.8 Enhanced Chemiluminescence</td>
<td>22</td>
</tr>
<tr>
<td>2.3 Management of <em>Escherichia coli</em> Strains</td>
<td>23</td>
</tr>
<tr>
<td>2.3.1 Strains and Growth Conditions</td>
<td>23</td>
</tr>
<tr>
<td>2.3.2 Preparation of Competent Cells</td>
<td>23</td>
</tr>
<tr>
<td>2.3.3 Heat-Shock Transformation</td>
<td>23</td>
</tr>
<tr>
<td>2.3.4 Plasmid Preparation</td>
<td>24</td>
</tr>
<tr>
<td>2.3.5 Restriction Digest</td>
<td>24</td>
</tr>
<tr>
<td>2.3.6 Cyanobacterial Transformation</td>
<td>24</td>
</tr>
<tr>
<td>2.3.7 Dot Transformation</td>
<td>25</td>
</tr>
<tr>
<td>3 Results</td>
<td>27</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>27</td>
</tr>
<tr>
<td>3.2 Verification of Strains</td>
<td>29</td>
</tr>
<tr>
<td>3.3 Physiological characteristics of mutants carrying amino acid substitutions at position 469 in CP47</td>
<td>29</td>
</tr>
<tr>
<td>3.3.1 Photoautotrophic Growth Curves</td>
<td>30</td>
</tr>
<tr>
<td>3.3.2 Oxygen Evolution</td>
<td>32</td>
</tr>
<tr>
<td>3.3.3 $77$ K Fluorescence Emission Studies</td>
<td>35</td>
</tr>
<tr>
<td>3.3.4 Fluorescence Induction</td>
<td>37</td>
</tr>
<tr>
<td>3.3.5 Detection of PS II assembly by Blue –Native PAGE</td>
<td>42</td>
</tr>
<tr>
<td>3.4 Identification of secondary mutation in pseudorevertant H469Qrev</td>
<td>44</td>
</tr>
<tr>
<td>3.4.1 Physiological characterisation of H469Yrev*$^{6}$</td>
<td>49</td>
</tr>
<tr>
<td>3.4.1.1 Photoautotrophic Growth Curve</td>
<td>50</td>
</tr>
</tbody>
</table>
3.4.1.2 Oxygen Evolution 50
3.4.1.3 77 K Fluorescence Emission 52
3.4.1.4 Fluorescence Induction 54
3.4.2 Dot Transformation 57

4 Discussion 59
  4.1 Physiological characteristics of mutants carrying amino acid substitutions at position 469 in CP47 60
  4.2 Identification of secondary mutation in pseudorevertant H469Qrev strain 65
  4.3 Conclusions and Future prospects 68

References 71

Appendix 79
List of Tables

2.1 List of *Synechocystis* sp. PCC 6803 strains used in the study ..........................14

2.2 List of oligonucleotides used in the study ......................................................17

3.1 Oxygen evolution rates of mutants created in the wild-type background and in the genetic background of H469Qrev pseudorevertant. Data are the average of at least two independent experiments (or at least 4 measurements) ± the standard error.........................31

3.2 Characteristics of 7 gene mutations in pseudorevertant H469Qrev .......................44

3.3 Oxygen evolution rates for wild type, H469Y, H469Yrev, H469Yrev<sup>+</sup>6 and H469Yrev<sup>+</sup>7 strains. Data are the average of at least two independent experiments (or at least 4 measurements) ± the standard error.................................................................50
List of Figures

1.1 Light-induced electron and proton transport reactions of oxygenic photosynthesis in cyanobacteria. Arrows representing the flow of electrons and protons are shown........1
1.2 1.9 Å crystal structure of photosystem II in a cyanobacteria isolated from the thermophilic cyanobacterium Thermosynechococcus vulcanus.........................3
1.3 Schematic illustration of PS II monomer in cyanobacteria. Adapted from Govindjee et al., 2010.................................................................4
1.4 Biogenesis of PS II in cyanobacteria. Adapted from Mabbit et al. (2014). Sequential attachment of preformed PS II sub-complexes are shown leading to formation of the active PS II dimer.................................................................5
1.5 Topological model of chlorophyll-binding protein, CP47....................................7
2.1 Modified Erlenmeyer flasks used for growing liquid cultures of the strains used for different physiological studies. ........................................................................15
3.1 Gel electrophoresis showing the PCR products obtained for confirming the mutations in psbB.................................................................27
3.2 Genomic DNA sequence of the psbB gene in the H469A, H469Arev, H469K, H469Krev, H469P, H469Prev, H469Q, H469Qrev, H469Y and H469Yrev strains as compared with the control .................................................................28
3.3 Photoautotrophic growth curves measured at 730 nm, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains ........................................................................30
3.4 Oxygen evolution traces measured in the presence of DCBQ and K₃Fe(CN)₆, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains .................................................................32
3.5 Oxygen evolution traces obtained in the presence of bicarbonate, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev, and d) psbB control, H469Y and H469Yrev strains .................................................................33
3.6 77 K fluorescence emission spectra obtained with 440 nm excitation and normalised to the PS I emission at 725 nm, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains

3.7 77 K fluorescence emission spectra obtained with 580 nm excitation and normalised to the PS I emission at 725 nm, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains

3.8 Fluorescence Induction Curves measured after illuminating dark-adapted cells with a constant actinic blue measuring light, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains

3.9 Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic blue measuring light in the presence of DCMU, wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains

3.10 Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic red measuring light, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains

3.11 Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic red measuring light in the presence of DCMU, wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains

3.12 PS II assembly. Solubilized thylakoids run on BN-PAGE gel (1) followed by western blotting for antibodies D1 (2), CP47 (3) and CP43 (4)

3.13 Gel electrophoresis showing PCR-derived products from either the H469Q or the H469Qrev strains for each of the 7 mutated genes identified by genomic sequencing. A) H469Q and B) H469Qrev DNA fragments
3.14 Genomic DNA sequence of the 7 genes carrying mutations in pseudorevertant H469Qrev .................................................................46
3.15 Restriction digest of plasmids containing the PCR-generated inserts corresponding to the genes with unique mutations in the H469Qrev strain and plasmid map of slr1055 showing the restriction site used in the corresponding digest shown in lane 1 in panel A ............................................................47
3.16 Cyanobacterial transformation of the H469Y strain .................................48
3.17 Photoautotrophic Growth curves measured at 730 nm from wild-type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains ........................................49
3.18 Oxygen evolution traces measured in the presence of DCBQ and K₃Fe(CN)₆ from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains .........................51
3.19 Oxygen evolution traces measured in the presence of bicarbonate from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains .........................52
3.20 77 K fluorescence emission spectra obtained with 440 nm excitation and normalised to the PS I emission at 725 nm from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains ..................................................53
3.21 77K fluorescence emission spectra obtained with 580 nm excitation and normalised to the PS I emission at 725 nm from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains ..................................................54
3.22 Fluorescence Induction Curves measured after illuminating dark-adapted cells with a) constant actinic blue measuring light and b) presence of DCMU from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains .................................................55
3.23 Fluorescence Induction Curves measured after illuminating dark-adapted cells with a) constant actinic red measuring light and b) presence of DCMU from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains .................................................56
3.24 Dot transformation of the genomic DNA from the H469Yrev+6 strain on other strains carrying a substitution for His-469. 1) H469A, 2) H469K, 3) H469P, 4) H469Q and 5) H469Y. 10 µL of ~1 µg of genomic DNA from the H469Yrev+6 strain was applied to the lawn of the other His-469 mutants embedded in 0.8% agar ........................................57
4.1 Organisation of histidine residues and their corresponding chlorophyll molecules located in the transmembrane α-helices VI of CP47 .................................................64
4.2 Genomic DNA sequence of the 6 genes introduced into the H469Yrev+6 strain that were originally found in the genomic DNA of H469Qrev pseudorevertant ......................67
Abbreviations

Å  Angstrom
Ala  Alanine
APC  Allophycocyanin
α  Alpha
BMF  Blue measuring flash
BN-PAGE  Blue-native polyacrylamide gel
bp  Base pair
BSA  Bovine serum albumin
Chl  Chlorophyll
CTAB  Hexdecyl trimethyl ammonium bromide
CtpA  C-terminal processing A
Cyt b₆f  cytochrome b₆f
Cyt b₅₅₉  Cytochrome b₅₅₉
DCBQ  2, 6-dichloro-1, 4-benzoquinone
DCMU  3, 4-dichloro-1, 1-dimethyl urea
Fd  Ferrodoxin
FNR  Ferrodoxin-NADP reductase
g  Gravitational force
Gln  Glutamine
GTO1  Glucose tolerant Otago 1
His  Histidine
kDa  kiloDalton
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>Mn4O7Ca</td>
<td>Manganese-oxygen-calcium cluster</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phycobilisomes</td>
</tr>
<tr>
<td>PC</td>
<td>Plastocyanin</td>
</tr>
<tr>
<td>PCC</td>
<td>Pasteur culture collection</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pheo</td>
<td>Pheophytin</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PQH2</td>
<td>Plastoquinol</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>PS I</td>
<td>Photosystem I</td>
</tr>
<tr>
<td>PS II</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>RC</td>
<td>Reaction centre</td>
</tr>
<tr>
<td>RMF</td>
<td>Red measuring flash</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA (buffer)</td>
</tr>
<tr>
<td>TfBI</td>
<td>Transformation buffer I</td>
</tr>
<tr>
<td>TfBII</td>
<td>Transformation buffer II</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
</tbody>
</table>
Tyr  Tyrosine
w/v  weight to volume
YZ   D1 tyrosine 161
YD   D2, tyrosine residues
µ    Micro (10^6)
Chapter 1: Introduction

1.1 Photosynthesis

Photosynthesis utilises solar energy to store chemical energy in chemical bonds in ATP and NADPH that is then available to generate carbohydrates from carbon dioxide and water; these reactions also result in the production of molecular oxygen as a byproduct of the splitting of water. The overall reaction is summarised as below

$$6\text{CO}_2 + 6\text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2$$

This photosynthetic process involves two independent series of reactions: (1) the light or thylakoid reactions, and (2) the dark or carbon fixation reactions. The light reactions occur in the specialised internal membranes of chloroplasts (or cyanobacterial cells) known as thylakoids and require two photosystems, Photosystem I (PS I) and Photosystem II (PS II), acting in series and linked by the cytochrome $b_{6f}$ (Cyt $b_{6f}$)

![Diagram of Photosynthesis](image)

Figure 1.1 Light-induced electron and proton transport reactions of oxygenic photosynthesis in cyanobacteria. Arrows representing the flow of electrons and protons are shown. Abbreviations: PS II, photosystem II; QA, primary plastoquinone electron acceptor; QB, secondary plastoquinone electron acceptor; P680, chlorophyll reaction centre of PS II; PQ, plastoquinone; PQH$_2$, plastoquinol; PC, plastocyanin; P700, chlorophyll reaction centre of PSI; Fd, ferrodoxin and FNR, ferrodoxin-NADP reductase.
complex and additional mobile electron carriers (Whitmarsh and Govindjee, 1999). In cyanobacteria, light is harvested by antenna complexes called phycobilisomes, present on the cytosolic side of the thylakoid membrane, and these light-harvesting complexes direct the light energy into the chlorophyll reaction centre of each photosystem — i.e., P680 and P700 in PS II and PS I, respectively (Grossman et al., 1993). Absorption of photons by PS II results in the reduction of the primary plastoquinone electron acceptor, QA and then the electron is transferred to the secondary plastoquinone electron acceptor, QB. After two electrons have been transferred fully reduced, plastoquinol (PQH2) is formed, which diffuses through the thylakoid membrane to reduce the Cyt b6f complex. Further passage of electrons from the Cyt b6f complex to the P700 chlorophylls of the PS I reaction centre is via plastocyanin. The plastocyanin molecule then re-reduces PS I to replace the electron that has been transferred from PS I to ferrodoxin (Fd) and then on to reduce NADP+ to finally produce NADPH utilising ferrodoxin-NADP oxidoreductase. In addition, while electron transport is taking place, a proton electrochemical gradient is generated across the thylakoid membrane through the release and translocation of protons into lumen accompanying the oxidation of water by PS II and the oxidation of plastoquinol by the Cyt b6f complex. The proton electrochemical gradient is then able to drive ATP synthase to produce ATP. The end products of the light reactions, NADPH and ATP, are required to power the dark or carbon fixation reactions, which take place in the stroma of the chloroplasts or the cytosol of cyanobacteria (Fig. 1.1). Fixation of carbon dioxide into sugars via the Calvin-Benson cycle is catalysed by ribulose-1, 5-bisphosphate carboxylase oxygenase (Rubisco) (Whitmarsh and Govindjee, 1995; 1999).

1.2 Photosystem II

1.2.1 Structure, composition and function

Photosystem II is found in the photosynthetic membrane with the oxygen-evolving site adjacent to the lumen and plastoquinone-binding site near the cytosol in cyanobacteria. This enzyme is sometimes referred as the light-driven water-plastoquinone oxidoreductase of photosynthesis. PS II is a highly conserved and ordered multi-
subunit protein complex that exists in a dimeric state in its fully assembled and functional form (Ferreira et al., 2004; Umena et al., 2011; Komenda et al., 2012; Vinyard et al., 2013; Suga et al., 2015).

Photosystem II consists of a central core reaction centre (RC) containing two proteins designated as D1 and D2, surrounded by two chlorophyll $a$-containing proximal antenna proteins designated as CP43 and CP47. The RC binds the cofactors which are involved in photochemical charge separation and electron transfer for the reduction of plastoquinone by water. The proximal antenna system helps to capture light directly or via the phycobilisome distal antenna thereby directing the absorbed excitation energy to the RC where charge separation takes place (Govindjee et al., 2010). Various detailed X-ray crystallographic PS II structures from thermophilic cyanobacteria are

![Figure 1.2](image_url)
available (Ferreira et al., 2004; Loll et al., 2005; Umena et al., 2011). The 1.9-Å crystal structure of the PS II dimer solved by Umena et al. (2011) from the thermophilic cyanobacterium *Thermosynechococcus vulcanus* is shown in Fig. 1.2. Each monomer of ~350 kDa contains 20 different protein subunits, 35 chlorophylls, 2 pheophytins, 11 β-carotenes, 2 plastoquinones, 2 hemes, 1 non-heme iron and the Mn$_4$CaO$_5$ cluster for the water-splitting reaction and oxygen production (Nickelsen and Rengstl, 2013; Suga et al., 2015).

As noted above, the core RC consists of two integral membrane proteins D1 (encoded by *psbA*) and D2 (encoded by *psbD*), along with two chlorophyll-binding proteins CP43 (encoded by *psbC*) and CP47 (encoded by *psbB*). In addition the photosystem
contains α- and β- subunits of cytochrome \(b_{559}\) (Cyt \(b_{559}\)) (encoded by \(psbE\) and \(psbF\)) along with some extrinsic proteins (PsbO, PsbU and PsbV) and low-molecular weight

Figure 1.4 Biogenesis of PS II in cyanobacteria. Adapted from Mabbit et al. (2014). Sequential attachment of preformed PS II sub-complexes are shown leading to formation of the active PS II dimer. Labels are: D1 and D2, reaction centre proteins; CP43 and CP47, chlorophyll–binding proteins of PS II and Cyt \(b_{559}\), cytochrome \(b_{559}\). The PsbH, PsbI, PsbK, PsbL, PsbM, PsbT, PsbX, PsbY, PsbZ, Psb27 and Psb30 low-molecular-weight proteins are also denoted by their corresponding letters or numbers.
proteins. A schematic illustration of a PS II monomer is shown in Fig. 1.3. The integral proteins D1 and D2 bind the four chlorophylls belonging to P680 of the RC, whereas the chlorophyll-binding proteins, CP43 and CP47, bind antenna chlorophyll molecules. Upon illumination, the excited P680\textsuperscript{*} transfers an electron to the pheophytin (Pheo) to form a radical state P680\textsuperscript{**} Pheo\textsuperscript{-} which is followed by the electron passing on to QA and QB, which are located on the D2 and D1 proteins, respectively. Plastoquinol, PQH\textsubscript{2}, formed from the double reduction of QB after two light-driven electron transfers, leaves the QB–binding site to join the plastoquinone pool and is replaced by another oxidised quinone. The radical P680\textsuperscript{**} is reduced by a tyrosine residue (YZ-160) of the D1 protein in nanoseconds thereby preventing a back reaction between P680\textsuperscript{**} and Pheo\textsuperscript{-}. The oxidised Yz is in turn reduced by electrons obtained from the Mn\textsubscript{4}CaO\textsubscript{5} cluster of the water-oxidising complex of PS II. Once four positive charges have accumulated on the Mn\textsubscript{4}CaO\textsubscript{5} cluster, an oxygen molecule is formed from two bound water molecules (Umena et al., 2011; Vinyard et al., 2013; Suga et al., 2015).

1.2.2 Biogenesis of PS II

Photosystem II assembly occurs in a sequentially and highly coordinated manner which is shown in Fig. 1.4. Assembly of PS II grows outwards from the reaction centre complex comprising of the D1, D2, Cyt b\textsubscript{559}, and PsbI subunits. A D2-Cyt b\textsubscript{559} pre-complex represents the initial phase of PS II biogenesis resulting in the formation of the initial RC sub-complex, following the addition of pD1 (precursor D1) and PsbI incorporation (Komenda et al., 2004; Nickelsen and Rengstl, 2013). Processing of pD1 at its C-terminus by a C-terminal processing protease (CtpA) takes place during RC complex formation to produce mature D1 (Nickelsen and Rengstl, 2013). Synthesis of the D1 protein is regulated by the availability of chlorophyll a (Sobotka, 2014). The CP47 pre-complex containing the core antenna CP47 protein and several low-molecular-weight PS II subunits (PsbH, PsbL and PsbT) is then incorporated into the RC complex to form the RC47 CP43-less complex. As PS II assembly advances, the chlorophyll-binding protein, CP43, along with Psb27 is then incorporated into the RC47 CP43-less complex to form an inactive PS II monomer (Nickelsen and Rengstl, 2013; Mabbit et al., 2014). Formation of the active PS II monomers requires the
dissociation of the Psb27 protein from the PS II monomer and the attachment of Mn$_4$CaO$_5$ cluster and the extrinsic proteins (PsbO, PsbU, PsbV and potentially PsbQ (Liu et al., 2014; Michoux et al., 2014)) to the lumenal surface of PS II (Bricker et al., 2012). Dimerisation of PS II then takes place between two active monomers along with the attachment of phycobilisome peripheral antenna to form a PS II super complex (Kouril et al., 2012; Nickelsen and Rengstl, 2013).

1.2.3 PS II Repair

Photosystem II catalyses a series of reactions which make it susceptible to light-induced photodamage resulting in a continuous repair mechanism to achieve sustained water-splitting activity. When PS II is damaged, the Mn$_4$CaO$_5$ cluster and the extrinsic proteins (PsbO, PsbU, PsbV and PsbQ) detach from the complex. The photodamaged D1 protein is then removed and replaced with the help of FtsH metalloproteases (Nixon et al., 2010; Nickelsen and Rengstl, 2013). A newly synthesised pD1 is then inserted into the RC47 CP43-less complex with subsequent attachment of CP43, the Mn$_4$CaO$_5$ cluster and the extrinsic proteins to re-form an active PS II dimer (Nickelsen and Rengstl, 2013; Mabbit et al., 2014).

Figure 1.5. Topological model of chlorophyll-binding protein, CP47. Modified from Barber et al., 2000). Histidine residues are highlighted in red.
1.3 Chlorophyll $a$-binding protein, CP47

The chlorophyll $a$-binding protein CP47, encoded by the psbB gene, has an apparent molecular mass of 47 kDa and is found in the chloroplast genome of higher plants and green algae and in the cyanobacterial genome. It is also sometimes referred as CPa1 and PS II-B (Bricker, 1990). As noted above, along with CP43 (encoded by psbC), CP47 serves as the internal or proximal antenna system of PS II. Fig. 1.5 shows that CP47 possesses six transmembrane $\alpha$-helices which are separated by five extrinsic loop domains (Vermaas et al., 1987; Bricker, 1990). The A, C and E loops are found in the lumenal space whereas the B and D loops are exposed to the stroma or cyanobacterial cytosol. The amino acid sequence of CP47 is highly conserved with up to 85% similarity found among cyanobacteria and plants (Bricker, 1990). CP47 bind 16 chlorophylls molecules, which are arranged as a double layer for energy capture and transfer to the reaction centre (Vasilév et al., 2001; Umena et al., 2011). Biochemically, CP47 is closely associated with the PS II RC and its removal requires harsh treatments with LiC1O$_4$ and high concentrations of dodecyl maltoside (Ghanotakis et al., 1989). Moreover, insertion or deletion of the psbB gene results in the loss of photoautotrophic growth and an absence of the PS II RC components from the thylakoid membrane; thus CP47 seems to play a major role in the functional assembly of PS II (Vermaas et al., 1988; Eaton–Rye and Vermaas, 1991).

1.3.1 Histidine residues in CP47

Histidine (His) residues found in the transmembrane regions of the core antenna complex of PS II serve as chlorophyll ligands in higher plants and cyanobacteria (Deisenhofer et al., 1985; Zuber and Brunisholz, 1991; Shen et al., 1993; Umena et al., 2011). Twelve conserved histidine residues are found within the predicted membrane-spanning regions of CP47. They are located either on the stromal or lumenal sides of the transmembrane helices (Barber et al., 2000). Earlier studies indicated that the imidazole nitrogen of these histidine residues might serve as an axial ligand for the magnesium ion of chlorophyll molecules in the purple bacterial reaction centre and light-harvesting complex II in plants (Kühlbrandt et al., 1994; McDermott et al., 1995).
as well as the chlorophyll molecules in PS I (Jordan et al., 2001). Moreover, a role for histidyl ligation of chlorophyll molecules in CP47 and CP43 was also suggested by mutagenesis studies (Eaton-Rye and Vermaas, 1992; Shen et al., 1993; Manna and Vermaas, 1997; Wu et al., 1999). Site-directed mutagenesis studies by Shen et al. (1993) and Shen and Vermaas (1994) also indicated that a number of histidine residues located in transmembrane α-helices I-IV and helix VI might serve as chlorophyll ligands. Replacement of these histidine’s by tyrosine residues showed a significant reduction in the ability of the corresponding mutants to grow photoautotrophically, as well as a decrease in their ability to evolve oxygen and reduction in PS II assembly (Eaton-Rye and Vermaas, 1992; Shen and Vermaas, 1994). It was suggested that the replacement of histidine to tyrosine resulted in the formation of Pheo by losing Mg$^{2+}$ from chlorophyll and or the binding of Pheo at a particular pigment-binding site of CP47 during biogenesis disrupted assembly and function (Shen and Vermaas, 1994). Moreover, it was postulated that the decrease might be due to the impaired energy transfer from Pheo to chlorophyll, or by radiation-less decay from Pheo (Shen and Vermaas, 1994). This could be due to the alteration of pigment binding which indicates their role in pigment-protein association (Shen et al., 1993).

1.3.2 Substitution of Histidine residues within helix VI of CP47 by Glutamine

In order to investigate whether the three conserved histidyl residues within helix VI serve as chlorophyll ligands, His-455, His-466 and His-469 were replaced with glutamine (Gln) by site-directed mutagenesis (Eaton-Rye and Vermaas, 1992). Gln can function as the fifth ligand to chlorophyll molecules in light-harvesting proteins (Wu et al., 1999). The subsequent phenotype of H455Q and H466Q cells was similar to the wild-type strain; however, the H469Q mutant showed a significant reduction in photoautotrophic growth and 80% reduction in assembled PS II reaction centres suggesting the substitution resulted in a destabilising effect within PS II that affected assembly or incorporation of CP47 into the thylakoid membrane. Furthermore, mutagenesis studies by Wu et al. (1999) at these histidine residues (H455T, H455Y and H469Y) and the double mutant, F430L-H466R showed complete loss of photoautotrophic growth which supported the fact that His-455 and His-466 are chlorophyll ligands. Moreover a spontaneous pseudorevertant, H469Qrev, was isolated
in the H469Q mutant background which restored photoautotrophic growth. DNA sequencing of both H469Q and H469Qrev strains confirmed the presence of desired mutation (CAT to CAG) at the His-469 position (Eaton-Rye and Vermaas, 1992). Furthermore, a number of the obligate photoheterotrophic strains (a psbB deletion, H469K, H469P and H469Y) at His-469 position were isolated in the wild-type and suppressor mutant backgrounds (Huang et al., 2001). When present in the the suppressor background the H469K, H469P and H469Y mutants were able to grow photoautotrophically, assemble functional PS II centres, and evolve oxygen. This in turn suggests that the introduction of lysine, proline and tyrosine at this position are unlikely to serve as axial ligands resulting in unassembled PS II centres (Wu et al., 1999; Huang et al., 2001). Furthermore it was confirmed that the change responsible for the phenotype of H469Qrev was not present in the psbB gene and H469Qrev was a pseudorevertant containing a suppressor mutation at a different location.

1.4 Experimental Model: *Synechocystis* sp. PCC 6803

Cyanobacteria are a common experimental model system to study photosynthesis and carbon and nitrogen assimilation (Eaton-Rye, 2011). Photosynthesis in cyanobacteria takes place within the cytoplasm where the electron transport chain is associated with the thylakoid membrane. In plants, light-driven photosynthetic reactions take place in the thylakoid membrane of the chloroplast. In spite of their differences in the thylakoid membranes structure, light-harvesting systems and the exact composition of the hydrophilic extrinsic proteins of PS II, PS II in cyanobacteria is homologous in composition and function to that of plants (Barry et al., 1994).

*Synechocystis* sp. PCC 6803 is a unicellular, non-nitrogen fixing cyanobacterium which is abundantly present in fresh water (Stanier et al., 1971). It can be easily transformed by exogenous DNA, and grows photoheterotrophically using glucose as a carbon source (Rippka et al., 1979; Grigorieva and Shestakov, 1982; Williams 1988). It was the first phototrophic organism to have its genome fully sequenced which revealed the genome structure, its gene constituents and their relative map positions.
Introduction

(Kaneko et al., 1996; Kaneka and Tabata, 1997). These characteristics were important for the development of gene replacement systems where genetic manipulation of the desired photosynthetic components could be easily achieved (Williams, 1988). In addition, the recent analysis of the primary transcriptome identified 4091 transcriptional units and various sRNAs that are related to photosynthesis and nitrogen metabolism as well as phosphate depletion and iron stress conditions (Kopf et al., 2014). Furthermore, a cyanobacterial genome database called CyanoBase has been established which acts as a central repository for gene structural and functional information (Ikeuchi and Tabata, 2001).

1.5 Objectives

The role of the histidine residue at position 469 of the chlorophyll-binding protein CP47 is currently not characterised. In order to understand its function, the current study was divided in two parts.

1.5.1 Physiological characterisation of strains carrying substitutions for His-469

In order to understand the significance of the histidine residue at position 469 of CP47, physiological characterisation of mutated strains with targeted substitutions will be carried out looking at a range of different aspects including: photoautotrophic growth; their ability to evolve oxygen; extent of PS II assembly determined by 77 K fluorescence emission spectroscopy, variable fluorescence induction studies, as well as Blue-native polyacrylamide gel (BN-PAGE) electrophoresis followed by western blotting.

1.5.2 Identification of secondary mutations in pseudorevertants retaining the introduced mutations at position 469

The physiological characterisation of the strains carrying substitutions at His-455, His-466 and His-469 has suggested that binding of Chl at His-469 might have a different role in CP47 incorporation into PS II than other chlorophyll-binding ligands in the proximal antenna. However, the exact function of His-469 remains uncertain. In order to identify the secondary mutation in the H469Qrev pseudorevertant, genomic
sequencing of the strain was carried out and seven unique mutations were detected. To evaluate these seven mutations, plasmids containing different combinations of these distinct mutations will be constructed and transformed into cyanobacteria. Different combinations of the introduced seven mutations found in the H469Qrev strain will be investigated to see if a particular set of mutations is required to restore photoautotrophic growth. The physiological characterisation of the transformed strains will be evaluated for photoautotrophic growth, ability to evolve oxygen and levels of PS II assembly. Furthermore the ability of the unique mutations from the H469Qrev strain to restore PS II activity and photoautotrophic growth to additional strains carrying His-469 substitutions (H469A, H469K, H469P, H469Q and H469Y) will be tested.
Chapter 2: Experimental Methods

2.1 Methods

2.1.1 General

The media or chemicals used in the experiments were made using distilled, deionised water and stored at room temperature or at 4°C. These solutions were autoclaved at 15 psi for 20 min wherever required. All DNA samples were stored at -20°C. In the course of this study, standard microbiological techniques were adopted, making use of a laminar flow hood wherever required to achieve a sterile setting.

2.1.2 Synechocystis sp. PCC 6803 Strains

All the cyanobacterial strains were obtained from the laboratory BG-11 15% glycerol stocks stored at -80°C and are shown in Table 2.1.

2.1.3 Media and Growth Settings: Synechocystis sp. PCC 6803

Cyanobacterial strains were grown on BG-11 solid media with appropriate antibiotics and 20 µM atrazine and 5 mM glucose. They were restreaked onto new plates every 2-3 weeks (Eaton-Rye, 2011). For physiological studies, strains were cultivated in BG-11 liquid media supplemented with 5 mM glucose and suitable antibiotics wherever necessary. The antibiotics used in this study were: 15 µg/mL chloramphenicol, and 25 µg/mL spectinomycin and kanamycin. Strains were removed using a sterile 1 mL serological pipette and inoculated into a modified conical flask containing 150 mL or 300 mL of BG-11, 5 mM glucose and suitable antibiotics. Fig. 2.1 shows the modified conical flask used for liquid cultures. Liquid cultures were kept in 30°C under continuous light at either 20 µE².s⁻¹ or 50 µE².s⁻¹ for BG-11 solid plate and liquid cultures, respectively. Liquid cultures were continuously bubbled using an aquarium pump with air filtered through a sterile 0.2 µm filter (Millipore Corporation, MA). Prior to bubbling, they were allowed to acclimate for 2-3 h without aeration.
Table 2.1 List of the *Synechocystis* sp. PCC 6803 strains used in the study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Glucose tolerant, referred as GTO1 (Glucose tolerant Otago 1)</td>
</tr>
<tr>
<td>Del psbB</td>
<td>The <em>psbB</em> gene in a wild-type strain has been replaced by a 2.0 kb spectinomycin-resistance cassette, from a BamHI site 259 nucleotides downstream from the <em>psbB</em> start codon and up to a NcoI site 499 nucleotides downstream of the <em>psbB</em> stop codon (Eaton-Rye and Vermaas, 1991).</td>
</tr>
<tr>
<td>Del psbA</td>
<td>The D1 protein is encoded by 3 <em>psbA</em> genes, in this triple deletion mutant, <em>psbA1</em> and <em>psbA3</em> have both been deleted by a <em>sacB</em>-introduced markerless deletion whereas <em>psbA2</em> was deleted by insertion of a chloramphenicol-resistance cassette.</td>
</tr>
<tr>
<td><em>psbB</em> control</td>
<td>Includes the addition of a kanamycin-resistance cassette, downstream from the <em>psbB</em> gene in a “wild-type” strain (Eaton-Rye and Vermaas, 1991).</td>
</tr>
<tr>
<td>H469A</td>
<td>Strain with an Ala substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469Arev</td>
<td>Pseudorevertant of H469A strain, still carrying the Ala substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469K</td>
<td>Strain with a Lys substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469Krev</td>
<td>Pseudorevertant of H469K strain, still carrying the Lys substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469P</td>
<td>Strain with a Pro substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469Prev</td>
<td>Pseudorevertant of H469P strain, still carrying the Pro substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469Q</td>
<td>Strain with a Gln substitution at His-469 within the <em>psbB</em> gene (Eaton-Rye and Vermaas, 1992).</td>
</tr>
<tr>
<td>H469Qrev</td>
<td>Pseudorevertant of H469Q strain, still carrying the Gln substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469Y</td>
<td>Strain with a Tyr substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
<tr>
<td>H469Yrev</td>
<td>Pseudorevertant of H469Y strain, still carrying the Tyr substitution at His-469 within the <em>psbB</em> gene.</td>
</tr>
</tbody>
</table>
2.1.4 Genomic DNA Extraction

Liquid cultures of the strains were grown for 2-3 days and then pelleted at 5000g for 5 min by centrifugation in a 50 mL Falcon tube. These pellets were washed twice with 5 M sodium chloride to remove polysaccharides and resuspended in 1 mL of TE (10 mM Tris/HCl, 1 mM EDTA, pH 8) and lysosome before incubating at 37°C for 1 h with periodic mixing. Then 0.5 M EDTA and proteinase K were added to the mix and the incubation was continued for another hour with periodic mixing. Following this step, 5 M NaCl and CTAB (hexadecyl trimethylammonium bromide) were added and the sample incubated at 65°C for 10 min after which they were pelleted at 10000g for 10 min. The supernatant was then extracted with 1:1 chloroform by spinning for 30 min and centrifuged for 5 min at 5000g to separate phases. The upper aqueous phase was precipitated with a double volume of 95% cold ethanol and then pelleted and the pellet was resuspended in TE and 10 mg/mL RNase A which was incubated at 37°C for 30 min. These were then extracted with an equal volume of phenol: chloroform and centrifuged to collect the aqueous phase. This process was repeated until the interface was clear. The aqueous phase was then precipitated with 1/10 volume of 3 M sodium acetate, pH 5 and twice the volume of cold 95% ethanol and kept at -20°C for 1 h.
DNA was pelleted by centrifuging at 12000g for 10 min at 4°C and washed with cold 70% ethanol, dried and resuspended in 75-100 µL of TE and stored at -20°C.

2.1.5 Polymerase Chain Reaction (PCR)

**Standard PCR:** Polymerase chain reaction (PCR) was carried out in 50 µL reactions, using Phusion High Fidelity Hot-start II DNA Polymerase from Thermo Scientific (USA) or Platinum Taq polymerase (Life Technologies, USA).

**Phusion High Fidelity Hot-start II DNA Polymerase:** This reaction consists of 10 µL 5X Phusion HF buffer, 1 µL 10 mM dNTPs, 0.5 µL of each 0.5 µM forward and reverse primers, 1µL DNA template and sterile MilliQ water to make the total volume to 49.5 µL before adding 0.5 µL of Phusion High Fidelity Hot-start II DNA Polymerase to the mix. Typical reaction settings were as follows: a) initial denaturation step at 98°C for 30 s, b) 30 cycles of denaturation at 98°C for 10 s, annealing for 30 s depending on optimum primer binding temperature, extension at 72°C for 30 s per kb of the template used and c) final extension at 72°C for 5 min.

**Platinum Taq polymerase:** This consisted of 5 µL 10X PCR buffer minus Mg, 1 µL 10 mM dNTP mixture, 1.5 µL 50 mM MgCl₂, 1 µL 10 µM forward and reverse primers, 1 µL DNA template and sterile MilliQ water to make the total volume to 49.5 µL before adding 0.5 µL Platinum Taq Polymerase to the mix. Typical reaction setup were as follows: a) initial denaturation step at 94°C for 2 min, b) 30 cycles of denaturation at 94°C for 30 s, annealing for 1 min depending on optimum primer binding temperature, extension at 72°C for 2 min and c) final extension at 72°C for 5 min.

These reactions were performed in an Eppendorf Mastercycler Gradient thermal cycler. The changes in the cycles of reactions, the annealing temperature and the initial extension time depended on the oligonucleotide used. Table 2 summarises all the oligonucleotides used in this study.

**Colony PCR:** The PCR reactions were carried out in 50 µL volume using Platinum Taq polymerase (Life Technologies, USA) to check segregation. The DNA template was obtained by picking a single colony from the respective strain and mixing in 20 µL of sterile MilliQ water. The initial denaturing step was extended from 2 to 5-10 min to ensure complete cell lysis.
Table 2.2 List of oligonucleotides used in the study.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>psbBF</td>
<td>5’AATAAAAAATTTAAAAACGTTTTTGACACAAAAACAC 3’</td>
</tr>
<tr>
<td>psbB-far-NCoI-rev</td>
<td>ACAGCCACTGACCTGTTG</td>
</tr>
<tr>
<td>slr1055 forward</td>
<td>ATCAAGGCTGGCGCTGAAATC</td>
</tr>
<tr>
<td>slr1055_F1</td>
<td>GTTGAAATTGGACCCAGCTAC</td>
</tr>
<tr>
<td>slr1055_F2</td>
<td>GAGTTGATCGGTGTTCTCC</td>
</tr>
<tr>
<td>slr1055_F3</td>
<td>ATCCTTCTGAAGCTACCACGCC</td>
</tr>
<tr>
<td>slr1055_F4</td>
<td>GTGGTGGTGAATTGTCTCC</td>
</tr>
<tr>
<td>slr1055 seqF</td>
<td>CAACGTCACCTAGCGGTAG</td>
</tr>
<tr>
<td>slr0104 forward</td>
<td>GCCACCGCATTATTATCTCTG</td>
</tr>
<tr>
<td>slr0104_F1</td>
<td>TGGATGCCCATACAACGACT</td>
</tr>
<tr>
<td>slr0104 seqF</td>
<td>CTTGCTAGCGGTATGATTG</td>
</tr>
<tr>
<td>slr0104 reverse</td>
<td>AGCAATTGAGGAAGTGAGCC</td>
</tr>
<tr>
<td>sll1496 forward</td>
<td>TCACTTATGCAGTGAGGAC</td>
</tr>
<tr>
<td>sll1496_F1</td>
<td>AACTGCAACATCGAGTCC</td>
</tr>
<tr>
<td>sll1496 seqF</td>
<td>CGATTTCGACAGAACACGAC</td>
</tr>
<tr>
<td>sll1496 reverse</td>
<td>TACGACTCTCTATGCTCC</td>
</tr>
<tr>
<td>sll0862 forward</td>
<td>GGAGAATGCGGAACACTGTG</td>
</tr>
<tr>
<td>sll0862 seqF</td>
<td>TTACCCAGCCGCAATGCC</td>
</tr>
<tr>
<td>sll0862 reverse</td>
<td>TACTCCGGATATACGAGGA</td>
</tr>
<tr>
<td>ssl5096 forward</td>
<td>CACAGCGCAAGAATTGAC</td>
</tr>
<tr>
<td>ssl5096 seqF</td>
<td>TTTGGTCAACTGCACACC</td>
</tr>
<tr>
<td>ssl5096 reverse</td>
<td>TTCTCTGACCGAGCTTGGG</td>
</tr>
<tr>
<td>slr0930 forward</td>
<td>TGCCACTCGCCCTTAAAGA</td>
</tr>
<tr>
<td>slr0930_F1</td>
<td>GTTCAGAGTTGGCGAGGACT</td>
</tr>
<tr>
<td>slr0930 seqF</td>
<td>ATGCTGACGAAGTACAGGCT</td>
</tr>
<tr>
<td>slr0930 reverse</td>
<td>TAGTTTCCACTGGTCGACT</td>
</tr>
<tr>
<td>sr0851 forward</td>
<td>ATCGTTCACCTCCACACT</td>
</tr>
<tr>
<td>sr0851_F1</td>
<td>CGGATATTGTCCACCGTTGCT</td>
</tr>
<tr>
<td>sr0851 seqF</td>
<td>GTTGATCGAGTGACTTGCTCC</td>
</tr>
<tr>
<td>sr0851 reverse</td>
<td>ACTGTTGGTCACCTGCCTCATT</td>
</tr>
</tbody>
</table>

A tailing: This step was required when ligating a Phusion PCR product into the pGEM-T-easy vector (Promega, USA). PCR products were purified using a Purelink quick PCR purification kit (Invitrogen, Germany). Reactions were as follows: 5 µL of purified PCR product, 1 µL 10X Platinum Taq buffer, 0.4 µL 50 mM MgCl2, 1.2 µL
dATP and 0.4 µL Platinum Taq polymerase and sterile MilliQ to make up the volume to 10 µL. Reaction conditions were 95°C for 30 s followed by 72°C for 30 min.

2.1.6 Gel Electrophoresis

Electrophoresis was performed on a 0.8% agarose gel using 1X Tris/Borate/EDTA (TBE) running buffer. Samples were made of 2 µL loading dye, 5 µL PCR product and sterile water to make the volume to 12 µL and the gel was run at 100 V for 50 min. Gels were stained in 10 mg/mL ethidium bromide for 5-10 min and visualised with a Bio-Rad Gel Doc EQ system using UV trans-illumination.

5X TBE: 54 g Tris, 27.5 g boric acid anhydrous and 20 ml 0.5 M EDTA, pH 8.0, made up to 1 L with distilled water.

Loading Dye: 0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol.

2.1.7 DNA Sequencing

DNA sequencing was completed at the Genetic Analysis Services located in the Department of Anatomy, University of Otago. All DNA samples that were to be sequenced were purified using a Purelink quick PCR purification kit (Invitrogen, Germany) as per manufacturer’s instructions. Samples were made by mixing template and primer in a total volume of 5 µL. Condition were as follows:

- Primer : 3.2 pmol/5µL
- PCR product: 1 ng/100 bp/5 µL
- Plasmid: 150 ng/5 µL for up to 4 kb. Use 200 ng/5 µL for larger plasmids.

2.2 Physiological Studies

2.2.1 Photoautotrophic Growth Curves

Bacterial strains were grown in liquid BG-11 cultures containing glucose and antibiotics until the OD at 730 nm was approximately 0.8. These cultures were poured
in to a 50 mL sterile Falcon tube and harvested at 2760g for 7 min at 25°C. This process was repeated twice to remove any trace of glucose and finally the pellet was resuspended in 5 mL BG-11. New 150 mL cultures containing antibiotics but no glucose were set up at an OD at 730 nm of 0.05 and the OD was measured every 24 h for 7 days.

2.2.2 Chlorophyll Analysis

The chlorophyll a concentration of the original cells was measured using 100% methanol or in the case of extracted thylakoids by using 80% acetone. Absorbance was measured at 663 nm using a Jasco V-550 UV-Visible spectrophotometer (1 mg/mL chlorophyll a has an absorbance of 82 at 663 nm).

2.2.3 Oxygen Evolution

Liquid cultures of the strains were grown until the OD at 730 nm was approximately 0.8-1.2. These cultures were harvested at 2760g for 7 min and resuspended to a chlorophyll a concentration of 5 µg/mL in sterile BG-11 with 25 mM HEPES-NaOH (pH 7.5) media. Fifteen milliliters of cells was then taken in a 50 ml conical flask and incubated for 30 min at 30°C room on an orbital shaker.

Oxygen evolution measurements were done using a Clark-type electrode (Hansatech, UK), which was maintained at 30°C by a recirculating water bath. One milliliter samples of 5 µg/mL chlorophyll a were placed in the electrode chamber either with 200 µM 2,6-dichloro-1,4-benzoquinone (DCBQ) and 1 M potassium ferricyanide (K3Fe(CN)6) or 15 mM sodium bicarbonate as the electron acceptors. The oxygen concentration was measured for 1 min at ambient light, 3 min under red light illumination and again at 1 min ambient light using an oxygen electrode control box (CB1D, Hansatech, UK). The FLS1 light source (Hansatech, UK), which was used as the light source for illumination, has a neutral density of 0.3 OD and 580 nm band pass filters (Melles Griot, USA) with an output of 5000 µE.m⁻².s⁻¹. For each strain, 2-3 independent experiments were performed where each measurement consisted of the mean of two technical repeats. The average of the biological repeats is shown, normalised to the baseline during the first minute of the measurement where the light is off.
The rate of oxygen evolution was calculated by using the formula below:

\[ \mu \text{moles } O_2/\text{mg Chl } a/\text{hour} = \]

\[ (0.235 \ \mu \text{moles } O_2/\text{ml}) \times (\text{sample volume, ml}) \times (60 \text{min/hour}) \times (\text{slope, mV/min}) \]

\[ \times (\text{calibration voltage, mV}) \times (\text{Chl } a, \text{ mg}) \]

Where 0.235 \ \mu \text{moles } O_2/\text{ml} \text{ refers to the quantity of } O_2 \text{ dissolved at } 30^\circ C, \text{ calibration voltage refers to the value for oxygen saturated water, attained by filling the reaction chamber with water and recording the trace of water equilibrated with atmospheric oxygen and slope is calculated from a 1 min widow typically between 30 s and 90 s after the actinic light is turned on.}

2.2.4 77 K Fluorescence Emission Studies

Cells were obtained in a similar manner as discussed in Section 2.2.3. Immediately prior to the measurement, the cells were diluted to 2.5 \ \mu \text{g/mL chlorophyll } a. \text{ For a measurement 0.5 mL of the cells were placed in a glass tube of 4 mm internal and 6 mm external diameter and snap frozen in liquid nitrogen. The frozen tube was then placed inside a modified glass liquid nitrogen Dewar in a Perkin-Elmer Fluorescence Spectrophotometer MPF-3L. The fluorescence emission spectrum from 600 to 800 nm was collected at an excitation wavelength of 440 nm with the excitation and emission slit widths set at 12 nm and 2 nm, respectively. The scan speed was set at 4 which matches to a rate of 100 nm min\(^{-1}\). The emission spectra were also collected at an excitation wavelength of 580 nm with the excitation and emission slit widths set at 8 nm and 2 nm, respectively.}

The raw spectra after baseline correction were normalised to PS I emission peak maxima at 725 nm. Each strain had two biological repeats measured, each measurement includes the mean of the two technical repeats.

2.2.5 Fluorescence Induction

For this measurement cells were also obtained in similar manner as mentioned earlier in section 2.2.3. A 2 mL sample was diluted to a chlorophyll concentration of 2.5 \ \mu \text{g/mL using BG-11 (pH 7.5) and dark adapted for 5 min. The cells were then placed in a quartz cuvette and illuminated with actinic light over a period of 5 s using a}
Fluorometer FL 3300 (Photon systems Instruments, Czech Republic). The variable fluorescence emission was determined using a blue measuring light (455 nm) or red measuring light (625 nm). When required, 40 µM 3, 4-dichloro-1, 1-dimethyl urea (DCMU) was added to the sample. The actinic voltage on the FL 3300 was set to 80% throughout the study. The data are processed by the FluorWin software supplied with the instrument.

2.2.6 Thylakoid Extraction and Solubilisation

Starter liquid cultures were grown in BG-11 with glucose and appropriate antibiotics to an OD at 730 nm of approximately 1.0 and then used to inoculate two 300 mL cultures for each strain at an OD 730 nm of 0.2. These cultures were grown for 24 h until the OD 730 nm was around 0.8 – 1.0. Cells were harvested at 5000g for 10 min and then resuspended in cell wash buffer (50 mM HEPES-NaOH pH 7.5, 20 mM CaCl$_2$, 10 mM MgCl$_2$, 1 mM 6-amino caproic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM benzamidine). The cells were then harvested by centrifuging at 8000g for 10 min and resuspended in disruption buffer (50 mM HEPES-NaOH pH 7.5, 20 mM CaCl$_2$, 10 mM MgCl$_2$, 800 mM sorbitol, 1 M betaine monohydrate, 1 mM 6-amino caproic acid 1 mM PMSF and 2 mM benzamidine). From this point, everything was carried out in darkness and at 4°C. Cells were then lysed by 5 cycles of bead beating at 4800 rpm for 20 s with a 5 min rest on wet ice between cycles. Beads were removed by centrifuging at 2000g for 2 min and any remaining unbroken cells were removed by washing with disruption buffer and centrifuging at 8000g for 5 min. Thylakoids were harvested by centrifuging at 60000g for 1 h in a Type 75Ti rotor and resuspended in disruption buffer and harvested again at 60000g for 25 min to wash away the phycobillisomes. Thylakoids were finally resuspended in solubilisation buffer (25 mM Bis Tris- HCl pH 7.0, 20% w/v glycerol and 0.25 mg mL$^{-1}$ Pefabloc C (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride)) and stored in liquid nitrogen at -80°C till use.

Extracted thylakoids were solubilised by drop wise addition of Solubilisation buffer (25 mM Bis Tris-HCl pH 7.0, 20% w/v glycerol and 0.25 mg mL$^{-1}$) and 3% β-dodecyl maltoside solution and incubated for 15 min on ice. Insoluble materials were then
removed by centrifuging at 4°C for 15 min at 12000g. The yield of solubilisation is calculated by the measuring the concentration of chlorophyll \( a \), which is then used for BN-PAGE.

### 2.2.7 Blue- Native PAGE

Blue-native PAGE was carried out using a Novex Bis-Tris Gel system (Life Technologies, USA). Twenty eight microliters of samples at 0.3 mg.mL\(^{-1}\) Chl \( a \) in solubilisation buffer and 1% \( \beta \)-dodecyl maltoside (Anatrace, U.S.A) were prepared along with 10 µL of 4X sample buffer (0.2 M BisTris, 0.064 N HCl, 40% glycerol, 0.2 M NaCl, 4% Ponceau S ) and 3 µL 5% Serva G-250. Ten microliters of solubilised thylakoids containing 2 µg Chl \( a \) (Section 2.2.6) were loaded onto a precast 3 – 12% Bis-Tris gradient gel (Life Technologies, USA). Electrophoresis was carried out at 80 V for 1 h at 4°C then increasing the voltage to 100 V and the gel was run for a further 30 min and then finally at 150 V for 2 h.

### 2.2.8 Enhanced Chemiluminescence

Proteins were electroblotted onto a 0.2 µm polyvinylidene difluoride membrane (Biorad, USA) using electroblot buffer at 50 V for 2 h in 4°C after destaining in water overnight. The membrane was then destained in 100% methanol for 2-3 min and blocked using 4% BSA for 1 h at room temperature, washed twice in water for 10 min each and incubated in primary antibodies at 4°C overnight on a shaker. The membrane was then washed three times with TBS + 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) and incubated with secondary antibody for 1-2 h with gentle rocking at room temperature. Finally the membrane was washed three times with TBS + 0.1% Tween 20 for 5 min each and incubated the membrane with freshly made ECL reagent (Abcam, UK) and imaged using a Fuji imager PS3000, set on chemiluminescence mode (-30°C).

**Electroblot buffer:** 25 mM Tris, 192 mM glycine and 10% w/v glycerol

**TBS (pH 7.4):** 137 mM NaCl, 5 mM KCl, 25 mM Tris

**1\(^{o}\) Antibody:** PsbA (D1), PsbB (CP47) and PsbC (CP43), Agrisera, Sweden.
2° Antibody (Anti-Rabbit Peroxidase Conjugate): 1 M Tris-HCl pH 7.4, 1 M KCl, BSA and 1:5 dilution of peroxide conjugate

2.3 Management of Escherichia coli Strains

2.3.1 Strains and Growth Conditions

Escherichia coli (E. coli) DH5α strain was grown in lysogeny broth (LB) made up of 1% bactotryptone, 0.5% yeast extract and 1% NaCl supplemented with 1.5% agar for solid plates. Fifty µg/mL ampicillin was used whenever necessary.

2.3.2 Preparation of Competent Cells

To prepare competent cells 10 mL of LB media was inoculated with a single colony of DH5α cells and kept at 37°C overnight on a shaker. A 2 mL aliquot of this culture was then added to 100 mL pre-warmed ψB media and grown for another 2 h until the OD at 600 nm was approximately 0.3 - 0.4. The cells were then chilled on wet ice for 5 min before centrifuging at 2760g for 10 min at 4°C and resuspended in 15 mL chilled transformation buffer I (TfBI). Cells were again centrifuged at 2760g for 10 min and resuspended in chilled transformation buffer II (TfBII). Two hundred microliter aliquots were then snap frozen and stored at -80°C.

ψB Media: 1% bactotryptone, 0.5% yeast extract, 10 mM KCl, pH 7.6, sterile 34 mM MgSO₄

Transformation Buffer I: 30 mM potassium acetate, 50 mM MnCl₂, 100 mM RbCl, 10 mM CaCl₂, 15% glycerol, pH 5.8 with 0.2 M acetic acid

Transformation Buffer II: 10 mM MOPS pH 7, 10 mM RbCl, 75 mM CaCl₂, 15% glycerol

2.3.3 Heat-Shock Transformation

A 200 µL aliquot of competent cells was added to either 10 µL pGEM-T Easy ligation product (Promega, USA) or 1 µL of plasmid and incubated on ice for 30 min. These cells were heat-shocked at 37°C for 2 min, then chilled on ice for another 3 min. An
800 µL volume of LB media was then added to the cells and the mixture incubated at 37°C for 90 min on a shaker. Cells were centrifuged at 12000g for 30 s before resuspending in 200 µL of LB media. A 50 µL aliquot was then plated onto selective media and grown for 12-16 h at 37°C.

2.3.4 Plasmid Preparation

A 2 ml volume of sterile LB media was inoculated with a single colony from the transformation and grown overnight along with appropriate antibiotics at 37°C. Plasmid DNA was extracted using a Purelink HiPure Plasmid Isolation Kit (Roche, Germany) as per manufacturer’s guidelines.

2.3.5 Restriction Digest

Restriction enzymes were obtained from either New England Bio labs, USA or Roche, Germany. A 10 µL reaction volume was made up of DNA, 10X restriction enzyme buffer and the restriction enzyme and incubated at different temperatures depending on the enzyme used. These reactions were loaded onto an agarose gel and the products of the digestions checked before sending for DNA sequencing of the appropriate region to confirm the resulting plasmid.

2.3.6 Cyanobacterial Transformation

Liquid cultures along with glucose and appropriate antibiotics were grown until the OD at 730 nm was approximately 0.4-0.8. Cells were then harvested by centrifuging at 2760g for 7 min and resuspended to an OD of 2.5 at 730 nm. In a sterile test tube, 1-3 µg plasmid or genomic DNA were mixed with 0.5 mL of this cell suspension and incubated at 30°C for 6 h under constant illumination of 20 µE·s⁻¹. A 200 µL aliquot of this suspension was then plated onto a BG-11 plate and grown photoautotrophically. After colonies appeared (typically 2-3 weeks), a single colony was picked and restreaked onto a fresh BG-11 or BG-11-glucose plate and this process was repeated at least four times to achieve homozygous cultures. PCR and sequencing of the appropriate region were carried out from isolated genomic DNA to confirm the mutation.
2.3.7 Dot Transformation

Liquid cultures were grown with glucose and appropriate antibiotics until the OD at 730 nm was approximately 0.4-0.6. Cells were harvested by centrifuging at 2760g for 7 min, washed twice with BG-11 to remove any traces of glucose and resuspended to an OD of 2.0 in BG-11. A 1 ml aliquot of cell suspension was then mixed with 3 mL of 0.8% agar and poured over the BG-11 plates (Dzelzkalns and Bogorad, 1988). Ten microliters of ~1 µg of DNA was dotted onto the agar/cells mix and the plates grown photoautotrophically. If cells were transformed, colonies appeared in 3-4 weeks.
Chapter 3: Results

3.1 Introduction

The replacement of His-469 with a glutamine residue within the helix VI of the CP47 protein has previously been shown to result in a major reduction in the amount of assembled PS II reaction centres and henceforth their corresponding photoautotrophic growth was reported to be retarded (Eaton-Rye and Vermaas 1992). Moreover, a pseudorevertant for the strain carrying the His to Glu substitution (H469Qrev) was able to grow photoautotrophically at a rate comparable to the control strain and the secondary mutation was not present in the psbB gene. The introduction of Ala, Lys, Pro and Tyr at the 469 position also resulted in unassembled PS II centres (Huang et al., 2001). In order to understand the role of the His-469 residue in CP47, all mutated

![Image of gel electrophoresis](image)

Figure 3.1 Gel electrophoresis showing the PCR products obtained for confirming the mutations in psbB. Lanes: wild type (1); ΔpsbB (2); psbB control (3); H469A (4); H469Arev (5); H469K (6); H469Krev (7); H469P (8); H469Prev (9); H469Q (10); H469Qrev (11); H469Y (12) and H469Yrev (13). The M lane is a 1 kb + ladder with sizes indicated. Samples were run on a 0.8% agarose gel.
strains (H469A, H469Arev, H469K, H469Krev, H469P, H469Prev, H469Q, H469Qrev, H469Y and H469Yrev) were studied using a range of physiological and biochemical measurements. Furthermore, the identity of secondary mutations in the H469Qrev pseudorevertant was also determined by sequencing the H469Qrev strain.

Figure 3.2 Genomic DNA sequence of the *psbB* gene in the H469A, H469Arev, H469K, H469Krev, H469P, H469Prev, H469Q, H469Qrev, H469Y and H469Yrev strains as compared with the control. The red highlighted region represents the corresponding change in amino acid coding for CAT codon confirming its substitution at His-469 of the CP47 protein.
which in turn revealed seven different gene mutations. Different combinations of these 7 mutations were transformed into cyanobacteria. The combination which developed the most colonies was then checked for the presence of the mutations and also the physiological characterisation of the transformed strain, with most colonies, H469Yrev\(^{+6}\) was evaluated. Finally the ability of the H469Yrev\(^{+6}\) strain to restore PS II activity to the various strains carrying substitutions for His-469 was tested using the dot transformation method (Section 2.3.7).

### 3.2 Verification of Strains

To verify whether all the strains carrying substitutions at position 469 of CP47 were still containing their respective mutations, genomic DNA was extracted from the mutants created either in the wild-type genetic background or in the genetic background of H469Qrev pseudorevertant, wild-type, the psbB deletion strain as well as the psbB control strain carrying a kanamycin-resistance cassette downstream of psbB gene (Section 2.1.3) for analysis. Polymerase chain reaction and sequencing of the strains were carried out to confirm the presence of the expected amino acid substitutions or other mutations. Fig. 3.1 shows the PCR-derived DNA fragments in an agarose gel following electrophoresis. Sequencing confirmed that the psbB control strain carried a CAT codon for histidine at the position encoding His-469 in the psbB gene. Similarly for the H469Q and H469Qrev strains a CAG codon, encoding a Gln at the same position, was confirmed (Fig. 3.2). Likewise for the H469A and H469Arev strains, the CAT codon was changed to GCT encoding alanine. In addition for the H469K mutant the CAT codon was confirmed to be changed to an AAA encoding lysine; and for the H469P and H469Y mutants, CCT encoding proline and TAT encoding tyrosine, respectively were confirmed. Furthermore the corresponding codons were confirmed for the H469Krev, H469Prev and H469Yrev strains.

### 3.3 Physiological characteristics of mutants carrying amino acid substitutions at position 469 in CP47

Various physiological assays were carried out to gain information about replacing His-469 in CP47 with Ala, Lys, Pro, Gln, and Tyr. These included how the strains grew photoautotrophically, their ability to evolve oxygen, low-temperature (77 K)
fluorescence emission spectroscopy (to provide information on energy transfer between phycobilisomes and the PS II reaction centre and the extent of PS II assembly), variable fluorescence induction studies to provide information on PS II activity and western blotting to provide details on the protein levels of D1, CP47 and CP43.

3.3.1 Photoautotrophic Growth Curves

Photoautotrophic growth curves for each strain were obtained by measuring the optical density at 730 nm every 24 h for 7 days (Section 2.2.1). Both wild type and the control strain showed similar photoautotrophic growth with a doubling time of ~16 h whereas no growth was observed in the psbB deletion strain (Fig. 3.3 a). This shows that the

Figure 3.3 Photoautotrophic growth curves measured at 730 nm, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
control strain possesses a phenotype similar to the wild-type and the presence of the kanamycin-resistance cassette downstream of \textit{psbB} did not alter its phenotype (besides conferring resistance to kanamycin). A clear decrease in photoautotrophic growth was seen in the H469A, H469K, H469P, H469Q and H469Y strains as compared to control, whereas the introduction of the amino acid changes into the genetic background of the H469Qrev strain led to a partial restoration of photoautotrophic growth for several mutants when compared to the control strain.

Table 3.1 Oxygen evolution rates of mutants created in the wild-type background and in the genetic background of H469Qrev pseudorevertant. Data are the average of at least two independent experiments (or at least 4 measurements) ± the standard error.

<table>
<thead>
<tr>
<th>Strains</th>
<th>DCBQ and K$_3$Fe (CN)$_6$</th>
<th>Bicarbonate</th>
<th>(\mu)mol O$_2$ (mg Chl a)$^{-1}$ h$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>441 ± 43</td>
<td>326 ± 20</td>
<td></td>
</tr>
<tr>
<td>(\Delta\text{psbB})</td>
<td>26 ± 6</td>
<td>15 ± 5</td>
<td></td>
</tr>
<tr>
<td>(\text{psbB Control})</td>
<td>499 ± 18</td>
<td>287 ± 24</td>
<td></td>
</tr>
<tr>
<td>H469A</td>
<td>477 ± 17</td>
<td>294 ± 59</td>
<td></td>
</tr>
<tr>
<td>H469Arev</td>
<td>197 ± 5</td>
<td>229 ± 27</td>
<td></td>
</tr>
<tr>
<td>H469K</td>
<td>35 ± 2</td>
<td>93 ± 4</td>
<td></td>
</tr>
<tr>
<td>H469Krev</td>
<td>167 ± 9</td>
<td>251 ± 65</td>
<td></td>
</tr>
<tr>
<td>H469P</td>
<td>28 ± 6</td>
<td>56 ± 3</td>
<td></td>
</tr>
<tr>
<td>H469P prev</td>
<td>118 ± 11</td>
<td>111 ± 13</td>
<td></td>
</tr>
<tr>
<td>H469Q</td>
<td>159 ± 12</td>
<td>238 ± 12</td>
<td></td>
</tr>
<tr>
<td>H469Q rev</td>
<td>418 ± 28</td>
<td>240 ± 26</td>
<td></td>
</tr>
<tr>
<td>H469Y</td>
<td>90 ± 3</td>
<td>160 ± 7</td>
<td></td>
</tr>
<tr>
<td>H469Y rev</td>
<td>288 ± 25</td>
<td>254 ± 39</td>
<td></td>
</tr>
</tbody>
</table>

Notably the H469A rev, H469Q rev and H469Y rev strains showed substantial recovery in photoautotrophic growth while the H469P prev and H469K rev strains essentially remained only able to support limited photoautotrophic growth (Fig. 3.3 b-d). Both the H469A rev and H469Q rev strains had a doubling time of \(~14\) h whereas the H469A and H469Q mutants had a doubling time of \(~48\) h and \(~84\) h, respectively. Moreover, the H469K, H469P and H469Y mutants showed a substantial increase in doubling time to
around 120-144 h. However, H469Yrev cells were able to grow with doubling times of ~22 h but the H469Krev, H469Prev strains only showed a limited ability to exhibit improved photoautotrophic growth with doubling times of approximately 42, and 90 h, respectively.

3.3.2 Oxygen Evolution

![Oxygen Evolution Diagram](image)

Figure 3.4 (a-d) Oxygen evolution traces measured in the presence of DCBQ and K₃Fe(CN)₆. a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
Oxygen evolution is a non-invasive measurement for probing electron transport where saturating light is used to catalyze the water-splitting reaction. The rate of oxygen evolution corresponds to the rate of turnover of PS II and follows the release of oxygen following the formation of an O-O bond during the water-splitting reaction; for each oxygen evolved, two water molecules are split and four electrons pass down the electron transport chain and four protons are released into the thylakoid lumen. PS II-

![Graphs showing oxygen evolution traces](image)

Figure 3.5 (a-d) Oxygen evolution traces obtained in the presence of bicarbonate, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev, and d) psbB control, H469Y and H469Yrev strains.
specific electron transport was measured in the presence of DCBQ and K$_3$Fe(CN)$_6$ (Section 2.2.2). Removal of the psbB gene essentially abolished oxygen evolution compared to wild type and the control strain (Table 3.1). Replacement of histidine at position 469 of CP47 with other amino acids also resulted in a drastic decline in the ability of the mutants to evolve oxygen (Fig. 3.4 a-d). This suggests that there is either a slowing down of water splitting or a reduced number of assembled PS II centres in these strains. Interestingly, the H469Arev and H469Qrev strains were able to evolve oxygen at a comparable level to wild type and the control strain producing rates of 477 and 418 µmol O$_2$ per mg chlorophyll $a$ per h, respectively. However, the H469Krev, H469Prev and H469Yrev strains exhibited a reduction in oxygen evolution rates by approximately 67%, 77% and 42%, respectively as compared with the control strain. Examples of oxygen evolution traces are presented in Fig. 3.4 (a-d) illustrating the kinetics and the effect of continued light exposure on the different strains.

Whereas PS II–specific electron transport was measured in Fig. 3.4 whole chain electron transport was measured in Fig. 3.5 by omitting DCBQ and K$_3$Fe(CN)$_6$ and adding bicarbonate. Bicarbonate was used to assess electron transport since addition of bicarbonate rises the amount of carbon dioxide available and the subsequent demand for ATP and NADPH which in turn results in an increased rate of electron transport. No significant differences in oxygen evolution were observed for wild type and the control strain. A characteristic delayed response to illumination is seen in the presence of bicarbonate-supported oxygen evolution traces which might be due to the activating of the Calvin-Benson cycle after a short period of illumination (Fig. 3.5 (a-d)). The H469Arev and H469Qrev strains were able to evolve oxygen at rates of 294 and 240 µmol O$_2$ per mg chlorophyll $a$ per h, respectively. Nevertheless the H469Krev, H469Prev and H469Yrev strains exhibited a reduction in oxygen evolution rates by approximately 12%, 62% and 12%, respectively as compared to control cells. While there was general agreement between whole chain and PS II-specific measurements in this assay, the H469Yrev mutant was able to support higher rates of oxygen evolution in the presence of bicarbonate when compared to the rates obtained for this mutant with the PS II-specific assay. This might suggest that native plastoquinone acceptors were
able to perform more efficiently in H469Yrev cells, in the presence of bicarbonate, than the DCBQ artificial quinone acceptor (Fig. 3.5 (d)).

3.3.3 77 K Fluorescence Emission

Since the decline in the rate of oxygen evolution might be due to changes in the levels of PS II assembly, the level of assembled PS II was detected by measuring fluorescence emission following 440 nm excitation of chlorophyll $a$. Fluorescence emission spectra consist of a major PSI emission peak at 725 nm and two minor peaks at 685 and 695 nm.

![Fluorescence Emission Spectra](image)

Figure 3.6 (a-d) 77 K fluorescence emission spectra obtained with 440 nm excitation and normalised to the PS I emission at 725 nm. a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prenv, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
nm representing the CP43 and CP47 core antenna proteins of PS II, respectively. The changes in fluorescence spectral intensity and their shapes were studied by normalising to the PSI emission peak at 725 nm (Fig. 3.6 (a-d)). The control strain showed a slight increase in the amplitude of the 695 nm peak as compared to wild type (Fig. 3.6a).

Removal of the psbB gene resulted in the complete loss of the 695 nm peak with concomitant increase in the 685 nm peak amplitude. Replacement of the histidine residue at the 469 position of the CP47 protein with other amino acids showed similar effects as that seen in the psbB deletion strain, indicating the inability of the PS II

Figure 3.7 (a-d) 77 K fluorescence emission spectra obtained with 580 nm excitation and normalised to the PS I emission at 725 nm, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
reaction centre to incorporate CP47 into the complex. Moreover, the introduction of amino acid changes into the genetic background of the H469Qrev pseudorevertant strain caused varying levels of assembled PS II centres for each strain based on their inability to restore CP47 emission peak at 695 nm along with their reduced oxygen evolution capacity as shown in Table 3.1. For H469Arev, H469Qrev and H469Yrev strains, fluorescence emission spectra were comparable to that of the control strain. However, the H469Krev strain showed limited capability to restore the 695 nm peak shoulder and the H469Prev strain was unable to restore the 695 nm peak.

77 K fluorescence emission was also used to study energy transfer and coupling between the phycobilisome antenna and PS II and PS I by using an excitation wavelength of 580 nm. Typical fluorescence emission from 580 nm excitation consists of a phycocyanin (PC) peak with a maximum emission at 650 nm, an allophycocyanin (APC) peak with a maximum emission at 665 nm, the CP43 emission peak at 685 nm and the CP47 peak with an emission maximum at 695 nm. The 695 nm peak shoulder was more evident in the control strain as compared to wild type. Deletion of the psbB gene showed an increase in the 685 nm peak amplitude with a small reduction in the 650 nm peak amplitude (Fig. 3.7 (a)). Similarly, replacement of His-469 with other amino acids showed a sharp rise in the 685 nm peak amplitude with subsequent loss of the 695 nm peak shoulder suggesting that there is a reduction of assembled PS II centres and/or decreased energy transfer from phycobilisomes in these strains. The fluorescence emission spectra from H469Arev looked similar to that of control cells, whereas H469Krev and H469Prev strains were unable to restore the 695 nm peak (Fig. 3.7 (b, c)). For H469Qrev and H469Yrev strains, the 685 nm peak amplitude was greatly reduced compared to their corresponding mutants and was at a comparable level to the wild type.

3.3.4 Fluorescence Induction

Variable fluorescence induction studies at room temperature were used as a non-invasive tool where light-induced or dark reversible changes in chlorophyll a fluorescence emission of the photosynthetic apparatus were studied, giving information about changes in the quantum yield, number of chlorophyll a in PS II as well as the
electron transport in photosystems. This fluorescence output is referred as the Kautsky transient or ‘OJIP’ transient where the O to J rise represents QA reduction, J to I plateau represents electron transfer through the chain and I to P rise represents complete filling up of all the electron acceptors. Fluorescence spectra were obtained with either a blue measuring flash (BMF) or a red measuring flash (RMF) in the presence or absence of DCMU. DCMU acts by hindering the electron transport at the quinone acceptors of PS.

Figure 3.8 (a-d) Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic blue measuring light, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
II by occupying the binding site for Q₆ thereby preventing photosynthesis. BMF gives evidence about the Q₆ oxidation state by measuring chlorophyll a fluorescence with 455 nm excitation whereas RMF provides phycobilisome-coupled fluorescence emission by using 625 nm excitation. Changes in the amplitude and spectral

Figure 3.9 (a-d) Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic blue measuring light in the presence of DCMU, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
characteristics obtained with BMF or RMF are not directly related because of their different gain and flash intensity settings.

Removal of the psbB gene resulted in significant reduction in variable fluorescence compared to that of the wild-type and control strains, both when measured in the presence or absence of DCMU (Fig. 3.8, 9(a)). However, no significant differences were observed between wild type and control strain. The OJIP fluorescence rise peaks at ~1 s for wild type and the control strain at P and then onwards fluorescence reduces

Figure 3.10 (a-d) Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic red measuring light, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
by ~20%. For the H469Arev and H469Qrev strains, characteristic induction curves were very similar to that of control when assessed using BMF. The mutants H469A, H469K, H469P and H469Y strains exhibited similar fluorescence induction characteristics as that of psbB deletion strain. However, H469Krev, H469Prev and H469Yrev strains, maximum amplitude (F-Fo) were reduced to approximately 76%, 86% and 82%, respectively as that of control strain which suggests less PS II was

Figure 3.11 (a-d) Fluorescence induction curves measured after illuminating dark-adapted cells with a constant actinic red measuring light in the presence of DCMU, a) wild type, ΔpsbB and psbB control, b) psbB control, H469A, H469Arev, H469K and H469Krev, c) psbB control, H469P, H469Prev, H469Q and H469Qrev and d) psbB control, H469Y and H469Yrev strains.
assembled in these strains (Fig. 3.8 (b-d)). Besides, the characteristic J-P rise was either significantly reduced or absent in the psbB deletion strain and in the H469A, H469K, H469P and H469Y strains and in some of the strains when placed under the genetic background of H469Qrev pseudorevertant (H469Krev, H469Prev and H469Yrev). Interestingly, the fluorescence amplitude in the P region was significantly different in the presence of DCMU with no defined pattern. However, the fluorescence yield was similar or increased in the presence of DCMU for wild type, control and H469Arev strains. Interestingly for H469Qrev, H469Krev and H469Yrev strains, the fluorescence yield was increased by the addition of DCMU (Fig. 3.9 (a-d)).

Similarly, no significant differences in fluorescence pattern using RMF was observed between wild type and the control strain although there was a slightly reduced amplitude in the control cells. The OJIP fluorescence rise peaks at ~1 s for both strains at the P level and then onwards fluorescence reduces by ~40% for wild type and ~38% for control, respectively. However, variable fluorescence was greatly reduced when the psbB gene was removed indicative of the absence of assembled PS II complexes. Variable fluorescence observed with RMF were more or less similar to that of the observed BMF for all the strains carrying the amino acid substitutions for His-469. However, the variable fluorescence observed was greatly reduced after the P peak had been reached. This reduction was enhanced in all strains carrying substitutions for His-469 with fluorescence reducing beneath the initial F₀ level except for H469Arev and H469Qrev strains (Fig. 3.10). No appreciable difference in variable fluorescence was observed in the presence of DCMU between BMF and RMF measurements except the dip of the curve below F₀ after the P peak (Fig. 3.11). This decline in fluorescence after P peak might be attributed to a reduction in phycobilisome coupling to PS II during the fluorescence induction measurement (Hwang et al., 2008).

3.3.5 Detection of PS II assembly by Blue –Native PAGE

Assembly of PS II complexes was checked by blue-native polyacrylamide gel electrophoresis (BN-PAGE) followed by western blotting (Fig. 3.12 (a-d)). The wild type and control strain were able to form the three major macromolecular PS II complexes (dimer, monomer and CP43-less monomer). Replacement of His-469 with
other amino acids resulted in the absence of these three major PS II complexes except for H469A and H469Q strains. Moreover, the introduction of amino acid changes into the genetic background of the H469Qrev pseudorevertant showed varying levels of assembled PS II complexes. Notably the H469Arev, H469Krev and H469Yrev strains were able to form assembled PS II complexes at levels relative to those seen in wild type and the control strain. In contrast, the H469Prev strain was unable to assemble beyond the CP43-less monomer stage suggests its inability to form mature assembled PS II complexes. Moreover, there was an increased accumulation of low molecular weight unassembled CP43 complexes which can be seen clearly with the α-CP43 antibody in the psbB deletion mutant, mutants in the wild-type background (H469A, H469K, H469P, H469Q and H469Y) but the level of assembly remained reduced in some of mutants when placed into the genetic background of the H469Qrev strain (H469Krev and H469Prev). Removal of the psbB gene resulted in the absence of the two major macromolecular PS II complexes (dimer and monomer) seen in wild type and control strains which was confirmed with the α-CP47 antibody. Furthermore, there was an enhanced accumulation of some intermediate complexes were seen when

probed with D1 antibody, probably similar in size to RC47 complex (CP43 less monomer) although these intermediate complexes showed less reactivity with α-CP47 antibody.

3.4 Identification of secondary mutation in pseudorevertant H469Qrev

Results of the physiological characterisation of strains carrying substitutions for the His-469 of CP47 revealed substantial evidence that this histidine of CP47 has a different role from other chlorophyll-binding ligands in this protein. However, the precise role of the His-469 remains unclear. Therefore the identification of secondary mutations within the H469Qrev will be critical for understanding the role of His-469 of CP47.

Table 3.2 Characteristics of the 7 gene mutations in pseudorevertant H469Qrev.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>slr1055</td>
<td>Magnesium protoporphyrin IX chelatase subunit H, chlH</td>
<td>Silent mutation, CTC-CTT</td>
</tr>
<tr>
<td>slr0104</td>
<td>Hypothetical protein</td>
<td>TTC-CTC</td>
</tr>
<tr>
<td>sll1496</td>
<td>Mannose 1-phosphate guanyltransferase</td>
<td>TGT-TAT</td>
</tr>
<tr>
<td>sll0862</td>
<td>Hypothetical protein</td>
<td>Stop codon, TGG-TGA</td>
</tr>
<tr>
<td>ssl5096</td>
<td>Unknown protein</td>
<td>Silent mutation, GCG-GCA</td>
</tr>
<tr>
<td>slr0930</td>
<td>Hypothetical protein</td>
<td>GGC-GTC</td>
</tr>
<tr>
<td>slr0851</td>
<td>Type 2 NADH dehydrogenase, ndbA</td>
<td>ACT-AGT</td>
</tr>
</tbody>
</table>

In order to identify the secondary mutation within H469Qrev, genomic DNA was isolated and purified using the Zymo Genomic DNA clean and concentrator (D4010) kit and was sent for sequencing to New Zealand Genomic Limited (NZGL). Sequencing of H469Qrev showed seven distinct gene mutations in the strain (Table 3.2). To examine these mutations, primers and sequencing primers were designed. Polymerase chain reaction and Sanger sequencing of the H469Q and H469Qrev mutants was carried out to confirm the presence or absence of the 7 mutations in both
Figure 3.13 Gel electrophoresis showing PCR-derived products from either the H469Q or the H469Qrev strains for each of the 7 mutated genes identified by genomic sequencing. A) H469Q and B) H469Qrev DNA fragments. The M lane is a 1 kb + ladder with sizes indicated. Lane: slr1055 (1); slr0104 (2); sll1496 (3); sll0862 (4); ssl5096 (5); slr0930 (6) and slr0851 (7) gene. Samples were run on 0.8% agarose gel. Primers used for amplifying the specific genes were the corresponding forward and reverse primers indicated in Table 2.2.

strains. Fig. 3.13 shows the PCR-derived H469Q and H469Qrev DNA fragments using different primers in an agarose gel electrophoresis.

Upon Sanger sequencing, no mutations were found in the H469Q-derived PCR products whereas they were confirmed in the PCR products obtained from the genomic DNA of the H469Qrev strain. Moreover, in addition to the 7 gene mutations indicated by genomic sequencing, some additional indels were also identified (Table 3.2). Sequencing of the PCR product conforming to the slr0104 gene showed deletion of 3 amino acids in addition to the mutation already identified (Table 3.2). Similarly, deletion of 17 amino acids along with the mutation region were detected by Sanger sequencing of the PCR product corresponding to the slr0851 gene whereas an
addition of 33 amino acids within the mutation region were seen in the sequencing of PCR product of sll1496 gene (Fig. 3.14).

Figure 3.14 Genomic DNA sequence of the 7 genes carrying mutations in pseudorevertant H469Qrev. The red highlighted region represents the corresponding change in sequence as confirmed by Sanger sequencing.
Ligation of the PCR products corresponding to the 7 genes into the pGEM vector were carried out and checked for errors by restriction digestion. Fig. 3.15 shows the gel electrophoresis after restriction digestion with EcoR1. Bands obtained were in agreement with the plasmid map generated by Snap Gene® (Version 2.6.2). For example, restriction digestion of the plasmid containing the PCR product corresponding to the slr1055 gene was expected to give a 7961 bp product (Fig. 3.15). Other plasmid maps are shown in Appendix 1. Each of the 7 plasmids was sequenced and in each case the presence of the expected mutations was confirmed. These plasmids were used to transform the H469Y strain which has reduced photoautotrophic growth on BG-11 plates and colonies were developed photoautotrophically after 3-4 weeks (Fig. 3.16). This was carried out so that H469Y cells were transformed with 1, 2, 3, 4, 5, 6, or all 7 of the plasmids corresponding to the gene mutations derived from PCR.
products (see Table 3.2). The particular combination transformed with plasmids 1

Figure 3.16 Cyanobacterial transformation of the H469Y strain A (1) Negative control; (2) with genomic DNA from the wild-type; (3) genomic DNA from the H469Qrev and (4) genomic DNA from the H469Yrev strains, and B(1) with the plasmids in Table 3.2 containing the 7 PCR-products corresponding to the unique mutations in the H469Qrev strain, (1) slr1055 gene; (2) slr1055 and slr0104; (3) slr055, slr0104 and sll1496 and (4) slr055, slr0104, sll1496 and sll0862 and C(1) with the plasmids in Table 3.2 containing the 7 PCR-products corresponding to the unique mutations in the H469Qrev strain, (1) slr1055, slr0104, sll496, sll0862 and sll5096; (2) slr1055, slr0104, sl1496, sll0862, ssl5096 and slr0930 and (3) slr1055, slr0104, sl1496, sll0862, ssl5096, slr0930 and slr0851.
through 6 (excluding the slr0851 gene) developed the most colonies compared to other combinations used. Then a single colony from this particular transformed strain was restreaked onto a fresh BG-11 plate containing glucose. The strain obtained in the genetic background of H469Y containing the combinations of 6 plasmids corresponding to the gene mutations was then designated as H469Yrev+6. Genomic DNA of H469Yrev+6 was then isolated and sequenced. Interestingly upon Sanger sequencing, no mutations were found in the 6 genes thought to have been transformed into the H469Yrev+6 strain. Physiological characterisation of the H469Yrev+6 strain was carried out to understand their photoautotrophic growth and also to identify the changes in the PS II assembly caused by the new suppressor present in the H469Yrev+6 strain.

### 3.4.1 Physiological Characterisation of H469Yrev+6

Physiological characterisation of the H469Yrev+6 strain was evaluated in terms of photoautotrophic growth, ability to evolve oxygen and levels of functional PS II centres.

![Photoautotrophic growth curves](image)

Figure 3.17 Photoautotrophic growth curves measured at 730 nm from wild-type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains.
and was compared with that of the wild-type, H469Y, and H469Yrev strains as well as a strain containing a particular combination of transformed PCR plasmids with all 7 unique mutations found in the H469Qrev strain (designated as H469Yrev+7).

### 3.4.1.1 Photoautotrophic Growth Curve

Both H469Yrev+6 and H469Yrev+7 strains showed similar photoautotrophic growth with a doubling time of ~16 h and at a comparable level as that of the wild-type strain (Fig. 3.17). Furthermore, a significant increase in their ability to grow photoautotrophically was seen as that of H469Yrev strain which has a doubling time of ~22 h. Thus it appears that the new suppressor present in H469Yrev+6 and H469Yrev+7 cells contributed to their substantial recovery in photoautotrophic growth.

### 3.4.1.2 Oxygen Evolution

Substitution of tyrosine at position 469 of the CP47 protein reduced the saturated rate of steady-state oxygen evolution in the presence of DCBQ and K$_3$Fe(CN)$_6$ by approximately 80%. However, the H469Yrev strain exhibited a reduction in oxygen evolution rates by approximately 35% as compared to the wild-type strain. Interestingly, the H469Yrev+6 and H469Yrev+7 strains were able to evolve oxygen at a comparable level to wild type giving a rate of 441 and 440 µmol O$_2$ per mg chlorophyll a per h, respectively (Fig. 3.18).

Table 3.3 Oxygen evolution rates for wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7. Data are the average of at least two independent experiments (or at least 4 measurements) ± the standard error.

<table>
<thead>
<tr>
<th>Strains</th>
<th>DCBQ and K$_3$Fe(CN)$_6$</th>
<th>Bicarbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol O$_2$ mg Chl a$^{-1}$/h</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>441 ± 43</td>
<td>326 ± 20</td>
</tr>
<tr>
<td>H469Y</td>
<td>90 ± 3</td>
<td>160 ± 7</td>
</tr>
<tr>
<td>H469Yrev</td>
<td>288 ± 25</td>
<td>254 ± 39</td>
</tr>
<tr>
<td>H469Yrev+6</td>
<td>441 ± 98</td>
<td>246 ± 60</td>
</tr>
<tr>
<td>H469Yrev+7</td>
<td>440 ± 18</td>
<td>239 ± 78</td>
</tr>
</tbody>
</table>
Whole chain electron transport was measured by adding bicarbonate and these traces are shown in Fig. 3.19. Oxygen evolution rates supported by bicarbonate were significantly improved for the strains carrying substitutions for His-469 compared to the rates observed when DCBQ was used as a PS II-specific electron acceptor.

The H469Yrev+6 and H469Yrev+7 strains exhibited a reduction in oxygen evolution rates by approximately 25% to that exhibited by the wild-type strain. Interestingly,

Figure 3.18 Oxygen evolution traces measured in the presence of DCBQ and K3Fe(CN)6 from wild-type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains.
bicarbonate-supported oxygen evolution remained better for the H469Yrev strain than that observed in either H469Yrev$^{+6}$ or H469Yrev$^{+7}$ cells.

3.4.1.3 77 K Fluorescence Emission

The relative change in the level of assembled PS II centres was measured by fluorescence emission experiments by using 440 nm and 580 nm excitation wavelengths. When the fluorescence emission spectra were normalised to the 725 nm peak arising from PSI, the H469Y strain showed an enhancement in the 685 nm emission with a corresponding loss of the 695 nm peak as compared to wild type.
However, H469Yrev cells showed a reduction in the 685 nm peak amplitude with the appearance of a shoulder at 695 nm. For the H46Yrev\textsuperscript{+6} and H46Yrev\textsuperscript{+7} strains, an improved PS II assembly was seen by a decreased emission from 685 nm with concomitant appearance of 695 nm peak shoulder (Fig. 3.20).

Fluorescence emission spectra collected from wild-type, H469Y, H469Yrev, H46Yrev\textsuperscript{+6} and H46Yrev\textsuperscript{+7} strains using excitation at 580 nm revealed changes in the phycobilisome coupled energy transfer for each of these strains (Fig. 3.21). As mentioned earlier in Section 3.3.3, the H469Y strain showed an increase in the 685 nm peak amplitude with a slight reduction in the 650 and 665 nm peaks as compared to wild type. However, H469Yrev showed marginal recovery in all of the above

Figure 3.20 77 K fluorescence emission spectra obtained with 440 nm excitation and normalised to the PS I emission at 725 nm from wild type, H469Y, H469Yrev, H469Yrev\textsuperscript{+6} and H469Yrev\textsuperscript{+7} strains.
mentioned emission peaks. For H469Yrev+6 and H469Yrev+7 cells, the amplitudes of 650 and 665 nm peaks were greatly reduced with associated increase in the amplitude of the 685 nm peak as that of the wild-type strain.

3.4.1.4 Fluorescence Induction

Variable fluorescence spectra were measured with either BMF or RMF in the presence or absence of DCMU (Figs. 3.22-3.23). For the H469Yrev+6 and H469Yrev+7 strains, significant reduction in the variable fluorescence was seen in all the strains where His-469 was replaced by a tyrosine residue. Besides, the characteristic J-P rise was either significantly reduced or absent in the H469Y and H469Yrev strains. (Fig. 3.22).
Interestingly, the fluorescence amplitude in the P region was different in the presence

Figure 3.22 Fluorescence Induction Curves measured after illuminating dark-adapted cells with a) constant actinic blue measuring light and b) presence of DCMU from wild type, H469Y, H469Y.rev, H469Y.rev+6 and H469Y.rev+7 strains.
of DCMU with no defined pattern. Moreover, the fluorescence yield was also greatly reduced for the strains carrying substitutions for His-469 in the presence of DCMU.

Figure 3.23 Fluorescence Induction Curves measured after illuminating dark-adapted cells with a) constant actinic red measuring light and b) presence of DCMU from wild type, H469Y, H469Yrev, H469Yrev+6 and H469Yrev+7 strains.
Variable fluorescence observed with RMF was more or less similar to that of the observed BMF for all the strains carrying the tyrosine substitution for His-469. Besides, the variable fluorescence observed was greatly reduced after the P peak had been reached but the extent of the reduction was increased for the strains carrying substitutions for His-469 with the fluorescence amplitude reducing below the initial F₀ level (Fig. 3.23). Moreover, no appreciable differences in the fluorescence induction characteristics were observed in the presence of DCMU between BMF and RMF measurements for strains containing substitutions for His-469 except the dip of the curve below F₀ after the P peak (Fig. 3.23(a)).

### 3.4.2 Dot Transformation

To test whether the H469Yrev⁺⁶ strain has the capability to restore PS II activity to the other strains containing substitutions for His-469 of CP47, the dot transformation technique (section 2.3.7) was used as described by Ermakov-Gerdes and Vermaas.
(1999). This technique depends on the fact that cyanobacteria can take up genomic DNA and integrate it into their genome by homologous recombination (Grigorieva and Shestakov, 1982). Cell suspensions of mutant strains (H469A, H469K, H469P, H469Q and H469Y) along with 0.8% top agar were poured onto BG-11 agar plates. Once the lawn of the mutant strains was dry, 10 µL of ~1 µg genomic DNA from the H469Yrev+6 strain was dotted onto the above mentioned mutant cell suspensions. Only cells that received external genomic DNA from H469Yrev+6 strain were transformed. Cell colonies appeared where the genomic DNA was dotted after 3-4 weeks demonstrating photoautotrophic growth had been restored (Fig. 3.24.) Concentrated cell colonies were seen in the H469A, H469K and H469P strains where genomic DNA from H469Yrev+6 strain was applied. However, in the H469Q and H469Y strains, only background cell colonies were seen on the spots where the genomic DNA from H469Yrev+6 strain was dotted.
**Chapter 4: Discussion**

Thirteen of the 16 chlorophyll molecules located in the transmembrane α-helices I-VI of CP47 are coordinated by histidine ligands (His-9, His-23, His-26, His-100, His-114, His-142, His-157, His-201, His-202, His-216, His-455, His-466 and His-469) (Eaton-Rye and Putnam-Evans, 2005). Substitution of the histidine residues within the transmembrane helices I-IV with tyrosine using site-directed mutagenesis not only exhibited a significant loss in their ability to evolve oxygen but also decreased photoautotrophic growth as well as decreased the number of assembled PS II centres. Furthermore, replacing histidine with asparagine resulted in a reduced impairment on PS II stability and function as compared to tyrosine substitution (Shen et al., 1993). This suggests that the phenotype obtained by tyrosine substitution resulted in the formation of pheophytin in the mutants, which in turn explained decreased antenna efficiency observed in these strains (Shen and Vermaas, 1994). Site-directed mutagenesis studies were carried out by Eaton-Rye and Vermaas (1992) on the then putative chlorophyll ligands His-455, His-466 and His-469 within helix VI, by converting them to glutamyl residues. The H455Q and H466Q mutants possessed phenotypes similar to the control strain whereas the H469Q mutant resulted in reduced photoautotrophic growth and fewer assembled PS II centres. Moreover, a spontaneous pseudorevertant obtained from the H469Q strain showed similar characteristics to that of the control. Gln was unable to replace His-469 and the number of assembled functional PS II centres in H469Q strain dropped to ~20% of that found in the wild-type strain. This suggests that the histidine residue at position 469 (or the binding of the corresponding chlorophyll) might have an important role related to PS II assembly and stability; furthermore the secondary mutation seen in the pseudorevertant (H469Qrev) was not present in the psbB gene. Besides, the introduction of Ala, Lys, Pro and Tyr at the position corresponding to His-469 in the CP47 protein resulted in obligate heterotrophic strains with a decreased number of assembled PS II centres and impaired photoautotrophic growth (Wu et al., 1999; Huang et al., 2001).
In order to understand the precise role of His-469 of CP47, physiological characterisation of the strains carrying substitutions for His-469 were studied by measuring their photoautotrophic growth, oxygen evolution rates and levels of functional PS II centres. Moreover, the identity of the secondary mutation in the H469Qrev pseudorevertant was studied by sequencing the strain and this identified 7 distinct gene mutations. In order to evaluate if these mutations were responsible for the H469Qrev phenotype, plasmids incorporating these seven mutations derived from PCR products were constructed and subsequently transformed, in various combinations of these 7 mutations, into cyanobacteria (specifically the H469Y strain that was severely impaired in photoautotrophic growth). The particular combination of transformed PCR plasmids (PCR products) which developed the most colonies was then selected and physiological characterisation of the strain was evaluated in terms of photoautotrophic growth, ability to evolve oxygen and levels of assembled PS II centres. Finally the ability of the genomic DNA from the corresponding transformed strain (H469Yrev*) to restore PS II activity to other strains carrying substitutions for His-469 (H469A, H469K, H469P, H469Q and H469Y) was tested.

4.1 Physiological characteristics of mutants carrying amino acid substitutions at position 469 in CP47

In this study, various mutations encoding the amino acids Ala, Lys, Pro, Gln and Tyr that had been introduced so as to create the corresponding mutant strains, in two different genetic backgrounds, were studied to understand the resulting phenotypes of each of these strains. The mutations had previously been introduced in the wild-type background and also into the genetic background of the H469Qrev pseudorevertant (Huang, 2003). To select for mutants a kanamycin-resistance cassette was positioned downstream of the \textit{psbB} gene, accordingly — in earlier work — a control strain had been developed into which only the kanamycin-resistance cassette was present and no additional mutations were present in \textit{psbB} (Eaton-Rye and Vermaas, 1991). Results presented here show that the \textit{psbB} control strain has similar photoautotrophic growth, oxygen evolution rate, and levels of assembled PS II complexes as seen by 77 K
fluorescence emission spectra and variable fluorescence induction studies as well as protein levels of D1, CP47 and CP43 as assessed by western blotting to that of the wild-type strain. Hence this work confirmed that the presence of the kanamycin-resistance cassette downstream of the \textit{psbB} gene in control cells didn’t alter its phenotype, relative to wild type, beyond introducing resistance to kanamycin (Figs. 3.3-3.5). Furthermore, this study confirmed that deletion of \textit{psbB} resulted in complete absence of photoautotrophic growth, lack of oxygen evolution and a virtual loss of PS II reaction centre components from the thylakoid membrane; hence it was confirmed that CP47 is essential for functional assembly of PS II as first reported in Vermaas \textit{et al.} (1988).

The five strains, H469A, H469K, H469P, H469Q and H469Y, exhibited reduced photoautotrophic growth and low oxygen evolution rates as compared to the wild-type and control strains (Figs. 3.3-3.5). In the case of H469Q strain, the oxygen evolution rate was reduced by 70\%, in agreement with Eaton-Rye and Vermaas (1992) and this was restored in the H469Qrev pseudorevertant. On the other hand, the introduction of amino acid changes into the H469Qrev genetic background resulted in a range of phenotypes whereby some resembled wild type or control while other were only marginally improved compared to their corresponding mutants in the wild-type background. For example, the H469Arev strain also showed similar photoautotrophic growth and rate of oxygen evolution as that of wild type and the control strain. Hence the genetic background able to recover the H46Q strain to be similar to wild type was also able to restore the mutant with an Ala residue at position 469. The expectation is that Gln substitutes for His as a chlorophyll ligand due to the presence of the amide nitrogen in its side chain. Since Ala lacks this side chain, the role of the suppressor mutation(s) in H469Qrev and H469Arev cells appears to not be associated with the axial ligation of Mg$^{2+}$ via nitrogen but instead rescues some other characteristic that is lacking when His is not present at position 469. Therefore these results suggest His at position 469 of CP47 has a distinct role compared to other histidine residues, for example His-455 and His-466 within helix VI of the CP47 protein. Additionally the H469Krev, H469Prev and H469Yrev strains could not evolve oxygen or exhibited decreased photoautotrophic growth, suggesting these mutants had impaired PS II assembly even in the pseudorevertant genetic background. However, the rate of oxygen
evolution supported by bicarbonate was moderately improved over that supported by the PS II-specific electron acceptor DCBQ in all the strains carrying substitutions for His–469 especially the H469Yrev strain (Fig. 3.5). This suggests that the native quinone from the plastoquinone pool is able to function more effectively than the artificial quinone in the Q_b site of PS II in the CP47 mutants and therefore His–469 of CP47 indirectly contributes to the conformation or accessibility of the Q_B-binding site. Amongst the different substitutions, the presence of Pro resulted in the least response to the presence of bicarbonate and Tyr showed the greatest difference between oxygen-evolution rates supported by either DCBQ or bicarbonate. This might be due to the characteristics of these different amino acids such as the cyclic nature of Pro and the available hydroxyl present on the Tyr residue. Moreover, it has been previously shown that a chlorophyll molecule in PsaB is coordinated by the oxygen atoms from a Tyr side chain in the cyanobacterial PS I reaction centre (Jordan et al., 2001). Thus whereas the pseudorevertant rescued the H469A mutant which lacks a nitrogen for Mg^{2+} coordination, coordination via the hydroxyl of Tyr may in fact contribute to the improvement observed in the H469Yrev strain. Nevertheless, in the wild-type background the H469A strain was less impaired than the H469Y mutant.

The 77 K fluorescence spectra obtained in Fig. 3.6 and Fig. 3.7 shows that wild type and the control strain possess a 695 nm peak originating from PS II which was absent in the psbB deletion strain, suggesting that the emission arises from a chlorophyll a molecule in CP47 (Tang and Satoh, 1984; Pakarsi et al., 1985; Vermaas et al., 1986) and the 685 nm peak arises from pigments associated with CP43 (Eaton-Rye and Vermaas, 1991, 1992). Enhanced fluorescence emission from the 685 nm peak was observed in the psbB deletion strain and the strains containing substitutions for His-469 in the wild-type background, indicative of a reduced number of PS II centres or progressive destabilisation of the assembled PS II reaction centres (Shen et al., 1993). Moreover, an increased emission from allophycocyanin b might contribute to the 685 nm emission peak because of the absence of assembled PS II centres in these strains (Eaton-Rye and Vermaas, 1991). Reduction in the 685 nm peak fluorescence emission with concomitant restoration of the 695 nm peak was observed in H469Arev, H469Qrev and H469Yrev strains upon 440 nm excitation suggesting the ability of these
strains to form assembled PS II centres (Fig. 3.6). This interpretation is further supported by their corresponding protein levels of D1, CP47 and CP43 as assessed by western blotting (Fig. 3.12). Moreover, upon 580 nm excitation, an increased fluorescence emission from phycobilisomes was seen in all strains carrying substitutions for the His-469 residue in the wild-type background suggesting that the phycobilisomes are not coupled to PS II (Fig. 3.7). Reduction or absence of the 695 nm peak in strains carrying substitutions for the His-469 residue suggests that His-469 is crucial for the stable incorporation of CP47 into the thylakoid membrane and hence essential for PS II stability.

The impaired growth of the mutants in the wild-type background (H469A, H469K, H469P, H469Q and H469Y) and some of the strains in the genetic background of H469Qrev pseudorevertant (H469Krev and H469Prev) were associated with their reduced level of assembled PS II centres as seen by their enhanced emission at 685 nm and lack of the 695 nm peak (Fig. 3.6). In addition a decreased PS II specific variable fluorescence using BMF and RMF (Fig. 3.8 and Fig. 3.10) and an absence or reduced levels of PS II dimers and monomers were also observed (Fig. 3.12). Additionally, the reduced levels of assembled PS II centres in these strains were also accompanied by an increased accumulation of unassembled CP43 sub-complexes, in agreement with their increased 685 nm emission seen by 77 K fluorescence emission spectra (Fig. 3.6). Furthermore, an enhanced accumulation of some intermediate complexes was seen when isolated thylakoids were probed with a D1 antibody whereas these putative intermediate complexes showed less reactivity with the α-CP47 antibody. This suggests that a subcomplex may be accumulating in these strains at a similar molecular weight to that of the CP43-less subcomplex but which lacked the CP47 protein. One possibility is that the reaction centre D1-D2-Cyt b559 subcomplex is present with the Ycf48 subunit still attached; however, to test this we would need an antibody against Ycf48 and so this remains to be followed up in future studies. The apparent reduced levels of CP47 in the mutants in the wild-type genetic background may either be due to increased turnover of the protein or a reduced rate of protein synthesis. It has also been suggested earlier that the absence of PS II proteins in mutants where putative
chlorophyll ligands had been targeted could be due to rapid turnover of CP47 or CP43 (Yu and Vermaas, 1990).

The results presented here suggest that the extent of the decrease in photoautotrophic growth, oxygen evolution capability and reduced number of assembled PS II reaction centres in mutants created in the wild-type background, clearly depends on the nature of the mutation introduced in agreement with earlier studies by Shen et al. (1993). Moreover, the secondary mutation in the H469Qrev pseudorevertant was able to partially recover the disruption caused by substitution for His-469 in agreement with measurements of oxygen evolution and photoautotrophic growth reported in Huang (2003). It would appear that the structural disruption caused by the substitutions introduced in place of His-469 might be a factor in the degree of restoration achieved by the secondary mutation in the H469Qrev pseudorevertant. Interestingly, Eaton-Rye and Putnam-Evans (2005) noted that chlorophyll 35 in CP47 is coordinated by His-

Figure 4.1. Organisation of histidine residues and their corresponding chlorophyll molecules located in the transmembrane α-helices VI of CP47. The distance between the histidine residues (His-455, His-466 and His-469) to its nearest chlorophyll molecule were calculated using using Protein Data Bank (PDB) coordinates 3WU2 from Umema et al 2011 in PyMol version 1.3 (DeLano Scientific, 2002).
469, which in turn is involved in transferring energy to the PS II reaction centre (Vasilév et al., 2001; Ferreira et al., 2004). Using the crystal structure of PS II complex from *Thermosynechococcus vulcanus* by Umena et al. (2011), histidine residues within helix VI of CP47 were matched to their closest chlorophyll molecule. The distance from the imidazole nitrogen of the histidine residues (His-455, His-466 and His-469) to the Mg$^{2+}$ of the chlorophyll molecule were calculated and are shown in Fig. 4.1. All three histidine residues within helix VI showed a distance of 2.2 Å from their nearest chlorophyll molecule which compares well with the usual co-ordination distances of 1.9-2.4Å originally seen in the bacterial reaction centre and LH2 antenna system (Deisenhofer et al., 1985; McDermott et al., 1995). Therefore the structural information confirms that His-469 can indeed serve as a ligand to its adjacent chlorophyll molecule but in this case Gln cannot support Mg$^{2+}$ ligation at this position, even though it can support Mg$^{2+}$ ligation at the His-455 and His-466 positions. Therefore, the substitutions for His-469 might be causing a structural change in the architecture of CP47 that prevents the correct folding of CP47 and that subsequently disrupts further PS II biogenesis.

### 4.2 Identification of secondary mutation in pseudorevertant H469Qrev strain

Genomic sequencing of the H469Qrev pseudorevertant was carried out to identify the gene responsible for the secondary mutation which in turn identified seven different mutations in the strain. Plasmids containing each of these distinct gene mutations were constructed and transformed in various combinations into cyanobacteria. The specific combination of 6 transformed gene plasmids (H469Yrev$^+$), developed the most colonies photoautotrophically, as compared to other combinations (Fig. 3.16). Surprisingly, using Sanger sequencing, no mutations were found in the 6 candidate genes thought to have been transformed into the H469Yrev$^+$ strain. However, the H469Yrev$^+$ strain showed improved photoautotrophic growth and oxygen evolution supported by DCBQ over that found in the H469Yrev strain and in fact the H469Yrev$^+$ strain exhibited rates comparable to those of the wild-type strain. Interestingly and in contrast, bicarbonate-supported oxygen evolution in the H469Yrev strain remained
above that of H469Yrev+6 cells (Fig. 3.19). Nevertheless the increased growth of the H469Yrev+6 strain was also associated with an improved level of PS II assembly compared to H469Yrev as seen by a decreased 685 nm emission and appearance of the 695 nm peak in low temperature fluorescence emission spectra obtained with 440 nm excitation (Fig. 3.20). The improved performance of H469Yrev+6 cells was further supported by an improved PS II specific variable fluorescence using BMF and RMF in Fig. 3.22 and Fig. 3.23, respectively. In contrast, an enhanced fluorescence emission from phycobilisomes was seen in H469Y, H469Yrev and H469Yrev+6 strains upon 580 nm excitation (Fig. 3.21). Thus it appears the phycobilisomes may not be correctly attached or able to efficiently transfer energy to PS II centres in any strain where His-469 has been substituted with a tyrosine residue.

To identify mutations able to restore photoautotrophic growth and oxygen evolution, the approach taken utilised the ability of a lawn of Synechocystis sp. PCC 6803 to take up DNA. In this method the DNA of interest is spotted onto a lawn of recipient cells and colonies arising from transformed cells are isolated. Once complete segregation has been confirmed, these cells can then be investigated using physiological assays to characterise their ability to grow and evolve oxygen (Grigorieva and Shestakov, 1982; Dzelzkalns and Bogorad, 1988). This approach was used to test whether the genomic DNA derived from the H469Yrev+6 strain was able to restore photoautotrophic growth and PS II activity to the other strains carrying substitutions for His-469 (H469A, H469K, H469P, H469Q and H469Y). It was found that the secondary mutation, in the H469Yrev+6 strain, was able to restore photoautotrophic growth to the H469A, H469K and H469P strains. Interestingly, the secondary mutation present in the H469Yrev+6 strain was incapable of restoring photoautotrophic growth to H469Q and H469Y strains (Fig. 3.24). Thus it appears that the new suppressor present in the H469Yrev+6 cells was able to restore photoautotrophic growth in the mutants which were not carrying putative chlorophyll ligands (i.e. Ala, Lys, Pro) whereas the two strains with Gln and Tyr – both of which have been shown to ligate chlorophyll – were not responsive to the suppressor introduced by transformation with the H469Yrev+6 genomic DNA.
The results here show that the mutation restoring photoautotrophic growth to the H469Yrev\textsuperscript{+6} strain was distinct from the mutation responsible for the H469Qrev pseudorevertant. The blue highlighted region represents the corresponding sequence in the H469Yrev\textsuperscript{+6} strain showing absence of the mutation as confirmed by Sanger sequencing.

Figure 4.2 Genomic DNA sequence of the 6 genes introduced into the H469Yrev\textsuperscript{+6} strain that were originally found in the genomic DNA of H469Qrev pseudorevertant.
pseudorevertant phenotype. Sanger sequencing of the 6 genes introduced into H469Yrev*6 that were originally identified in from the genomic DNA of H469Qrev revealed that the mutations had not been incorporated by the H469Yrev*6 cells. However, there are multiple copies of the chromosome in Synechocystis sp. PCC 6803 and it is possible that the transformed H469Yrev*6 cells were heterozygous and although predominantly carrying the wild-type allele, having at least one copy of the introduced mutation was sufficient to support recovery. Sanger sequencing results showing the genomic DNA sequence from the 6 genes introduced into H46Yrev*6 strain are shown in Fig. 4.2. However, the inability of the isolated genomic DNA to restore H469Q and H469Y together with the Sanger sequence result (Fig. 4.2) does not support this interpretation. Moreover, levels of assembled PS II centres, as seen by 77 K fluorescence emission spectroscopy and variable fluorescence induction studies were also different for the H469Qrev and the H469Yrev*6 strains. Sequencing of the transformed strain, H469Yrev*6 should be carried out to identify the gene (or genes) responsible for rescuing photoautotrophic growth in the H469A, H469K and H469P cells.

4.3 Conclusions and Future Prospects

This study presents the physiological characteristics of strains carrying substitutions for His-469 of CP47 that had been created in either the wild-type genetic background or in the genetic background of the previously obtained H469Qrev pseudorevertant. The strains in the wild-type background showed loss of photoautotrophic growth, decreased oxygen-evolving capability and fewer assembled PS II reaction centres, indicative of the disruptive effect caused by swapping out His-469 for other amino acids. Hence the His at position 469 of CP47 plays an important role in the proper incorporation of this subunit during assembly of PS II. In the course of this study a second pseudorevertant (H469Yrev*6) was obtained that restored photoautotrophic growth to the H469A, H469K and H469P strains but not the H469Q or H469Y cells. This suggests that there are different mutations in the H469Yrev*6 cells and the H469Qrev strain that can restore photoautotrophic growth to cells with mutations at position 469 of CP47. Sequencing of the genomic DNA from the H469Yrev*6 strain
might provide information about the gene or genes responsible for recovering photoautotrophic growth in the three strains where the introduced amino acid is not expected to act as a chlorophyll ligand. Moreover, chlorophyll a fluorescence studies designed to look at electron transfer between the $Q_A$ and $Q_B$ plastoquinone electron acceptors should be carried out to understand the putative conformational changes of the $Q_B$-binding site observed in the various mutants (Figs. 3.4 and 3.5). Additionally, the possibility that a reaction centre D1-D2-Cyt $b_{559}$ subcomplex accumulates in strains where His-469 has been replaced by other amino acids (Fig. 3.12) should be investigated. Given the apparent size of this putative “stalled” assembly complex, we have speculated that Ycf48 may be present and this could be tested by additional BN-PAGE studies employing an antibody raised against the Ycf48 protein.
REFERENCES


APPENDIX

Plasmid Maps of 1) slr004, 2) sll1496, 3) sll0862, 4 ssl5096, 5) slr0930 and 6) slr0851 showing the restriction site used in the study.

1)

2)