A gene required for the regulation of photosynthetic light harvesting in the cyanobacterium *Synechocystis* PCC6803

A thesis submitted for the degree of Doctor of Philosophy by

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THESIS ABSTRACT

In cyanobacteria, state transitions serve to regulate the distribution of excitation energy delivered to the two photosystem reaction centres from the accessory light harvesting system, the phycobilisome. The trigger for state transitions is the redox state of the cytochrome $b/f$ complex/plastoquinone pool. The signal transduction events that connect this redox signal to changes in light harvesting are unknown. In order to identify signal transduction factors required for the state transition, random cartridge mutagenesis was employed in the cyanobacterium *Synechocystis* PCC6803 to generate a library of random, genetically tagged mutants. The state transition in cyanobacteria is accompanied by a change in fluorescence emission from PS2. By using a fluorescence video imaging system to observe this fluorescence change in mutant colonies it was possible to isolate mutants unable to perform state transitions. Five such mutants were isolated and in all cases disruption of the gene sll1926 found to account for the phenotype. sll1926 has only one significant homologue in current databases, a gene in *Anabaena* PCC7120. A hydropathy analysis would suggest that both proteins are membrane associated. An insertional inactivation mutant of sll1926 ($\Delta$sll1926) is specifically unable to perform state transitions: no impairments in electron transport, in light harvesting, or in reaction centre/phycobilisome assembly or function are evident. The product of sll1926 may therefore be a signal transduction factor. sll1926 inactivation in mutants lacking photosystem 1 or photosystem 2 leads to changes in phycobilisome coupling with the remaining photosystem. This strongly supports a mobile phycobilisome model for state transitions and suggests that state transitions are brought about by changes in the affinity of the phycobilisomes for both photosystems. $\Delta$sll1926 has a doubling time similar to the wild-type at all but very low light intensities. This implies that a major physiological role of state transitions is to maximise the efficiency of light utilisation.
To the Memory

of

Bernard F.G. Levy

1911 - 1993
ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Conrad Mullineaux for all his advice and guidance throughout my Ph.D. I would also like to thank him for his many helpful comments during the preparation of this thesis.

I would also like to thank Dr. Mark Ashby for all his advice and help.

In addition I would like to thank Dr. Adam Watson, Dr. David Stevens, Dr. Nickie Gumpel and Dr. Kat Madagan for their practical advice and Prof. Nugent and Dr. Nick Fisher for help with sequence analysis. I am grateful to Laura Winskill for DNA sequencing and Bose Felix for the preparation of media. I would also like to thank Elinor Thompson and Chris Emlyn-Jones for their thorough proof-reading of this thesis.

I am grateful to Dr. Nick Mann and Julie Scanlan for help with phospholabelling experiments and also Dr. Peter Nixon and Paulo Silva for help with photoinhibition experiments. I am also grateful to Wim Vermaas (Arizona State University) for providing the *Synechocystis* PCC6803 PS2' mutant and Christiana Funk (Stockholm University) for providing the *Synechocystis* PCC6803 PS1' mutant.

Finally, I am grateful to the BBSRC for funding this research.
ABBREVIATIONS

ATP.............................Adenosine triphosphate
CD...............................circular dichroism spectroscopy
chla................................chlorophyll a
cytf............................cytochrome b(6)f complex
DBMIB........................2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCBQ..........................2,6-dichloro-p-benzoquinone
DCMU..........................3-(3,4-dichlorophenyl)-1,1'-dimethylurea
DNA...................deoxyribonucleic acid
EDTA..........................diaminoethanetetra acetic acid (disodium salt)
EPR..........................electron paramagnetic resonance spectroscopy
FRAP..........................fluorescence recovery after photobleaching
FTIR..........................fourier transform infra-red spectroscopy
HQNO..........................2-7-heptyl-4-hydroxyquinoline N'-oxide
IPTG..........................isopropyl-β-D-thiogalactopyranoside
Km.............................kanamycin resistance gene
LHCII........................light harvesting complex II
mRNA........................message RNA
NADPH................nicotinamide adenine dinucleotide phosphate
NDH-1...................type-1 NADPH dehydrogenase
NDH-2................type-2 NADPH dehydrogenase
NMR........................nuclear magnetic resonance
ORF........................open reading frame
PCC........................pasteur culture collection
PCi........................inducible phycocyanin gene set (cpcB2A2)
PCc........................constitutive phycocyanin gene set (cpcB1A1)
PS1........................photosystem 1
PS2........................photosystem 2
Q........................near-cytoplasmic plastoquinol binding site of the cytf complex
Q........................near-luminal plastoquinol binding site of the cytf complex
RNA........................ribonucleic acid
RNAse H....................ribonuclease H
SDH........................succinate dehydrogenase
SDS.............................sodium dodecyl sulphate
SDS-PAGE........................SDS polyacrylamide gel electrophoresis
TES........................N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
Tris........................tris[hydroxymethyl]aminomethane
X-gal........................5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
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Chapter 1

INTRODUCTION
CHAPTER 1: INTRODUCTION

1.1 Prologue
All organisms must have the ability to adapt to changing environmental conditions in order to survive. From the complex physiological responses of a mammal to changing temperature, to the response of a bacterium to changing nutrient availability, organisms must all constantly balance the needs of their internal chemistry with the less ordered world around them. Within bacteria, these adaptive responses range from rapid and transient alterations in motility to long-term global reorganisations of gene expression and cell morphology. Within photosynthetic bacteria such as the cyanobacteria, and indeed within higher photosynthetic organisms, regulation of the photosynthetic apparatus is an important adaptive response for survival. By responding to changes in the wavelength and intensity of light cyanobacteria can maximise the efficiency of its utilisation.

1.2 Cyanobacteria and the model organism *Synechocystis* PCC6803
The cyanobacteria form one of the 11 major eubacterial phyla (Castenholz 1989, Woese 1987). They are an ancient group of photosynthetic, oxygen-evolving prokaryotes, which are thought to have contributed to the oxygen content of the atmosphere (Blankenship & Hartman 1998, Des Marais et al. 1992). Cyanobacteria exist in marine as well as freshwater environments. Some nitrogen-fixing species exist in symbiosis with plants, such as cycads (gymnosperms) (Lindblad & Bergman 1990). Some species also exist in symbiosis with fungi to form lichens (Paulsrud et al. 1998, Paulsrud & Lindblad 1999).

Cyanobacteria, like the chloroplasts of higher plants, have two photosynthetic reaction centres, photosystem 1 (PS1) and photosystem 2 (PS2). Indeed, there is much convincing evidence that chloroplasts evolved from a cyanobacterium-like ancestor (see Section 1.3). This, as well as the ease of genetic manipulation of cyanobacteria make them excellent model organisms for studying oxygenic photosynthesis. For several reasons *Synechocystis* PCC6803, a unicellular freshwater cyanobacterium, is particularly useful in this respect.

*Naturally Transformable.* Like several other species of cyanobacterium, *Synechocystis* PCC6803 has a natural DNA uptake system (Barten & Lill 1995, Thiel 1994). This means that to transform cells, DNA is simply mixed into a liquid culture.

*High efficiency of recombination.* This allows the targeted interruption, deletion or modification of genes (see Chapter 3).
**Genome sequence.** The complete genome sequence of *Synechocystis* PCC6803 is now available (Kaneko *et al.* 1996). This allows the targeted interruption, deletion or modification of previously uncharacterised genes (Vermaas 1996). This also facilitates the identification of the sites of lesions in random mutants (see Section 3.6).

**Heterotrophic growth.** *Synechocystis* PCC6803 can grow under a number of conditions ranging from fully photoautotrophic to fully heterotrophic modes (Vermaas 1996). Photoautotrophic growth requires the functional presence of both photosystems. In the absence of PS2 (Vermaas *et al.* 1990), PS1 (Smart *et al.* 1991), or of both photosystems (Shen & Vermaas 1994), *Synechocystis* PCC6803 can grow, provided there is an alternative carbon source available such as glucose. This allows the construction of photosynthetic mutations which would be lethal under photoautotrophic conditions.

It is interesting to note that *Synechocystis* PCC6803, although capable of heterotrophic growth in the absence of photosystems, cannot grow heterotrophically in complete darkness (Anderson & McIntosh 1991). There would appear to be a requirement for illumination which is independent of photosynthetic electron transport.

### 1.3 Cyanobacteria and the evolution of the chloroplast

The idea that plant cells resulted from an endosymbiotic event between autonomous green cells and a colourless host was first proposed by Schimper in 1883 (Schimper 1883). Comparisons of gene arrangement, expression and sequences from photosynthetic bacteria and plastids have since consistently demonstrated the cyanobacterial ancestry of plastids (Douglas 1994, Douglas 1998). For example, the complete plastid genome sequences from several photosynthetic eukaryotes (Douglas & Penny 1998, Hallick *et al.* 1993, Kowallik *et al.* 1995, Reith & Munholland 1995, Stirewalt *et al.* 1995, Sugiuara 1992 Wakasugi *et al.* 1997) are now available, as well as that of the cyanobacterium *Synechocystis* PCC6803 (Kaneko *et al.* 1996). This has allowed the analysis of gene order and gene complement as a means of tracing evolutionary relationships. This is a potentially more powerful way of reconstructing evolutionary events than the analysis of individual gene sequences since the latter is often complicated by lateral gene transfers (Delwiche & Palmer 1996). From the approximately 3000 predicted genes encoded by the *Synechocystis* PCC6803 genome only 100-200 equivalents are found on the plastid genomes. This suggests that following endosymbiosis of a cyanobacterium-like ancestor by an ancestral eukaryote there was a massive loss of genes from the cyanobacterial genome. Some gene transfer to the nucleus would have occurred (Martin *et al.* 1998), as well as the loss of redundant genes. Despite this gene loss, the gene content of plastids is remarkably similar. This has been interpreted as supporting a monophyletic origin of primary plastids; that is, primary plastids originated from a single cyanobacterial ancestor.
Analysis of the published plastid genome sequences can also be used to demonstrate patterns in gene loss as well as how gene distribution in extant photosynthetic organisms resulted from a monophyletic evolutionary event (Martin et al. 1998).

1.4 Light harvesting and photosynthesis

Photosynthetic organisms such as cyanobacteria convert light energy (photons) into chemical energy by a process that consumes carbon dioxide and releases oxygen: photosynthesis. This process is fundamental to life on earth: the oxygen released is the terminal electron acceptor for respiration. Photosynthetic life is also the primary producer in food chains.

The first step in photosynthesis is the absorption of light by photosynthetic antenna pigments. The photosynthetic pigments such as the chlorophylls are highly conjugated molecules, that is, they possess alternating single and double C-C bonds. This feature allows such pigments to absorb light strongly (Rabinowitch & Govindjee 1969). Absorption of light involves the conversion of photons (quanta of light energy) into the energy of excited electrons within pigment molecules. This energy can then be transferred rapidly to other pigment molecules. Where it is difficult for such rapid energy transfer to occur (for example in a chlorophyll solution), the energy of the excited electron can be converted into other forms by slower competing processes. One of these processes is the re-emission of the energy as light, or fluorescence (Clayton 1980). Within photosynthetic light harvesting systems, pigment molecules are typically attached to a protein framework within (or associated with) the thylakoid membrane. The orientations and proximities of the pigment molecules within this framework are finely tuned to maximise transfer of energy between them and minimise energy loss by competing processes such as fluorescence. The primary mechanism of energy transfer between the pigments is resonance energy transfer or Förster resonance transfer (Förster 1965). This process occurs through the interaction of the molecular orbitals of participating pigments in a manner analogous to the interactions of mechanically coupled pendulums of similar frequencies. Another mechanism, known as exciton coupling, may occur when chromophores are brought into closer contact (Cantor & Schimmel 1980). In this process groups of pigments may share delocalised excitation energy as if they are one pigment.

Once light energy has been converted to excitation energy by these pigments, it is transferred to the photosystem reaction centres. In oxygenic photosynthetic organisms the photosynthetic apparatus contains three main transmembrane electron transfer complexes, PS2, PS1, and a cytochrome $\text{b}_f \ (\text{cyt} \ b_f)$ complex (Figure 1.1). Within the PS2 and PS1 reaction centres the harvested excitation energy causes a charge separation between a primary donor (P) and a primary acceptor (A). On excitation, P becomes a powerful
Figure 1.1: An overview of the cyanobacterial photosynthetic apparatus. Solid arrows denote the flow of electrons. Abbreviations: PC, phycocyanin; AP, allophycocyanin; D1 and D2, core polypeptide dimer of PS2; cyt b₅₅₉, cytochrome b₅₅₉; Mn, manganese cluster of the oxygen evolving complex; 9 and 33, 9- and 33-kDa subunits of the oxygen evolving complex; CP43 and CP47, 43-and 47-kDa chlorophyll protein complexes; P₆₈₀, chlorophyll centre which is photo-oxidised in PS2; Pheo, Pheophytin primary electron acceptor; Q₃, quinone secondary electron acceptor; Q₁, a plastoquinone bound to PS2 which accepts two electrons from Q₃ and equilibrates with the PQ/PQH₂ pool; PQ/PQH₂, plastoquinone pool; cyt b₆, a cytochrome containing low-and high-potential heme centres which are involved in Q-cycle electron flow from plastoquinol bound at the Q₁ site to plastoquinone bound at the Q₃ site; FeS, an iron-sulphur redox centre; PC, plastocyanin; C₅₅₃, cytochrome c₅₅₃; PsaA and PsaB, related chlorophyll binding proteins which form the core of PS1; F₇₀₀, the chlorophyll which is photo-oxidised in PS1; A₁, A₂, F₆₅₂, bound redox intermediates of PS1; Flvd, flavodoxin; Fd, ferredoxin; FNR, ferredoxin/flavodoxin NADPH oxidoreductase.
reductant (P*). Interaction of P* with the acceptor A causes a charge separation to take place:

\[ P + A \Rightarrow P^* + A \Rightarrow P^+ + A^- \]

In PS2 and PS1 the primary donors are chlorophyll molecules. In PS2 the primary donor may consist of two reaction centre chlorophylls termed P680. They are termed P680 due to their bleaching near 680nm. The electron removed from P680 is ultimately transferred to a membrane pool of plastoquinone. P680 is re-reduced by a water-oxidising complex resulting in the evolution of molecular oxygen. In PS1, similar events lead to the reduction of NAD(P) by ferredoxin or flavodoxin and the oxidation of plastocyanin or cytochrome c by the PS1 reaction centre chlorophyll P700 (Nugent 1996). The photosystems consist of protein frameworks that position the cofactors necessary for electron transport. The arrangement of these cofactors is such that the photochemical trapping of excitation energy is highly efficient. It also allows vectorial electron transfer across the membrane. This vectorial electron transfer, linked to proton transfer, generates an electrochemical potential across the membrane which is coupled to processes such as the synthesis of ATP by the ATP synthase. The electron transfer events in PS1 and PS2 are linked by the cytbf complex. This complex, containing cytochromes and a Rieske iron-sulphur centre, catalyses the oxidation of plastoquinol, and the reduction of plastocyanin or cytochrome c.

To understand in detail how the photosynthetic apparatus functions it is necessary to have detailed structural knowledge of the complexes of which it consists. A structure at 4Å resolution of PS1 is now available (Krauss et al. 1996, Schubert et al. 1997). Work is being carried out to obtain similar high resolution structures for PS2 (Barber & Kühlbrandt 1999, Rhee et al. 1998) and cytbf (Mosser et al. 1997). The high resolution structure of the purple bacterial reaction centre was solved in 1984 by X-ray crystallography (Deisenhofer et al. 1984). In the future such understanding may be used in the design of artificial solar cells based on natural photosynthesis. This will exploit billions of years of evolutionary design to generate energy in a highly efficient and environmentally-friendly way (Cogdell & Lindsay 1998).

1.5 The phycobilisome
In cyanobacteria and the eukaryotes Rhodophyta and Glaucocystophyta the main accessory light harvesting system is a large soluble protein-bilin complex present on the cytoplasmic surface of the thylakoid membrane called the phycobilisome (Grossman et al. 1993, MacColl 1998, Sidler 1994) (Figure 1.1). The bilin chromophores, linear tetrapyrrole pigments, are bound to the protein framework via thioester linkages. The major classes of phycobiliproteins present in phycobilisomes, as determined by their
absorption spectra are phycoerythrin \((A\text{max} = 490-579\text{nm})\), phycocyanin \((A\text{max} = 615-640\text{nm})\) and allophycocyanin \((A\text{max} = 650-655\text{nm})\). Phycoerythrocyanin \((A\text{max} = 567\text{nm})\) can replace phycoerythrin as a pigment in some organisms. The structure of the phycobilisome varies between species, but typically includes a core of allophycocyanin with phycocyanin rods radiating from it. In *Synechocystis* PCC6803 these rods contain only phycocyanin. In some species such as *Calothrix* PCC7601 phycoerythrin is also present. The absorption and fluorescence characteristics of these phycobiliproteins allows for highly efficient resonance energy transfer down the rod pigments to the allophycocyanin core. From there, energy passes through long-wavelength fluorescing pigments of the phycobilisome called terminal emitters to the chlorophyll antennae of the photosystems.

The X-ray crystal structures of members of the phycobiliprotein family have been determined to atomic resolution (for review, see Betz 1997). With such structural information it is possible to begin to determine how the phycobiliproteins form such an efficient light harvesting system (Betz 1997).

1.6 Respiration in cyanobacteria

Within the cyanobacteria aerobic respiration occurs within the same membrane system as photosynthesis. This is contrary to the situation in eukaryotes where aerobic respiration and photosynthesis occur predominantly in different organelles. Within the thylakoid membrane of cyanobacteria, the photosynthetic and respiratory electron transport pathways both utilise the plastoquinone pool and cytb\(f\) (Scherer *et al.* 1988, Schmetterer 1994). Recent research would indicate that succinate dehydrogenase (SDH) has a central role in electron transport to the plastoquinone pool in *Synechocystis* PCC6803 (Howitt *et al.* 1999). In contrast, type-1 NADPH dehydrogenase (NDH-1) would appear to have a more minor role and type-2 NADPH dehydrogenase (NDH-2) has no detectable catalytic role. Figure 1.2 illustrates probable principal respiratory electron-transport events in *Synechocystis* PCC6803. Electrons flow from succinate (SDH) and NADPH (NDH-1) into the plastoquinone pool. From there electrons flow through cytb\(f\) to soluble electron carriers (Manna & Vermaas 1997), which deliver electrons to a cytochrome aa\(_3\)-type cytochrome c oxidase.
Figure 1.2: Respiratory and photosynthetic electron transport in *Synechocystis* PCC6803. Photosynthetic electron transport proceeds from PS2 to the plastoquinone pool and from there through cytbf to PS1. Respiratory electron transport appears to occur predominantly from succinate, through the plastoquinone pool, cytbf and to cytochrome oxidase (see text for details).
1.7 Adaptation of the cyanobacterial photosynthetic apparatus to light of varying wavelength and intensity

1.7.1 Introduction

In cyanobacteria, PS1 monomers typically contain approx. 100 chlorophyll $a$ (chl$a$) molecules (Schubert et al. 1997) whereas PS2 monomers typically contain 40 (Rögner et al. 1996). PS2 forms dimers in vivo (Boekema et al. 1995). There is some evidence that PS1 forms trimers in vivo (Kruip et al. 1994). The majority of chl$a$ in the thylakoid membrane is therefore associated with PS1. The extent of this will clearly vary with the PS1:PS2 ratio, which can range from 3.9-0.7 depending on illumination and growth conditions (Murakami 1997) (see Section 1.7.2). The absorption spectrum of chl$a$ has maxima in the blue (approximately 430nm) region (the Soret band) and in the red (670-700nm) region (Clayton 1980). These reflect different electronic transitions which occur within the chlorophyll molecule. The phycobilisomes absorb in the 450-660nm region of the spectrum (MacColl 1998). In its natural environment a cyanobacterium encounters light of varying wavelength. For example, a cyanobacterium undergoing wind-driven vertical mixing in a marine environment will encounter different wavelengths of light at different depths, due in part to self-shading. This can lead to alterations in the relative amount of light energy absorbed by the two photosystems. This in turn can lead to changes in relative turnover of the photosystem reaction centres. Differing light intensities can also alter relative reaction centre turnover. This is because the trapping speeds of the two photosystem reaction centres are unequal. The PS1 reaction centre traps excitation energy much faster than that of PS2 (Beddard et al. 1975, van Grondelle et al. 1994). This means that PS2 reaction centre turnover will reach a maximum level at a lower light intensity than PS1. Thus, at higher light intensities, turnover of the PS1 reaction centre will be faster than that of PS2. These changes in relative reaction centre turnover brought about by light of varying wavelength and intensity may not correspond to the metabolic demands of the cell at a particular time.

Cyanobacteria have evolved several mechanisms to adapt to light of changing wavelength and intensity and hence maximise its utilisation. Long-term adaptation involves changes in photosystem gene expression leading to changes in the PS2/PS1 ratio. These changes occur on a timescale of hours to days and compensate for persistent imbalances in photosystem turnover (Fujita 1997). Short-term adaptation or the state transition, involves alterations in excitation-energy distribution from the phycobilisome to the photosystems and occurs on a timescale of seconds to minutes (van Thor et al. 1998).
Complementary chromatic adaptation is observed in phycoerythrin-containing cyanobacteria and adjusts the amounts of phycocyanin and phycoerythrin in the phycobilisomes in response to green (absorbed strongly by phycocyanin) and blue (absorbed strongly by phycoerythrin) light (Allen & Matthijs 1997). Other mechanisms also exist which adjust the amounts of phycocyanin in the phycobilisome rods in response to changes in light intensity (Westermann & Wehrmeyer 1995). Before discussing the state transition in more detail, some of these other mechanisms will be discussed.

1.7.2 Photosystem stoichiometry changes
This mechanism entails changes in the photosystem ratio which occur on a timescale of hours to days and compensate for persistent imbalances in reaction centre turnover (Fujita 1997). These changes in photosystem ratio seem to result from changes in the expression of PS1 (Fujita & Murakami 1987). Thus, under red and/or high intensity illumination which tends to increase PS1 turnover, the amount of PS1 per cell will decrease (hence the PS2/PS1 ratio will increase). Under yellow illumination, which is absorbed strongly by the phycobilisomes and so tends to increase PS2 turnover, the amount of PS1 will increase (hence the PS2/PS1 ratio will decrease).

The inhibitor HQNO inhibits cytochrome b oxidation at the near-cytoplasmic plastoquinol binding site of the cytbf complex (Qc) (Matsuura et al. 1988). This inhibitor was found to decelerate the increase in PS1 seen under yellow "PS2" light in the cyanobacterium Synechocystis PCC6714 (Murakami & Fujita 1993). The decrease in PS1 seen under red "PS1" light however was found to be unaffected. This led to the suggestion that the HQNO-sensitive cytochrome b oxidation reaction at the Qc site of the cytbf complex was the trigger for the stimulation of PS1 formation (Murakami & Fujita 1993). This regulation of PS1 expression is thought to be at the level of translation or assembly of PS1 since PsaA/B mRNA levels were found to be unaffected by light quality (Aizawa & Fujita 1997). Although genes have been identified which affect the regulation of photosystem stoichiometry (Hihara et al. 1998, Hihara & Ikeuchi 1999, Wilde et al. 1995), the signal transduction system which mediates it remains unknown. The isolation and characterisation of mutants disrupted in this process is perhaps the best way forward in understanding its molecular basis.

1.7.3 Complementary chromatic adaptation and the two-component system for bacterial signal transduction
Complementary chromatic adaptation is a mechanism which adjusts the pigment composition of the phycobilisomes in response to different wavelengths of light (Engelmann 1902, Gaidukov 1903). It occurs in cyanobacteria that contain the red pigment phycoerythrin in addition to the blue phycocyanin in their phycobilisomes.
(Boresch 1921). Under red light which is strongly absorbed by phycocyanin, the phycocyanin content of the phycobilisome rods will increase ($\lambda_{\text{optimum}} = 650-660\text{nm}$). Conversely under green light which is absorbed strongly by phycoerythrin, the phycoerythrin content of the phycobilisome rods will increase ($\lambda_{\text{optimum}} = 540-550\text{nm}$). This presumably serves to maximise light absorption by the phycobilisome under light of changing wavelength.

Whereas the signal transduction systems behind most of the cyanobacterial light adaptation mechanisms remain poorly understood, that of complementary chromatic adaptation is very well characterised. This system is based on the widespread mechanism for bacterial signal transduction, the two component system (Parkinson & Kofoid 1992). A whole range of adaptive responses employ the two-component system. For example metabolic responses to carbon, nitrogen, and phosphate availability, chemotaxis, and physiological responses to changes in medium osmolarity (Stock et al. 1989). This system has also been found in several eukaryotes (Alex & Simon 1994, Imamura et al. 1998, Loomis et al. 1998).

These systems typically consist of a sensory histidine kinase and a response regulator (Figure 1.3). The histidine kinase becomes autophosphorylated on a histidine residue in response to a signal. The histidine kinase then phosphorylates an aspartate residue on the response regulator. This response regulator then mediates the response. Response regulators typically have an alpha helix-beta strand structure, with the phosphorylated aspartate on a surface-exposed loop between the first alpha helix and first beta strand. An example of such response regulators is CheY which in *Escherichia coli* dictates clockwise or anticlockwise rotation of the flagellum and hence mediates chemotaxis. A high resolution X-ray crystal structure of CheY now exists (Stock et al. 1993).

Much of the work done on the molecular basis of complementary chromatic adaptation has been done in the cyanobacterium *Calothrix* sp. strain PCC7601. In this organism the genes encoding the phycocyanin, phycoerythrin and linker proteins whose expression changes during complementary chromatic adaptation have been isolated and characterised (Grossman 1990, Grossman et al. 1988). The genome has a phycocyanin gene set which accumulates message constitutively (*cpcB1A1*) and one that only accumulates mRNA in red light (*cpcB2A2*). These gene-sets are denoted constitutive and inducible (PCc and PCi). Message from the phycoerythrin gene-set (*cpeBA*) only accumulates at high levels in green light. The abundance of these mRNAs reflects the polypeptide composition of the phycobilisome at a particular time. Furthermore, since the stability of these transcripts is unaffected by light quality, control would appear to be at the level of transcription.
Figure 1.3: The two-component system for signal transduction. A sensor histidine kinase becomes autoprophosphorylated on a histidine residue in response to a signal. The histidine kinase then phosphorylates an aspartate residue on the response regulator. This response regulator then mediates the response.
Insights into the molecular machinery of many processes is gained through the generation and analysis of mutants. Various mutants in complementary chromatic adaptation have been made (Bruns et al. 1989). Red mutants are locked in a green light mode and have high levels of phycoerythrin but no detectable levels of inducible phycocyanin in green and red light. Blue mutants conversely have high levels of phycocyanin, but normal levels of phycoerythrin under green and red light conditions. In green mutants inducible phycocyanin expression is normal but the phycoerythrin genes are never activated and in black mutants there are moderate amounts of phycocyanin and phycoerythrin which are again indifferent to light quality. By complementation studies on the red mutant it was possible to identify the disrupted gene in this mutant (Chiang et al. 1992). This gene, designated \( rcaC \), encoded a protein with strong sequence identity to the Bacillus subtilis PhoP protein, a member of the superclass of regulatory proteins associated with bacterial two component regulatory systems (Seki et al. 1987). Unlike most response regulators however, RcaC contained a potentially phosphorylatable aspartate residue at its N terminus and C terminus (Chiang et al. 1992). Complementation studies on the black mutant similarly revealed the gene disrupted in this mutant, \( rcaE \). This gene encoded a protein with sequence homology to the chromophore attachment domain of plant phytochrome at its N-terminus. Phytochromes are photoreceptors found in higher plants that mediate a wide variety of responses (Rüdiger & Thümmler 1994). At its C-terminus RcaE had homology to histidine kinases typical of two-component systems (Kehoe and Grossman 1996). This suggested that RcaE was a chromatic-adaptation sensor histidine kinase and RcaC was its (or one of its) response regulators. Recently, new classes of mutants have revealed the presence of another response regulator involved in complementary chromatic adaptation, RcaF (Kehoe and Grossman 1997). RcaF is similar in size and structure to SpoOF, a small response regulator in B. subtilis which is part of a four-step phosphorelay. Four-step phosphorelay systems are modified two-component systems, which contain four phosphoacceptor modules instead of two. In the four-step phosphorelay systems characterised thus far, phosphate has been shown first to pass from a histidine residue (H1) on a histidine kinase module to an aspartate residue (D1) on a response regulator module. The phosphate is then passed to a second histidine (H2) and finally to a second aspartate (D2). As well as the discovery of RcaF, RcaC is known to have a domain with strong sequence similarity to the histidine kinase H2 sites of a number of four-step phosphorelay systems, in addition to the two response-regulator domains (Appleby et al. 1996). This led Kehoe and Grossman to propose a model for complementary chromatic adaptation signal transduction which involved a four-step phosphorelay mechanism (Grossman & Kehoe 1997, Kehoe & Grossman 1997) (Figure 1.4). In green light, RcaC remains unphosphorylated and transcription occurs from \( cpeBA \) but not from PCI (this may be due to phosphatase activity of RcaE). Red light causes RcaE (H1) to phosphorylate the first response regulator RcaF (D1). RcaF then phosphorylates
Figure 1.4: Four-step phosphorelay mechanism for complementary chromatic adaptation. Red light stimulates phosphoryl group flow through the pathway which leads to the induction of phycocyanin synthesis and the repression of phycoerythrin synthesis. Green light prevents phosphoryl group flow through the pathway, possibly by promoting phosphatase activity of RcaE. This stimulates phycoerythrin synthesis and represses phycocyanin synthesis (see text for details). Black circle: possible light-sensing domain. Hatched circle: consensus DNA-binding motif of RcaC. H1 and H2 are respectively the primary and secondary histidine kinase domains of the four-step phosphorelay whilst D1 and D2 are respectively the primary and secondary response regulator domains.
the histidine kinase domain of RcaC (H2). This in turn phosphorylates one of the RcaC response regulator domains (D2) [site-directed mutagenesis would indicate that this is primarily the N-terminal aspartate residue (Kehoe and Grossman 1995)]. Phosphorylated RcaC then activates transcription from PCi and switches off transcription from cpeBA. The phytochrome-like domain of RcaE may be the (or one of the) photosensors which mediates signal transduction.

RcaC may bind to the promoters of these genes directly. Indeed, RcaC has a DNA-binding motif (Kehoe & Grossman 1997) (Figure 1.4). However, the kinetics of changes in the transcriptional activity of the two gene-sets on transfer of cells from red light to green light or visa versa are markedly different (Grossman et al. 1994). This suggests that if RcaC is involved in promoter binding it is in concert with other regulatory proteins.

The possibility of interactions between promoters of the phycocyanin and phycoerythrin genes and transcriptional regulators can be investigated through gel retardation assays (a DNA-protein complex has an electrophoretic mobility in a polyacrylamide gel matrix different from that of DNA alone). Using such an approach, protein binding to the putative cpeBA promoter was observed. Two proteins were subsequently identified as binding separately to this region: RcaA and RcaB (Sobczyk et al. 1993). A third protein was found that bound to the inducible phycocyanin operon (PCi), RcaD (Sobczyk et al. 1994). The binding properties of RcaA and RcaD appeared to be regulated by phosphorylation. RcaD has been purified to homogeneity and protein sequences used to clone rcaD. This gene is co-transcribed with another gene, orfP, which possesses a GTP-binding domain. The role of rcaD and orfP in complementary chromatic adaptation is currently being investigated by insertional inactivation (Luque et al. 1999). Similar work will lead to the characterisation of RcaA and RcaB.

1.7.4 The prokaryotic phytochromes

The phytochrome family of chromoproteins are common to plants and act as light detectors mediating a large variety of responses from shade avoidance to flowering (Quail et al. 1995). The phytochrome itself is a cytosolically located dimer. The N-terminal region ligates a chromophore, phytochromobilin which enables the chromoprotein to convert from the red-absorbing Pr form ($\lambda_{max}$ approx. 665nm) into the far-red absorbing Pfr form ($\lambda_{max}$ approx. 730nm) upon light absorption. The signal is transduced via the C-terminal region by a mechanism which is poorly understood in plants (Quail et al. 1995).

Open-reading frames (ORF's) encoding proteins with similarities to phytochrome and bacterial sensor kinases have been found in cyanobacteria. The putative sensor for
complementary chromatic adaptation RcaE has such similarities (see Section 1.7.3). In the cyanobacterium *Synechocystis* PCC6803 there are several ORFs with homology to genes for sensor histidine kinases and to phytochrome (Kaneko *et al.* 1996). The ORF denoted slr0473 has homology to phytochrome (36% identity to the *phyC* gene product of *Arabidopsis thaliana*) and has the phytochrome highly-conserved chromophore-binding domain (Hughes *et al.* 1996). Heterologous expression of this protein (Cph1) in *E. coli* and reconstitution of the protein with phycocyanobilin was achieved. Spectrophotometric analysis of this chromoprotein after exposure to red and far red light showed it to have two interconvertible forms like phytochrome. Using Raman spectroscopy these forms were also found to have remarkable structural similarity to the plant phytochrome equivalents (Remberg *et al.* 1997). A gene just downstream of slr0473, slr0474 (*rcpl*) was found to encode a protein related to the CheY superfamily of bacterial response regulators. Biochemical analysis demonstrated that Cph1 mediated the far-red/red reversible phosphorylation of this response regulator (Yeh *et al.* 1997). The function of this light activated signal transduction pathway is unknown however.

The *plpA* gene of *Synechocystis* PCC6803 has limited similarity with phytochrome (around the chromophore attachment site of the tobacco *phyB* gene). Inactivation of this gene caused an inability to grow photoautotrophically under blue light although under red and far-red light growth rates were the same as for the wild-type (Wilde *et al.* 1997). Several other genes with limited similarity to the chromophore attachment domain of plant phytochrome have been identified (Kaneko *et al.* 1996). Insertional inactivation mutants of these genes show differences in growth rate and pigment content to the wild-type under various light conditions (Wilde *et al.* 1999). Such genes may code for photoreceptors required for the light regulation of growth. Study of phytochrome-mediated signal transduction in cyanobacteria such as *Synechocystis* PCC6803 may shed some light on phytochrome mediated signal transduction in plants.

### 1.7.5 The state transition in cyanobacteria

The state transition occurs on a rapid timescale (seconds to minutes). It serves to adjust the distribution of excitation energy transferred to the photosystem reaction centres from the phycobilisomes under different light conditions (van Thor *et al.* 1998). For example, under illumination that tends to increase PS1 reaction centre turnover relative to PS2 (red light and/or high intensity light), there is a high efficiency of excitation-energy transfer from the phycobilisome to PS2. This is state 1. If light is then changed to that which tends to increase PS2 reaction centre turnover relative to PS1 (for example yellow light, which is absorbed strongly by the phycobilisomes), a proportion (approx. 40%) of this energy is transferred to PS1. This is state 2.
The trigger for the state transition is the redox state of the plastoquinone pool or cythf, an effective monitor of relative photosystem turnover (see Section 1.8.1). Thus, under light that increases PS1 turnover, cythf/plastoquinone is oxidised and state 1 is induced. Under light that increases PS2 turnover, cythf/plastoquinone becomes reduced and state 2 is induced. In cyanobacteria the respiratory and photosynthetic electron transport chains intersect at the plastoquinone pool (see Section 1.6). Thus, in the dark or under very low light intensities, respiratory electron flow reduces the plastoquinone pool/cythf and brings about state 2. If these conditions persist, then the regulation of photosystem stoichiometry (which is triggered by the redox state of cythf) will lead to an increase in the amount of PS1 (Fujita 1997).

1.7.6 Interaction between signal transduction systems

The principal mechanisms currently known by which cyanobacteria adapt their photosynthetic apparatus to light of changing wavelength and intensity have now been discussed. The signal transduction systems controlling these processes are studied independently since the phenomena themselves are observed independently. It must be remembered, however, that these light adaptation processes act in concert to tune the photosynthetic apparatus to its light environment. Given this, a degree of interaction between these signal transduction systems probably occurs. Indeed, rather than individual signal transduction systems, light adaptation, and indeed the rest of the cellular machinery, may represent a complex interacting regulatory network. There is in vivo evidence to suggest that bacterial signal transduction pathways interact with one another, a phenomenon known as crosstalk (Schneider et al. 1991). This has led to the idea of "neural" signal transduction networks in bacteria (Hellingwerf et al. 1995, Hellingwerf et al. 1998). An individual signal transduction pathway can produce a given output signal for a given input signal. The interaction between individual signal transduction systems leads to the possibility that they may form a complex, regulatory network. Such networks, like the neural networks of the human brain, would have the ability to modify output signals in response to changing input signals. In other words they would have a primitive learning ability. The state transition, the subject of this thesis, will now be discussed in more detail.

1.8 The state transition and signal transduction

1.8.1 Cyanobacteria

In contrast to green plant chloroplasts, the molecular basis for the signal transduction events which control the state transition in cyanobacteria remain poorly characterised.

It has been shown that the trigger for the state transition in cyanobacteria is the redox state of the plastoquinone pool or cythf. Mullineaux and Allen (1986) used a modulated
fluorescence measuring system to observe the state transition in *Synechococcus PCC6301*. They compared the ability of DCMU, which completely inhibits PS2 turnover, to bring about state 1 in starved and unstarved cells. They found that in starved cells the addition of DCMU is sufficient to bring about a state 1 transition (under the weak modulated phycocyanin excitation the cells are in state 2). In unstarved cells, DCMU addition was not sufficient to bring about a state 1 transition. State 1 could only be induced by illumination which increased PS1 turnover. Respiratory electron transport into the plastoquinone pool was found to be decreased in starved cells in comparison with unstarved cells. Thus, in unstarved cells, it was more difficult (i.e. required illumination which increased PS1 turnover) to bring about state 1. This implied that the signal for the state transition was the redox state of the plastoquinone pool or a closely associated electron carrier.

Mullineaux and Allen (1990) also used a variety of chemical and illumination treatments to oxidise and then reduce the plastoquinone pool in *Synechococcus PCC6301*. They then observed the effects of such treatments on the state transition by taking 77K fluorescence emission spectra. They found that when the plastoquinone pool was oxidised, state 1 was induced. When the plastoquinone pool was reduced, state 2 was induced.

Vemotte *et al.* (1990) also observed that, in starved cells, although the plastoquinone pool becomes oxidised, the cells can remain in state 2. It was suggested that in such starved cells, although the plastoquinone pool becomes oxidised, cytbf remains reduced. It would therefore appear to support the idea that cytbf rather than the plastoquinone pool provides the trigger for the state transition. In cyanobacteria, cytbf is required for cell viability. Therefore, the construction of cytbf inactivation mutants to study this problem [as in *Chlamydomonas reinhardtii* (see Section 1.8.2)] is impossible.

It has been suggested that, as in green plant chloroplasts, the state transition in cyanobacteria involves phosphorylation. Allen *et al.* (1985) working with *Synechococcus PCC6301*, correlated the transition to state 2 (as shown by 77K fluorescence emission spectra) with the phosphorylation of two proteins: a 15kDa thylakoid-membrane-associated protein and an 18kDa soluble protein later identified as β-phycocyanin (Harrison 1990). Using similar techniques in the red alga *Porphyridium cruentum*, Biggins *et al.* (1984) could not find such a correlation between protein phosphorylation and the state transition. A more detailed discussion of this issue is presented in Section 6.1.
1.8.2 Green plant chloroplasts

The state transition also occurs in green plant chloroplasts (Keren & Ohad 1998). In green plant chloroplasts, contrary to the situation in cyanobacteria, PS2 and PS1 are localised in different regions of the thylakoid membrane. PS2 is found predominantly in the tightly appressed granal stacks, whereas PS1 is found in the non-appressed stroma lamellae regions (Anderson 1986). In green plant chloroplasts the state 1-state 2 transition is believed to be brought about by the phosphorylation of the light harvesting system LHCII and its migration from PS2 in the PS2-rich granal stacks to PS1 in the PS1-rich non-appressed stroma lamellae region (Larsson et al. 1986, Allen 1992, Gal et al. 1997). There is much evidence to suggest that the redox state of cytbf is the trigger for the state transition in the green plant chloroplast. For example, mutants of C. reinhardtii that lack cytbf complexes are unable to phosphorylate LHCII and are locked in state 1 regardless of the redox state of the plastoquinone pool (Gal et al. 1990, Lemaire et al. 1986, Wollman & Lemaire, 1988). Similar studies have revealed a similar situation in higher plants (Gal et al. 1988, Vener et al. 1997). In the thylakoid membrane, various redox-controlled kinase activities have been detected (Gal et al. 1997). Insights have recently been gained of how the redox poise of cytbf is transduced into changes in LHCII phosphorylation and hence changes in excitation energy distribution.

Vener et al. (1997) used a transient lowering of the pH in a spinach thylakoid suspension in darkness to reduce the plastoquinone pool and hence the high-potential path of the cytbf components (cytochrome f and the Rieske Fe-S center) and plastocyanin. This reduction coincided with the activation of an LHCII kinase. This activation was shown to correlate with the presence of the EPR signal at g = 2.03, considered to represent the interaction of plastoquinol with the near-luminal oxidising site of cytbf (Qo). Indeed, a flash of light predicted to cause a single turnover of PS1 and to oxidise plastocyanin, the high potential components of cytbf and plastoquinol bound at the Qo site did result in kinase deactivation. In addition, studies with the quinone analogue DBMIB, which displaces plastoquinol from the Qo site showed that occupancy of the Qo site by plastoquinol is linearly related to kinase activation (Vener et al. 1997).

Zito et al. (1999) also made a site directed mutant in the conserved PEWY sequence in the petD gene of C. reinhardtii (PEWY - PWYE). This sequence has an important role in the formation and function of the Qo site. These mutants were shown to be incapable of binding plastoquinol and were locked in state 1. These studies suggest that binding of plastoquinol to the the Qo site of cytbf is specifically required for activation of the LHCII kinase(s) and the transition to state 2. The molecular basis for how occupation of the Qo site by plastoquinol is transduced into LHCII kinase(s) activation remains unknown. However, the existence of detailed structural data makes it possible to propose models for
how this occurs. The structures of the chloroplast Rieske protein (Zhang et al. 1996), the chloroplast cytochrome f protein (Martinez et al. 1994) and the mitochondrial bc1 complex (Xia et al. 1997) have been solved to high resolution. The bc1 complex and cytbf are analogous in their general structure and function. This means that information gained from the high resolution structure of bc1 can be extrapolated to cytbf, for which detailed structural data is more sparse. Structural data combined with mutagenesis and mechanistic studies show that on binding of plastoquinol to the Qo site of the cytochrome bc1 complex, large conformational changes in the Rieske Fe-S subunit occur (Zhang et al. 1998). Vener et al suggested that an analogous conformational change in cytbf may mediate kinase activation on binding of plastoquinol to the Qo site (Vener et al. 1998).

As a signal transduction system this is quite unique among characterised systems. Vener et al. (1997) drew an analogy with classical receptor-ligand signal transduction. In such systems a ligand binds to a (usually membrane-bound) receptor. A conformational change within the receptor then mediates a response, for example via other proteins. In the case of the cytbf complex and state transitions, cytbf could be seen as the receptor and plastoquinol as the ligand. This situation however is unique in that all the components are probably membrane bound and acting within the membrane. Also, the receptor is cytbf, a major electron-transport complex.

The molecular identity of the LHCII kinase(s) remain unknown. Several groups are working towards the identification of these signal transduction components. Kruse et al. (1999a,b) have tackled the problem by using insertional mutagenesis. They transformed the arg7 (argininosuccinate lyase) gene into an arginine-requiring (arg7cw15mt) strain of C. Reinhardtii. The arg7 gene randomly integrates into the nuclear genome creating random, tagged mutants which can then be selected by their ability to grow on medium lacking arginine (Debuchy et al. 1989, Purton & Rochaix 1995). The state 2-1 transition in C. reinhardtii (as in cyanobacteria) is accompanied by an increase in the level of fluorescence emission from PS2 (Wollman & Delepelaire 1984). Using screening methods based on this fluorescence change, five mutants specifically unable to perform wild-type state transitions were isolated (Kruse et al. 1999b). Measurements of the rate of in vivo LHCII peptide phosphorylation showed one of these mutants (stm1) to be defective in LHCII phosphorylation. Other groups have used similar strategies to isolate state transition mutants in C. reinhardtii (Fleischmann et al. 1999). Analysis of such mutants may reveal an LHCII kinase or similar signal transduction component. Similar work may be possible in A. thaliana (Allen et al. 1995).

Phosphatase activity responsible for the dephosphorylation of LHCII has also been detected. This activity seems to be independent of light as well as of redox control (Elich et
al. 1997). Recently an immunophilin-like protein has been identified which has a potential phosphatase binding domain and influences thylakoid protein dephosphorylation (Fulgosi et al. 1998). The gene for this protein appears to be widespread among photosynthetic organisms, including *Synechocystis* PCC6803 (Kaneko et al. 1996).

In the past, phosphorylation-induced changes in the interactions of photosynthetic complexes, such as LHCII with PS2, were hypothesised to occur by long-range electrostatic effects (Allen 1992). However, recent structural studies of phosphoproteins involved in photosynthesis favour a molecular recognition model whereby phosphorylation-induced conformational changes alter the interactions of photosynthetic complexes (Allen & Nilsson 1997). The major part of the structure of non-phosphorylated LHCII has been resolved (Kühlbrandt et al. 1994) and, based on this structure and the results of CD, NMR and FTIR spectroscopy, a mechanism for the phosphorylation-induced structural changes accompanying the state transition has been proposed (Nilsson et al. 1997). Phosphorylation of a threonine (Thr-5) near the N-terminus causes the local formation of an alpha helix around this residue. This structural change reduces protein-lipid interactions around the phosphorylation site and allows the association of this helix with a positively-charged region between two other helices in the LHCII monomer. This conformational change results in the dissociation of LHCII trimers into monomers and their detachment from PS2 and diffusion to the PS1-rich stroma lamellae region (i.e. state 2). In addition Zer et al. (1999a,b) have shown that light activates LHCII for phosphorylation by exposing the Thr-5 phosphorylation sites of LHCII to kinases.

### 1.9 The molecular basis for the cyanobacterial state transition

#### 1.9.1 Introduction

In green plant chloroplasts there is much evidence for a mobile model for state transitions. In cyanobacteria this issue has been much more contentious. Within cyanobacteria a wide variety of techniques have been used to determine how the interaction of pigment-protein complexes changes during the state transition. Two main models for how the state transition occurs in cyanobacteria have been proposed (Figure 1.5). The spillover model for the state transition suggests that the phycobilisome stays perpetually associated with PS2 (Biggins & Bruce 1989). The transition from state 1 to state 2 is brought about by an increase in the flow of excitation energy directly from the PS2 chla antenna to the PS1 chla antenna. This would be triggered for example by conformational changes occurring in the photosystems. The mobile model for the state transition proposes that on the transition from state 1 to state 2 a proportion of the phycobilisomes associated with PS2 dissociate and become re-associated with PS1 (Allen 1992). Below are summarised some of the techniques used in analysis of the state transition, and their findings.
Figure 1.5: Models for the state transition in cyanobacteria. In the mobile model, the transition from state 1 to state 2 entails the detachment of a proportion of phycobilisomes from PS2 and their reassociation with PS1. In the spillover model, the phycobilisome stays perpetually attached to PS2. The state 1-to-state 2 transition is brought about by an increase in the flow of excitation energy directly from the PS2 to the PS1 chla antenna.
1.9.2 Fluorescence spectroscopy

Fluorescence spectroscopy is a useful technique by which to dissect the paths of excitation energy transfer within the photosynthetic apparatus in the different states. For steady state fluorescence measurements assignments of fluorescence emission to particular pigments are fairly straightforward. At room temperature the yield of fluorescence from PS1 is very weak compared with that at 77K. This is due to the fast trapping of energy by P700: estimates of 20ps have been made for the trapping lifetime in *Synechocystis* PCC6803 (Bittersmann & Vermaas 1991). At 77K however there is prominent fluorescence emission in the 715-735nm region (Butler 1961, Goedheer 1964). This originates from a small population of red-shifted chlorophylls that can transfer excitation energy uphill to P700. This energy transfer pathway is efficient at room temperature. At lower temperatures, however, the pathway becomes less efficient, resulting in an increase in fluorescence from these red-shifted chlorophylls (Wittmershaus et al. 1992).

In the fluorescence emission spectra of *Synechocystis* PCC6803 cells at 77K with phycocyanin excitation (600nm), PS1 emission is at 725nm. As well as this peak, there is also a broad peak at 650nm originating from a mixture of phycocyanin and allophycocyanin emission. There is also a peak at 680nm that originates from the terminal emitters of the phycobilisome as well as PS2. A shoulder is present at 690nm that corresponds to the PS2 core complex (Su et al. 1992) (Figure 3.7). In the fluorescence emission spectra of *Synechocystis* PCC6803 cells at 77K with chlorophyll excitation (435nm), phycocyanin and allophycocyanin fluorescence is lost (Su et al. 1992) (Figure 4.3). The wavelength of these peaks is species-dependent. At room temperature these fluorescence bands are broadened, and (in the case of PS1) lost, making fluorescence assignments more difficult.

The state transition was first observed independently by Murata (1969) in the red alga *P. cruentum*, and by Bonaventura and Myers (1969) in the green alga *Chlorella pyrenoidosa*. The phenomenon was first observed in cyanobacteria by Fork and Satoh (1983). Murata (1969) found that the 77K fluorescence emission spectra of *P. cruentum* with chlorophyll excitation as well as with phycocyanin excitation changed, depending on how the cells were preilluminated prior to freezing. When cells were preilluminated with green light, which is absorbed strongly by the phycobilisomes, emission from the PS2 components was lower (state 2) than if the cells were preilluminated with far-red light, which is absorbed strongly by PS1 (state 1). Similar changes were later observed in cyanobacteria (Fork & Satoh 1983). The simplest explanation for this difference in PS2 fluorescence between the two spectra was a change in excitation energy transfer between chla of PS2 and chla of PS1. The 'spillover' model for the state transition was therefore proposed (Murata 1969) (Figure 1.5). In this model the phycobilisome remains perpetually attached.
to PS2. The state 1-state 2 transition is then brought about by an increase in excitation energy transfer ('spillover') from the PS2 chla antenna to the PS1 chla antenna. Since Murata's observation much evidence has accumulated in support of an alternative mobile model for the state transition (see below). In this model, the state 1-2 transition is brought about by the decoupling of phycobilisomes from PS2 and their reassociation with PS1. This mobile model however does not explain the change seen in the 77K fluorescence emission spectrum with chlorophyll excitation. Some authors have suggested that this change is related to photosystem oligomerisation (Meunier et al. 1997). It has also been suggested that it is due to a photosystem-associated antenna protein which changes energy transfer characteristics during the state transition (Mullineaux 1992). Shen & Vermaas (1994) made a mutant strain of *Synechocystis* PCC6803 in which genes from both PS1 and PS2 were inactivated such that no stable photosystem assembly occurred. In these circumstances chlorophyll with a 77K fluorescence emission maximum at 678nm became prominent. It was suggested that this may represent a chlorophyll antenna to PS2 (Shen & Vermaas 1994). Such an antenna which changes its energy transfer characteristics during the state transition may account for the change seen in the 77K fluorescence emission spectrum with chlorophyll excitation; see Section 9.2.1 for a more detailed discussion of this point.

**1.9.3 Picosecond time-resolved fluorescence spectroscopy**

Picosecond time-resolved fluorescence spectroscopy can be used to measure the time it takes for fluorescence emitted from a particular pigment pool to decay, following an excitation flash. The fluorescence decay time of a particular pigment pool is related to the speed at which excitation energy is transferred through that pigment pool to other pigment pools. The technique can therefore be used to measure the speed of excitation energy transfer from one pigment pool to another. This has an important application with regard to the state transition. Using this technique it should be possible to measure changes in spillover from the PS2 to PS1 chla antennae, as well as changes in phycobilisome-to-photosystem energy transfer during the state transition. Mullineaux et al. (1990) found that in the cyanobacterium *Synechococcus* PCC6301, the state transition had no effect on the lifetime of fluorescence-decay components assigned to PS2. This suggested that a change in spillover from PS2 to PS1, which would be predicted to alter such lifetimes, does not occur.

**1.9.4 Flash-induced photo-oxidation of P700**

Flash-induced photo-oxidation of P700 can be used to measure the relative absorption cross-section of PS1 in the different states. Cell cultures are adapted to either state 1 (blue light) or state 2 (dark). When subjected to a flash of excitation, P700 will become momentarily oxidised and then re-reduced. This change in P700 redox state can be
monitored by measuring the change in absorption at 700nm. The flash intensity dependence of these absorption transients, measured for excitation flashes of different wavelength, can be used to construct an excitation spectrum for PS1.

In dark-adapted (state 2) *Synechococcus* PCC6301, the excitation spectrum showed a large peak corresponding to absorption by phycobilins. This showed that efficient coupling exists between the phycobilisomes and PS1. In blue-light-adapted (state 1) cells, the peak corresponding to absorption by phycobilins was reduced. This showed that the antenna size of PS1 is reduced in state 1 compared with state 2 (Mullineaux 1992). Furthermore, it was found that closure of PS2 reaction centres had no detectable effect on the absorption cross-section of PS1. This suggested that spillover of excitation energy from PS2 to PS1 does not occur (Mullineaux 1992). In a PS2-deficient mutant of *Synechocystis* PCC6803, efficient energy transfer occurs from the phycobilisomes to PS1 (Mullineaux 1994). These findings suggested direct phycobilisome to PS1 excitation energy transfer and the existence of a specific phycobilisome-PS1 complex.

1.9.5 Fluorescence recovery after photobleaching
Recent data has been obtained using a variant on the technique of fluorescence recovery after photobleaching (FRAP). This technique entails photobleaching a line across a cyanobacterial cell. The diffusion of the phycobilisomes and photosystems back into the bleached area can then be selectively monitored with a confocal microscope by observing their endogenous fluorescence. Due to its large size the cyanobacterium *Dactylococcopsis salina* is ideal for this purpose (cells are typically 4-8\(\mu\)m across and 35-80\(\mu\)m long). Whereas PS2 showed no detectable diffusion on the timescale of the measurements (20 seconds-20 minutes), the phycobilisomes diffused quite rapidly (diffusion coefficient 6 x 10\(^{-11}\)cm\(^2\) s\(^{-1}\)) (Mullineaux et al. 1997). These results suggested that the association of the phycobilisomes with the photosystems is weak and transient, and that the permanent PS2-phycobilisome complex proposed in the spillover model for state transitions does not exist.

1.9.6 Electron microscopy
Electron microscopy studies of cyanobacterial thylakoid membranes would suggest that state transitions involve a major re-organisation of the thylakoid membrane. Freeze-fracture electron micrographs show that PS2 is often organised as long, parallel rows of dimers in the thylakoid membrane (Mörschel & Schatz 1987, Nilsson et al. 1992). Similar studies have shown the phycobilisomes to form similar rows, which associate with the PS2 rows (Giddings et al. 1983). By conducting such ultrastructural studies on cells pre-adapted to either state 1 or state 2 conditions it was observed that in state 2 the proportion of these rows is reduced compared to state 1 (Olive et al. 1986). Furthermore, these
1.9.7 The use of molecular genetics to understand the cyanobacterial state transition.

In combination with techniques such as spectroscopy and microscopy, molecular genetics is a very powerful tool which may be used to gain an understanding of how the photosynthetic apparatus functions and is regulated.

A mutant of *Synechococcus* PCC 7002 devoid of phycobilisomes and any detectable phycobiliproteins has been made (Bruce *et al.* 1989). This mutant contains insertional inactivations of *apcA* and *apcB* (which encode the α and β subunits of allophycocyanin) and a spontaneous secondary mutation in the *cpc* locus. A state transition could be observed in this mutant in the 77K fluorescence emission spectrum with chlorophyll excitation. Preferential excitation of short wavelength chla was used to induce state 2, and carotenoid and long-wavelength chla to induce state 1. Similar illumination induced state transitions in the wild-type. This study shows that the phycobilisomes are not required to obtain state transitions as observed in the 77K fluorescence emission spectrum with chlorophyll excitation.

Another study has been made on a mutant with a termination codon created by a base substitution within the *apch* gene (Su *et al.* 1992). Although this mutant completely lacks phycobilisome core components, it retains phycocyanin rods with rod linker proteins. 77K fluorescence emission spectra with phycocyanin excitation showed that although PS2 fluorescence levels were greatly reduced compared with wild-type, PS1 fluorescence levels were comparable. The PS1 excitation spectrum of the mutant also matched a phycocyanin absorption spectrum. These observations suggested direct energy transfer from phycocyanin to PS1. The authors suggested that this phycocyanin-PS1 chlorophyll excitation energy transfer route may have a physiological role in the wild-type, possibly in state transitions.

Several mutants exist that are unable to perform state transitions, for example an *ndhB*-defective mutant in *Synechocystis* PCC6803 (Schreiber *et al.* 1995). In this mutant no functional NADPH dehydrogenase is assembled. 77K Fluorescence emission spectra of this mutant with phycocyanin as well as chlorophyll excitation show that it is permanently in state 1 (van Thor *et al.* 1998). Pulse modulated measurements of the PS2 variable fluorescence also show this (Schreiber *et al.* 1995). The trigger for state transitions is believed to be the redox state of plastoquinone or cythf (Mullineaux & Allen 1990, Vernotte *et al.* 1990). The absence of state transitions in this *ndhB*-defective mutant is
therefore probably a result of its altered electron transport properties. NADPH dehydrogenase donates electrons to plastoquinone. Its absence would therefore be expected to lead to a more oxidised plastoquinone pool and hence state 1.

Insertional inactivation of the apcD gene leads to the inability to perform state transitions. The apcD gene encodes the APC-B subunit, a minor component of the phycobilisome core and one of the terminal emitters of fluorescence. This was originally observed in *Synechococcus* PCC7002 (Zhao et al. 1992). It has since been observed in *Synechocystis* PCC6803 as well (Ashby & Mullineaux 1999a,b). Insertional inactivation of the apcF gene in *Synechocystis* PCC6803 also leads to the inability to perform state transitions (Ashby & Mullineaux 1999a,b). apcF encodes the β*18.5* subunit, another minor component of the phycobilisome core and terminal emitter of fluorescence. The inability of ΔapcD and ΔapcF to perform state transitions is presumably due to an alteration in energy transfer from the phycobilisomes to the reaction centres. Ashby and Mullineaux (1999a,b) constructed the ΔapcD and ΔapcF mutants in *Synechocystis* PCC6803 wild-type and in PsbD1CD2*, a mutant lacking PS2 (Vermaas et al. 1990). 77K Fluorescence spectra with phycocyanin excitation for these mutants showed that APC-B (ApcD) has a relatively minor role in energy transfer from the phycobilisomes to the photosystems in comparison to β*18.5*(ApcF) (Ashby & Mullineaux 1999a,b).

Structural models have been proposed for how phycobilisomes associate with the photosystems (Bald et al. 1996). These interactions, however, remain poorly understood. For example, it is unknown which subunits of PS1 and PS2 are required for interaction with the phycobilisomes. In order to isolate genes required for energy transfer from the phycobilisomes to PS1, Ashby and Mullineaux (1999c) generated random, genetically tagged mutants in a strain of *Synechocystis* PCC6803 lacking PS2 (PsbD1CD2) using random cartridge mutagenesis (Chauvat et al. 1989, Labarre et al. 1989). They screened the library of mutants for increased phycobilisome fluorescence using a fluorescence video imaging system. Increased phycobilisome fluorescence would be indicative of disruption in phycobilisome-PS1 energy transfer. A mutant was isolated with this characteristic, and the gene slr0115 found to be disrupted. Insertional inactivation mutants of slr0115 and a homologue slr0947 were generated in the wild-type and PsbD1CD2* backgrounds. 77K Fluorescence emission spectra were used to study phycobilisome-to-photosystem energy transfer in these mutants. This showed that Δslr0115 seemed to be disrupted in phycobilisome-to-PS1 energy transfer whereas Δslr0947 was disrupted in phycobilisome-to-PS2 energy transfer. These genes have similarity to OmpR-type DNA binding regulator proteins. It was therefore suggested that they represented members of two-component
signal transduction systems which serve to regulate the expression of a factor or factors involved in phycobilisome-to-photosystem energy transfer (Ashby & Mullineaux 1999c). As such, this adaptation mechanism presumably augments the rapid control of the state transition. For example, under red light, which is absorbed strongly by PS1, cells would move into state 1 in a matter of seconds. If these light conditions persisted long enough for changes in gene expression, PS1 levels decrease as a result of long-term changes in photosystem stoichiometry. In concert with this, the increased energy transfer to PS2 and decreased energy transfer to PS1 are augmented with changes in the expression of factors involved in phycobilisome-to-photosystem energy transfer.

1.10 The physiological role of the state transition
It was originally suggested by Murata (1969) that the state transition serves to increase the efficiency of light utilisation in photosynthesis. One way this may happen is through regulation of the ATP/NADPH ratio (Turpin & Bruce 1990, Vallon et al. 1991).

Two basic modes of electron transport are possible in oxygenic photosynthetic organisms: linear and cyclic electron transport. During linear electron transport, electrons flow from PS2, through cyt bf to PS1 and finally reduce NADP+. During cyclic electron transport, electrons from PS1 flow to the plastoquinone pool/cyt bf and cycle around PS1. This cyclic electron flow around PS1 generates ATP, but not NADPH (Bendall & Manasse 1995, Fork & Herbert 1993). In the cyanobacterium Synechocystis PCC6803, NAD(P)H dehydrogenase appears to have a central role in cyclic electron flow (Mi et al. 1992, 1994). Cyclic electron flow in this organism thus would appear to involve the donation of electrons from PS1-reduced NADPH into the plastoquinone pool via NAD(P)H dehydrogenase. Other pathways for cyclic electron flow may also exist in cyanobacteria. For example, electron flow may occur from ferrodoxin to the plastoquinone pool via a ferrodoxin-plastoquinone oxidoreductase activity (Manasse & Bendall 1993). It has been suggested that state 2 may favour cyclic electron transport and hence ATP production. Thus, a function of state transitions may be the regulation of the ATP/NADPH ratio.

Bulte et al. (1990) employed various treatments to decrease ATP levels in cells of the green alga C. reinhardtii. They demonstrated that this ATP depletion resulted in the transition to state 2. This ATP depletion was also shown to be accompanied by an increase in reduction of the plastoquinone pool. This is thought to be due to activation of starch degradation by low ATP levels. This raises the NADPH concentration and feeds electrons into the plastoquinone pool via an NADPH/plastoquinone oxidoreductase (Godde 1982). The redox state of cyt bf, which accepts electrons from plastoquinone, is thought to be the trigger for state transitions (Vener et al. 1997, Zito et al. 1999). Thus, the state 2-inducing effect of ATP depletion observed may have been due to increased reduction of cyt bf. Bulte
and colleagues however claimed that reversion to state 1 of cells placed in state 2 by ATP depletion specifically required restoration of ATP levels in addition to the reoxidation of cytbf. This implied that the state transition was specifically triggered by changes in ATP concentration in addition to the redox state of cytbf. Thus, when ATP levels become depleted, state 2 would be induced. This would increase cyclic electron flow around PS1 which helps restore ATP levels.

Vallon et al. (1991) studied the thylakoid distribution of cytbf in state 1 and state 2 in C. reinhardtii and maize. They found that the proportion of cytbf in the PS1-rich stroma lamellae regions was significantly increased in state 2 compared with state 1. It was therefore suggested that in state 2, a PS1-cytbf complex forms that favours cyclic electron flow and hence ATP generation.

PS2 is particularly sensitive to oxidative damage, especially on overexcitation (Clarke et al. 1993, Keren & Ohad, 1998). It has been suggested therefore that state transitions may have a photoprotective role (Horton & Lee 1985, Mullineaux & Allen 1990). Under conditions of PS2 overexcitation, the state 1-2 transition would decrease the absorption cross section of PS2 and so limit damage to PS2. There is some evidence that a state 1-2 transition occurs at high light intensities. Studying the cyanobacterium Synechococcus PCC6301, Rouag & Dominy (1994) claimed that under high light intensities a partial reversal of the state 1 transition occurred.

A study of the state transition in a cyanobacterial bloom in a shallow estuarine system yielded similar findings. The ratio of far-red (>700nm) light to total photosynthetically-available radiation was found to increase with increasing depth, as other wavelengths were attenuated by dissolved organic matter and pigments (Schubert et al. 1995). A cyanobacterium undergoing wind-driven vertical mixing in this environment would experience light conditions varying from white light near the water surface, to far-red enriched light at moderate depths and eventually to darkness. 77K Fluorescence emission spectra of samples taken from various depths showed that state 1 prevailed at moderate depths where light is far-red enriched. State 2 prevailed at the lower dark depths. Under the higher white-light conditions near the water surface however, cells were found to adapt to state 2.
1.11 The experimental approach used in this thesis

It is clear that in cyanobacteria the state transition is triggered by the redox state of the plastoquinone pool/cytbf. The signal transduction events that link this redox signal to changes in excitation energy distribution however are unknown.

In order to gain an understanding of the molecular basis for signal transduction, the intention was to identify genes required for the state transition in *Synechocystis* PCC6803. As discussed in Section 1.2, *Synechocystis* PCC6803 has many advantages as a model organism. The approach was to employ random cartridge mutagenesis in this cyanobacterium to generate a library of random, genetically-tagged mutants. The state 1-2 transition is accompanied by a decrease in fluorescence emission from PS2 (Fork & Satoh 1983). Using a fluorescence video imaging system to observe this change of fluorescence in mutant colonies it would be possible to isolate mutants unable to perform state transitions. The identification of the lesions in state transition mutants would then pinpoint genes required for the state transition. Some of these genes may code for signal transduction factors.
Chapter 2

EXPERIMENTAL PROCEDURES
CHAPTER 2: EXPERIMENTAL PROCEDURES

Routine DNA manipulations were performed according to Sambrook \textit{et al.} (1989). The complete sequence of the \textit{Synechocystis} PCC6803 genome was searched and analysed at the CyanoBase www site (http://www.kazusa.or.jp/cyano/). Many of the procedures detailed here are also described in Emlyn-Jones \textit{et al.} (1999).

2.1 Media and growth conditions

The cyanobacterium \textit{Synechocystis} PCC6803 (glucose tolerant strain) was grown in BG11 medium (Castenholz 1988) supplemented with 10mM NaHCO$_3$. Mutants lacking PS2 or PS1 were grown in BG11 medium supplemented with 5mM glucose. Cultures were grown in an illuminated orbital incubator at 30°C. Cultures were grown at a light intensity of approx.10μE.m$^{-2}$.s$^{-1}$ unless specified otherwise. \textit{E.coli} strains were grown in LB medium (Sambrook \textit{et al.} 1989) in an orbital incubator at 37°C. For BG11 and LB, solid media contained 1.5% Bacto agar (Difco). Antibiotics were added to media where appropriate, at concentrations of 50μg/ml (kanamycin), 25μg/ml (chloramphenicol), 100μg/ml (ampicillin) and 50μg/ml (rifampicin).

2.2 Molecular biology techniques

2.2.1 Reagents and enzymes

Restriction endonucleases and other DNA modifying enzymes were purchased from New England Biolabs, Stratagene, Promega and Boehringer Mannheim. All common laboratory reagents were purchased from Sigma Chemical Co.

2.2.2 Plasmids

The cloning vectors pBluescript SK+ and pBluescript II SK+ were purchased from Stratagene. The cloning vector pGEM-5Zf(+) was purchased from Promega. pUC-4K (kanamycin resistance (Km$^R$) gene from Tn903 cloned with polylinker site) was purchased from Pharmacia Biotech. The plasmids pBSEPKan and pBSSK were made by M. Ashby (University College London, U.K.). pBSEPKan was made by cloning the Km$^R$ gene from Tn903 (pBR691) into the EcoRI/PstI sites in pBluescript SK+. pBSSK was made by cloning the Km$^R$ gene (SalI) from pBSEPKan in reverse orientation into pBluescript II SK+.
2.2.3 Oligonucleotides
Oligonucleotides were custom synthesised by Perkin-Elmer Applied Biosystems. The oligonucleotides used in this thesis are listed in Table 2.1.

2.2.4 Transformations

2.2.4.1 Synechocystis PCC6803
Synechocystis PCC6803 transformations were performed according to Porter (1988).

2.2.4.2 E.coli
XL-2 blue competent cells (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F proAB lacZAM15 Tn10 (TetR) Amy CamR]) (Stratagene) were used for transformations according to the instructions of the manufacturer. Where high efficiencies of transformation were required (for example the marker rescue (Section 3.6)), XL-2 blue ultracompetent cells (Stratagene) were used. Using these cells, transformation efficiencies of 5 x 10^6 colonies per μg plasmid DNA are possible. Where a high efficiency of transformation is not crucial (for example a standard molecular cloning step), XL-2 blue competent cells were used. Transformation efficiencies of 10^6 colonies per μg plasmid DNA are typical for these cells. XL-2 blue competent cells were made using the protocol of Hanahan (1985).

2.2.5 Preparation of DNA

2.2.5.1 Genomic DNA from Synechocystis PCC6803
Pure genomic DNA to be used for PCR was prepared using the DNeasy Plant Mini-kit (Qiagen), with the following modifications: 50ml of dense cell culture was harvested by centrifugation and resuspended in 400μl TES buffer (50mM NaCl, 5mM EDTA, 5mM Tris pH 8.5). 100μl lysozyme (500mg/ml in H2O) was added and the sample incubated at 37°C for 15 minutes with regular mixing. The DNeasy plant mini kit protocol was then followed from step 2. Genomic DNA to be used for restriction digests was prepared using a standard miniprep procedure (Porter 1988).

2.2.5.2 Plasmid DNA from E.coli
Plasmid DNA was prepared from E. coli using the Qiagen Mini and Midi kits. Where it was necessary to perform large numbers of plasmid mini preps, a protocol designed by A. Barakati (Queen Mary & Westfield College, U.K.) and based on the Qiagen protocols was used. This protocol is as follows: Cells were harvested from 2ml
## PCR PRIMERS

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## SEQUENCING PRIMERS

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<td>M13 reverse</td>
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Table 2.1: Oligonucleotides used in PCR and for sequencing.
culture. The cell pellet was resuspended in 150μl Qiagen buffer P1 containing 10mg/ml RNAse H (P1 is 10mM EDTA, 50mM Tris-HCl pH 8.0). 150μl Qiagen buffer P2 was added and the mixture inverted and incubated at room temperature for 5 minutes (P2 is 200mM NaCl, 1% SDS). 150μl pre-chilled Qiagen buffer P3 was added, and the mixture incubated on ice for 15 minutes (P3 is 3.0M potassium acetate pH 5.5). The sample was centrifuged at 20,000g in a microfuge for 15 minutes. One millilitre of ethanol was added to the supernatant and the sample was incubated on ice for 10 minutes and then centrifuged at 20,000g in a microcentrifuge for 10 minutes. The pellet was resuspended in 300μl TE buffer (1mM EDTA, 10mM Tris-HCl pH 8.0) and 150μl of a 7.5M ammonium acetate solution added. The sample was incubated on ice for 10 minutes and centrifuged at 20,000g in a microcentrifuge for 5 minutes. One millilitre of ethanol was added to the supernatant and the sample incubated on ice for 10 minutes. The sample was then centrifuged at 20,000g in a microcentrifuge for 10 minutes. The supernatant was removed and 0.5ml 70% ethanol was added to the pellet. The sample was centrifuged at 20,000g in a microcentrifuge for one minute and the supernatant removed. The pellet was washed with absolute ethanol, dried, and resuspended in TE buffer.

2.2.6 Gel purification of DNA fragments
DNA fragments were recovered from agarose gels using a protocol based on the Geneclean kit (BIO 101). In this protocol, glassmilk was replaced with a less expensive silica preparation (Boyle & Lew 1995).

2.2.7 DNA ligations
Cloning ligations were performed in 10μl reaction volumes with 100ng vector and approximately 3-fold the number of insert molecules. 1 unit of T4 DNA ligase was used and all ligations were incubated at 16°C overnight. For the analysis of ligations by blue-white selection, ligations were transformed into competent cells and plated onto LB containing IPTG (30μM), the chromogenic substrate X-gal (30μg/ml) and ampicillin (100μg/ml) (see also Sambrook et al. 1989).

2.2.8 PCR
"Synechocystis" PCC6803 genomic DNA prepared using the Qiagen DNeasy kit (see Section 2.2.5.1) was used for PCR. PCR was performed in a Perkin-Elmer GeneAmp PCR system 2400 using the Expand High Fidelity PCR system (Boehringer Mannheim) according to the manufacturers instructions. For whole-cell PCR, a small inoculum of cells

48
taken from an agar plate was suspended in 50μl sterile distilled H$_2$O, and 5μl used in reactions.

2.2.9 Sequencing
Automated dye-terminator sequencing was carried out by Laura Winskill using a Perkin-Elmer ABI Prism 377 DNA sequencer.

2.2.10 Preparation of RNA from Synechocystis PCC6803
A 50ml culture was harvested and the pellet resuspended in 0.6ml TEN.SDS (0.5M NaCl, 0.01M EDTA, 0.2% SDS, 0.2M Tris-HCl pH 8). The sample was then subjected to three freeze-thaw cycles (37°C-liquid nitrogen) and then 0.6ml phenol:chloroform :isoamylalcohol (25:24:1) was added. The sample was centrifuged at 20,000g in a microfuge for 2 minutes. 1.4ml ethanol was added to the supernatant and the sample centrifuged at 20,000g in a microcentrifuge for 10 minutes. The pellet was washed in 70% ethanol, dried, and resuspended in RNAse-treated H$_2$O. The techniques and precautions routinely observed when working with RNA are as described in Sambrook et al. (1989).

2.2.11 Southern and Northern blotting

2.2.11.1 Southern blotting
Agarose gel electrophoresis of restricted genomic DNA was carried out as described in Sambrook et al. 1989. Transfer of DNA to a Hybond-N nylon membrane (Amersham Life Science) by capillary blotting and hybridisation of probe to the membrane were carried out according to the Amersham Life Science protocol. Radiolabelled DNA probes were made using the Prime-It random primer kit (Stratagene). [$\gamma$-$^{32}$P]-CTP used for radiolabelling of probes was purchased from Amersham Life Sciences. Hybridisation was carried out in bottles (Hybaid) using a dual hybridisation oven (Hybaid). After hybridisation, membranes were autoradiographed using X-ray film (Kodak) and intensifying cassettes (Genetic Research Instruments). Typical incubations for autoradiography were 16 hours at -80°C.
2.2.11.2 Northern blotting

Agarose gel electrophoresis of RNA (approx. 50μg/lane) was carried out as described in Sambrook et al. 1989. Capillary blotting, hybridisation and autoradiography were carried out as for Southern blots (Section 2.2.11.1).

2.3 Biochemical and biophysical techniques

2.3.1 Screening for state transition mutants using a fluorescence video imaging system

Random cartridge mutagenesis was employed in Synechocystis PCC6803 to generate a library of random, genetically-tagged mutants (see Chapter 3). The state 2-1 transition in cyanobacteria is accompanied by an increase in the level of fluorescence emission from PS2 (Fork & Satoh 1983). Using a fluorescence video imaging system to observe this change in fluorescence from mutant colonies, it was possible to isolate mutants from the library which are unable to perform state transitions.

The set-up for fluorescence video imaging is illustrated in Figure 2.1. Petri dishes, each containing approximately 500 mutant colonies, were illuminated with an excitation light source (Intralux 6000-1: Volpi) screened with a 620nm short-pass edge filter (Ealing), combined with a fibre optic ring light (SMD: Volpi). This excitation is absorbed strongly by the pigments of the phycobilisomes. Fluorescence emission from PS2 was detected with a TM765 monochrome CCD camera (Pulnix) screened with a Schott RG665 glass filter. State 2 images were taken after 10 minutes dark adaptation, and state 1 images were taken after 1 minute illumination with a light source (High-light 2000) screened with a Schott RG665 glass filter. The images were transferred to a computer with an SNP-8 frame grabber (DataCell) and processed using OPTIMAS 5.0 software (Optimas Corporation) in order to highlight colonies not performing state transitions. The images were processed as follows:

i) The state 1 image (Figure 2.2a) was divided by the state 2 image (Figure 2.2b) to yield a ratio image (state 1/state 2) (Figure 2.2c). In this image, colonies performing state transitions show ratio values greater than 1 (typically about 1.15), whereas the background and any colonies not performing state transitions show ratio values of 1.

ii) The (state 1/state 2) ratio image was rescaled to produce a map showing the positions of colonies performing state transitions. The background was set to zero, and ratio values greater than 1 were set to the maximum pixel value of 255 (Figure 2.2d).
iii) The original state 1 image was rescaled in a similar way to produce a map showing the positions of all the colonies on the plate. The background was set to zero, and fluorescence values above a threshold were set to the maximum pixel value (Figure 2.2e).

iv) Colonies not performing state transitions were highlighted by subtracting the state transition map from the colony map (Figure 2.2f).

2.3.2 77K Fluorescence emission spectra and room temperature fluorescence timecourse measurements

77K Fluorescence emission spectra and room temperature fluorescence timecourse measurements were made in a Perkin-Elmer LS50 luminescence spectrometer. For 77K measurements, cells (5μM in BG11 medium) were injected into 4mm-diameter silica tubes. They were then adapted to state 1 or state 2 as for colonies on plates (see Section 2.3.1) and frozen in liquid nitrogen. The excitation and emission slit widths were 5nm. Room temperature fluorescence measurements were carried out with liquid cultures (5μM in BG11) in a 3ml cuvette. The light 1 was as for colony state 1 adaptation, and the excitation and emission slit widths were 10nm. All measurements were repeated at least once in order to ensure reproducibility.

2.3.3 Absorption measurements

Absorption spectra were measured in an Aminco DW2000 spectrophotometer. Measurements of chla concentration and cell density were made in a Unicam UV2 spectrophotometer.

2.3.4 Pigment content analysis

Chla concentrations of liquid cultures were estimated from the absorption of methanol extracts at 665nm (Porra et al. 1989). The cell densities of liquid cultures were estimated from apparent absorption at 750nm (cell scattering). These measurements were used to calculate chla per cell. The ratio of phycocyanobilin/chla was estimated from cell absorption spectra using the formulae of Myers et al. (1980). This was used to calculate phycocyanobilin per cell.

Thylakoid membranes for PS1 estimation were isolated as described in Mullineaux (1992). An absorption spectrum of the phycobilin-containing supernatant from the preparation was...
Figure 2.1: Screening for state transition mutants using a fluorescence video imaging system. Fluorescence images of mutant colonies, dark adapted to state 2 or red light adapted to state 1 are processed in order to identify those showing no difference in fluorescence between the states (i.e. those unable to perform state transitions).
Figure 2.2: The processing of fluorescence images using OPTIMAS 5.0 software.
used to calculate the phycocyanin/allophycocyanin ratio (Bennett & Bogorad 1973). Phycobilisome content could then be estimated on the assumption that there are 66 allophycocyanin subunits per phycobilisome core. PS1 content was estimated spectrophotometrically. Thylakoid membranes [prepared as described in Mullineaux (1992)] were homogenised and resuspended to a chla concentration of 10μM. An absorption-difference spectrum was then recorded for membranes in the presence of ascorbate or ferricyanide (2mM). The concentration of P700 was estimated from the maximum absorption difference (at approx.702nm) using an extinction coefficient of 64mM⁻¹.cm⁻¹ (Hiyama & Ke 1972).

PS2 was assayed from the binding of ^14C-labelled atrazine (Chow et al. 1990). Whole cells were incubated for 5 minutes in the dark with different concentrations of ^14C-labelled atrazine. The samples were then centrifuged and the supernatants separated from the cell pellets. The supernatants were mixed with a scintillation cocktail (Sigma-Fluor) and counted in a scintillation counter. The amounts of atrazine bound by the cells were estimated by reference to the counts from a series of known dilutions of atrazine. The PS2 content of the samples was assumed to be equivalent to the saturating amount of atrazine bound. This was estimated by weighted linear regression on a double-reciprocal plot of atrazine added versus atrazine bound using Sigma plot for windows version 5.0. Note that this assay will detect any PS2 centre with a quinone binding site. This may lead to discrepancies with other assays, which detect only fully functional PS2 centres.

2.3.5 77K Fluorescence emission spectra of intact phycobilisomes
Intact phycobilisomes were prepared from Synechocystis PCC6803 according to the method of Glazer (1988). After unloading from the sucrose density gradient, phycobilisomes were diluted in buffer (1M sucrose, 0.75 M K₂HPO₄ pH 7.0). 77K fluorescence emission spectra were then taken as for whole cells (Section 2.3.2).

2.3.6 Preparation and analysis of thylakoid membranes
Thylakoid membranes were prepared according to the method of Murata & Omata (1988). Membranes were analysed by SDS-PAGE on a 15% polyacrylamide (38:1 acrylamide/bis-acrylamide) gel (Laemmli 1970). Proteins were visualised by Coomassie staining.

2.3.7 In vitro [γ-³²P]-ATP protein labelling
The procedure for in vitro [γ-³²P]-ATP-labelling experiments was based on that of Tsai (1997). Membrane and soluble fractions were prepared from French-pressed cells by a method similar to that of Murata & Omata (1988). Assay of protein concentration was performed using the Bio-Rad DC protein assay kit. Membrane and soluble fractions (1mg
of protein in 36μl of 20mM TES buffer pH 7.0) were incubated at 30°C in the dark for 10 minutes in the presence of sodium dithionite (10mM), duroquinol (0.5mM), MgCl₂ (5μM), ATP (0.75μM) and 1μl [γ-³²P]-ATP (5μCi/μl, 3000Ci/mmol). The reaction was stopped by adding 13.3μl of SDS-PAGE gel sample buffer (8mM EDTA, 8% SDS, 20% β-mercaptoethanol, 0.5M Tris pH 6.8) and then heating at 100°C for 5 minutes. Samples were run on a 10-30% polyacrylamide gradient (38:1 acrylamide/bis-acrylamide) gel (Laemmli 1970) and Coomassie stained before autoradiography.

2.3.8 Oxygen evolution/uptake measurements
Oxygen evolution/uptake measurements were performed in an oxygen electrode (Rank Brothers) at 30°C using liquid cultures at 20μM chlα. Oxygen evolution was measured with saturating illumination and oxygen uptake was measured in the dark.

2.3.9 Measurements of photoinhibition
Measurements of photoinhibition were based on the protocol of Nixon et al. (1995). Cultures were grown until late exponential phase. Cells were then harvested and resuspended to a chlα concentration of 25μg/ml. Cells, with and without the protein synthesis inhibitor lincomycin (final concentration 100 μg/ml) were stirred in flat glass dishes at 30°C and subjected to heat-filtered white light of approximately 1600μE.m².s⁻¹. PS2 activity was monitored every hour by measuring the rate of oxygen evolution under saturating light in the presence of the electron acceptors ferricyanide (2mM) and DCBQ (1mM).

2.3.10 Growth experiments
For growth experiments, 100ml cultures in 500ml flasks were used. Filters were purchased from Stage Electrics. Medium red (Rosco) was used for red light (the transmission spectrum for this filter is shown in Figure 2.3a). A combination of Lee green and Deep straw (LEE filters) was used for yellow light (the transmission spectrum for this combination is shown in Figure 2.3b). The yellow light was designed to be absorbed strongly by the phycobilisomes, which absorb in the 450-660nm region of the spectrum (MacColl 1998). The red light was designed to be absorbed strongly by chlα, which absorbs in the approximately 430nm and 670-700nm regions of the spectrum (Clayton 1980). A 0.6 neutral density filter (Lee filters) was used for very low white light intensities (2μE.m².s⁻¹).
Figure 2.3: Transmission spectra for a) red filter and b) yellow filter.
Cell density was estimated by measuring the light scattering at 750nm in a spectrophotometer (Unicam UV2). The measurement was calibrated by counting cells in a haemocytometer: an OD$_{750}$ of 1.0 corresponds to $1.52 \times 10^8$ cells/ml. Cultures were started at an OD$_{750}$ of about 0.1 and were measured every hour for cultures grown under high intensity light, approximately every 12 hours for cultures grown under moderate intensity light and approximately every 24 hours for cultures grown under low and very low intensity light (Table 8.1). Doubling times were estimated from growth curves by a non-linear regression using Sigma Plot for Windows version 5.00. Typically 4 or 5 data points from the exponential phase of growth were used for such an analysis.

2.3.11 Detection of 6HRpaC by dot blot analysis and purification of 6HRpaC

Thylakoid membranes were prepared according to the method of Murata and Omata (1988). These were subjected to dot-blot analysis according to the Qiagen protocol (The Qiaexpress detection and assay handbook) using a hexahistidine antibody (Qiagen). Detection was with an ECL Western blotting detection kit (Amersham Life Sciences). For purification of RpaC from 6HRpaC, the Qiagen Ni-NTA affinity chromatography protocol under denaturing conditions was followed (a handbook for high-level expression and purification of 6xHis-tagged proteins), with the following modifications. One litre of dense cell culture was harvested and resuspended in 10ml buffer B (8M Urea, 0.1M Na$_2$PO$_4$, 0.03% (w/v) Dodecyl Maltoside, 0.01M Tris-HCl, pH 8.0). The sample was passed through a french press at 23,000 psi. Protocol 9 (preparation of cleared lysates under denaturing conditions) was then followed from step 3), and then Protocol 14 (batch purification under denaturing conditions) followed. 0.03% (w/v) Dodecyl Maltoside was added to buffers A, B and C.
Chapter 3

THE IDENTIFICATION OF A GENE REQUIRED FOR THE STATE TRANSITION IN *SYNECHOCYSTIS* PCC6803
CHAPTER 3: THE IDENTIFICATION OF A GENE REQUIRED FOR THE STATE TRANSITION IN *SYNECHOCYSTIS PCC6803*

3.1 Introduction

As discussed in chapter 1, the molecular basis for the changes in excitation energy distribution that occur during state transitions in cyanobacteria has been a subject of extensive investigation. It is clear that in cyanobacteria the state transition is triggered by the redox state of the plastoquinone pool/cytb/f. The signal transduction factors linking this redox signal with changes in excitation energy distribution are unknown.

The intention was to identify signal transduction factors required for the state transition in *Synechocystis* PCC6803. As discussed in Chapter 1, *Synechocystis* PCC6803 has many advantages as a model organism. The approach was to generate a library of random, genetically tagged mutants. This library would then be screened for the inability to perform state transitions using a fluorescence video imaging system. The identification of the lesions in state transition mutants would then pinpoint genes required for the state transition. Some of these genes may code for signal transduction factors.

3.2 Transposon mutagenesis

An initial attempt to generate random, genetically tagged mutants in *Synechocystis* PCC6803 was made using transposon mutagenesis. The transposon used was Tn5. This transposon has been used to generate successfully random mutants in several species of cyanobacteria (Cai *et al.* 1997, Campbell *et al.* 1997, Cohen *et al.* 1994, Ernst *et al.* 1992), and other bacteria (Belas *et al.* 1991, Kunte & Galinski 1995, Singer & Finnerty 1984).

Transposon mutagenesis in *Synechocystis* PCC6803 was carried out by conjugation from *E. coli*. The transposon Tn5 is present on a plasmid construct pAM1037 in a donor strain of *E. coli* AM1037 (obtained from S. Golden, Texas A&M University, Texas, U.S.A.). This donor strain of *E. coli* was mixed with a conjugative strain of *E. coli* AM1460, and a rifampicin resistant strain of *Synechocystis* PCC6803 RR4. Mating of AM1037 with AM1460 generates a conjugative strain of *E. coli* capable of transferring pAM1037 to RR4. Once in RR4, the Tn5 gene in pAM1037 can insert into the genome. Tn5 contains a kanamycin resistance (KmR) gene which allows the selection of mutants. The ratios of AM1037, AM1460 and RR4 were optimised for mutagenesis by A. Smith (University College London, U.K.). The RR4 strain of *Synechocystis* PCC6803 was obtained by selection of the wild-type on rifampicin containing medium. It presumably contains a spontaneous mutation in the β- subunit of RNA polymerase, the site of rifampicin binding. The RR4 strain of *Synechocystis* PCC6803 was used so that the *E. coli* strains could be
eliminated after mutagenesis. Using this procedure photoautotrophic Km<sup>r</sup> mutants were generated at a frequency of approximately $10^5$.

A characteristic of transposon insertion is that it is not always random. Depending on the transposon and the host species, certain 'hotspots' for transposon insertion can exist. For example, in *Acinetobacter* sp., Tn5 was found to insert into a single location (Singer & Finnerty 1984). This behaviour is probably caused by homology between transposon sequences and a site(s) in the host genome. Depending on the level of homology this would promote recombination between the transposon and the particular site(s), leading to non-random transposon insertion (Calos & Miller 1980).

Southern analysis was performed on 10 of the RR4 Tn5 mutants to establish whether Tn5 insertion was random (Figure 3.1). Mutant genomic DNA was digested with *Bg*I (which has one site within the Tn5 sequence) and Tn5 used as a probe. For all the mutants, the two bands obtained were of the same molecular weight. This showed that insertion of Tn5 was at one site in the genome only, i.e., it appeared to be completely non-random.

The alternative method of random cartridge mutagenesis was therefore chosen to generate a random mutant library.

### 3.3 Random cartridge mutagenesis

This method has been used successfully to generate random mutants in *Synechocystis* PCC6803 (Baier *et al.* 1999), as well as in other naturally transformable bacteria (Morrison *et al.* 1984, Niaudet *et al.* 1982).

Random cartridge mutagenesis entails the ligation of digested genomic DNA to an antibiotic resistance gene (Figure 3.2). This generates a whole range of different ligation products consisting of the antibiotic resistance gene with genomic sequences ligated at one or both ends. The ligation products are then transformed into host cells. Homologous recombination between sequences flanking the antibiotic resistance gene and their corresponding sequences in the host genome then results in insertion of the antibiotic resistance gene into the host genome (Figure 3.2). An analysis of the mechanism of antibiotic resistance gene insertion has been made in *Synechocystis* PCC6803 (Chauvat *et al.* 1989, Labarre *et al.* 1989). It is proposed that the predominant mechanism of insertion is by double crossover/gene conversion between sequences flanking the antibiotic resistance gene in a linear construct, and homologous sequences in the host genome. Thus, if the sequences flanking the antibiotic resistance gene are contiguous on the host genome, double crossover/gene conversion could result in an insertion of the Km<sup>r</sup> gene. If the sequences flanking the antibiotic resistance gene are a distance apart on the host
Figure 3.1: Southern analysis of 10 Tn5 mutants of *Synechocystis* PCC6803. Genomic DNA was digested with *Bg*II. Blots were probed with pAM1037.
Figure 3.2: Random cartridge mutagenesis. Genomic restriction fragments (black) are ligated to an antibiotic resistance gene (white). Homologous recombination (here a double crossover) with the host genome (dotted) leads to insertion of the antibiotic resistance gene.
genome, double crossover/gene conversion could result in an insertion, but with the deletion of the sequences between the two flanks (as depicted in Figure 3.2).

Mutants generated using this method can be selected with antibiotic. *Synechocystis* PCC6803, like many other cyanobacteria has multiple copies of its genome (Labarre *et al.* 1989). An initial mutation is usually limited to one genome copy only. If this mutation is non-lethal, segregation of genome copies driven by antibiotic selection will eventually lead to homoplasmiccy (i.e. cells containing no wild-type genome copies). If the mutation is lethal but recessive, the cells can only survive in a heteroplasmic state (i.e. cells containing wild-type and mutant genome copies). If the mutation is lethal and incompletely recessive, transformants will be unable to survive. This is because the presence of the mutation is lethal, and the absence of the antibiotic resistance gene is also lethal.

Random cartridge mutagenesis has several advantages for our application over other forms of mutagenesis:

1) With transposon mutagenesis, hotspots for transposon insertion sometimes exist within the genome (see Section 3.2). With random cartridge mutagenesis such hotspots for antibiotic resistance gene insertion are unlikely to exist.

2) Mutants generated by random cartridge mutagenesis can be selected on the basis of antibiotic resistance. With, for example, chemical mutagenesis, the absence of such means of selection can make the full segregation of transformants to homoplasmiccy difficult.

3) Random cartridge mutants are genetically tagged. This makes the identification of the site of antibiotic resistance gene insertion a simple matter. In *Synechocystis* PCC6803 the entire genome sequence is available (Kaneko *et al.* 1996). This makes such a process even simpler (see Section 3.6).

4) The use of random mutagenesis means no assumptions are made. An alternative approach to identifying signal transduction factors required for the state transition would be to make insertional inactivation mutants of likely genes. For example, the two-component regulatory system is widespread (although not universal) among prokaryotes. There are many putative response regulators and histidine kinases within the *Synechocystis* PCC6803 genome sequence which may have a role in state transition signal transduction (Kaneko *et al.* 1996). The disadvantage of such an approach is that it is necessary to make assumptions about the molecular basis of signal transduction.
3.4 Generation of a mutant library in *Synechocystis* PCC6803

Using random cartridge mutagenesis, five mutant libraries were constructed using five different restriction enzymes: *BamHI, EcoRI, PstI, SalI* (which all cut at a 6-base recognition site) and *Sau3A* (which cuts at a 4-base recognition site).

Within the *Synechocystis* PCC6803 genome there are a total of 3217 putative open reading frames: 3168 potential protein coding genes and 49 potential structural RNA genes (Kotani & Tabata 1998). The average length of an open reading frame is 966 base pairs. It is possible to make a rough estimate of the probability that a gene will contain a *BamHI, EcoRI, PstI, SalI* or *Sau3A* restriction site, and therefore that a lesion is possible in that gene:

6-base recognition sequences (*BamHI, EcoRI, PstI* or *SalI*) would be predicted to occur every: $4^6 = 4096$ bp

4-base recognition sequence (*Sau3A*) would be predicted to occur every: $4^4 = 256$ bp

The predicted frequency of *BamHI, EcoRI, PstI, SalI, Sau3A* sites is:

$$\frac{1}{4096} + \frac{1}{4096} + \frac{1}{4096} + \frac{1}{4096} + \frac{1}{256} = \frac{1}{205} \text{ bp.}$$

The probability of obtaining a lesion in a 966 bp gene is therefore:

$$1 - \left(\frac{204}{205}\right)^{966} = 0.99$$

The probability of obtaining a lesion in a 300bp gene is:

$$1 - \left(\frac{204}{205}\right)^{300} = 0.77$$

It is therefore likely that lesions are possible in a large proportion of the *Synechocystis* PCC6803 genes.

To make the mutant libraries, the Km\(^R\) was excised from pUC4K with each of the five restriction enzymes *BamHI, EcoRI, PstI, SalI* and *Sau3A*. The 1.4 kb fragment containing the Km\(^R\) gene was then gel-purified. Approx. 2µg of gel purified Km\(^R\) gene was then ligated to approx. 2µg of wild type genomic DNA cut with the corresponding restriction
enzyme (ligation as for standard cloning ligation, Section 2.2.7). The ligation product was then transformed into wild-type *Synechocystis* PCC6803 and mutants were selected on BG11 plates containing kanamycin. It was predicted that any mutants specifically affected in state transition signal transduction would grow photoautotrophically.

It has since been shown that a mutant specifically affected in the state transition grows photoautotrophically and with a similar doubling time to the wild-type at all but very low light intensities (see Chapter 8). Mutants that are indirectly disrupted in the state transition, such as those disrupted in electron transport, would be less likely to grow photoautotrophically and so would be selected against.

For each transformation, approximately 20,000 mutant colonies were obtained. These colonies were grown for 2 weeks. After this time the mutants were replated and grown for a further 2 weeks. This replating was achieved by resuspending mutant colonies together in medium added directly to the plate. Cells were then inoculated onto fresh plates. The number of cells, N, plated after resuspension, was greater than the number of colonies resuspended:

\[ N = \ln(1-P) / \ln(1-f) \]

where p is the fraction of mutants to be retained on resuspension and plating (0.99 is adequate for these purposes), and f = 1/ total number of mutant colonies resuspended (Sambrook *et al.* 1989). This was to ensure that mutants were not lost in the process of resuspension and plating. For example, if 2000 mutant colonies were resuspended from a plate, and 2000 cells inoculated onto fresh plates, 37% (740) of mutants would be lost.

It was predicted that 4 weeks would be adequate time to allow for segregation of mutants specifically affected in state transition signal transduction (Vermaas 1996). It has since been shown that an insertional inactivation mutant specifically affected in the state transition segregates to homoplasmicity within 4 weeks (see Section 3.7).

After time allowed for segregation the mutant colonies were again resuspended and plated out at approximately 500 cells per plate for screening.

**3.5 Screening the mutant library for the inability to perform state transitions**

Using a fluorescence video imaging system it is possible to observe the characteristic change in PS2 fluorescence seen on the state transition in mutant colonies, and so isolate state transition mutants (Section 2.3.1).
The Synechocystis PCC6803 genome is 3,573,470 bp. in length (Kaneko et al. 1996). It is possible to make a rough estimate of the number of mutant colonies it is necessary to screen to cover all possible mutants:

The predicted number of a specific 6-base recognition sequence in the genome is:

\[
\frac{3,573,470}{4096} = 872
\]

The predicted number of a specific 4-base recognition sequence in the genome is:

\[
\frac{3,573,470}{256} = 13960
\]

Assuming that these are the number of possible mutants that can be generated using a particular restriction enzyme, the number of mutant colonies, \(N\), that must be screened in order to cover all these possible mutants is given by:

\[
N = \frac{\ln(1-P)}{\ln(1-f)}
\]

Where \(P\) is the fractional coverage of all possible mutants required (for these purposes 0.99), and \(f = 1/\) estimated number of restriction sites in genome (mutants) (Sambrook et al. 1989).

For 6-base recognition sequences: \(N = 4013\)

For 4-base recognition sequences: \(N = 64,240\)

Approximately 20,000 mutant colonies were screened for each restriction enzyme library. In the case of the BamHI, EcoRI, PstI, and SalI libraries, the number of mutants screened greatly exceeded \(N\). In the case of the Sau3A library, the number of mutants initially generated as well as the number of mutants screened was insufficient to cover all possible mutants.

The initial screen highlighted approximately 100 colonies. These were streaked out and screened again using the fluorescence video imaging system. A subset of these mutants was then subjected to detailed spectroscopic analysis [77K fluorescence emission spectra and room-temperature fluorescence time course measurements (as in Section 3.8)] to confirm their inability to perform state transitions.

Eight mutants were subsequently confirmed as being unable to perform state transitions: M3 (PstI), M4 (PstI), M6 (EcoRI), M9 (PstI), M10 (SalI), M11 (EcoRI), M13 (EcoRI)
and M14 (PstI). These mutants were subjected to detailed Southern analysis. This confirmed that mutants M3, M4, M9 & M14 contained identical lesions, i.e. were descendants of the same mutant (data not shown). There were therefore five mutants with unique lesions: M3 (PstI), M6 (EcoRI), M10 (Sali), M11 (EcoRI) and M13 (EcoRI). The sites of the lesions in these state transition mutants were determined by marker rescue.

3.6 Determination of the sites of disruption in state transition mutants.
The sites of disruption in state transition mutants were determined by marker rescue. First, mutants were subjected to Southern analysis to identify a single genomic restriction fragment containing the Km\textsuperscript{R} gene suitable for cloning (data not shown). Genomic restriction fragments of this approximate molecular weight were extracted from an agarose gel slice and purified. These fragments were then ligated into an appropriate cloning vector. Ligations were set up with various vector/insert ratios. Each ligation was then transformed into \textit{E.coli} XL-2 blue competent cells and plated onto LB ampicillin with X-gal and IPTG in order to establish by blue white selection the optimum vector/insert ratio for ligation. The optimum ligation was then transformed into \textit{E.coli} XL-2 blue ultracompetent cells and plated onto LB kanamycin. This selected for transformants containing the cloned genomic fragment with the Km\textsuperscript{R} gene. The genomic fragments flanking the Km\textsuperscript{R} gene were then sequenced. The position of these sequences in the \textit{Synechocystis} PCC6803 genome could then be determined from the complete genome sequence (Cyanobase, see Chapter 2). Each mutant was marker-rescued as follows:

- M4: approx.1.7 kb \textit{BfuI} fragment cloned into \textit{NdeI} site of pGEM-5Zf(+) 
- M6: approx.2.1 kb \textit{SacI}/\textit{SacII} fragment cloned into \textit{SacI}/\textit{SacII} sites of pBluescript SK(+) 
- M10: approx.1.6 kb \textit{BfaI} fragment into cloned into \textit{NdeI} site of pGEM-5Zf(+) 
- M11: approx.1.8 kb \textit{BfaI} fragment into cloned into \textit{NdeI} site of pGEM-5Zf(+) 
- M13: approx.1.6 kb \textit{BfaI} fragment into cloned into \textit{NdeI} site of pGEM-5Zf(+) 

The genomic sequences flanking the Km\textsuperscript{R} gene in the rescued plasmids were determined using primers pk3 and pk7 (Km\textsuperscript{R} gene), primers T7 and M13 reverse [pGEM-5Zf(+)] and primers T7 & T3 [pBluescript SK(+)].

In Figure 3.3 the positions and orientations of the genomic sequences flanking the Km\textsuperscript{R} gene in the five mutants are shown. In the case of M6, M11 and M13 the sequences flanking one side of the Km\textsuperscript{R} gene corresponded to positions within the ORF slr2005. The
Figure 3.3: The positions of the genomic sequences flanking the Km<sup>R</sup> gene in state transition mutants. Numbers in bold show the positions of flanking sequences derived from slr2005 (see text for details).
sequences flanking the other side corresponded to positions from 143-1954 kb away. These lesions could not represent insertional deletions: a deletion of 143 kb would certainly be lethal. The possibility was therefore considered that homologous recombination between exactly homologous sequences had occurred with the host genome on one side of the construct only. The sequences flanking the other side of the construct may have recombined with a region of weak homology, or may have become inserted by some illegitimate recombination event. This may have led to an insertion or insertional deletion. The flanking sequence common to M6, M11 and M13 was that containing slr2005 sequences. It seemed likely therefore that this flank was the site of homologous recombination between exactly homologous sequences and that the slr2005 locus was the true insertion site of the Km\(^{R}\) gene in these mutants.

In the case of mutant M10, the sequence flanking one side of the Km\(^{R}\) gene was 5 kb 3' from slr2005. The sequence flanking the other side of the Km\(^{R}\) gene was 282 kb 5'. Such a deletion would be lethal. As above, however, it is possible that homologous recombination occurred on one side of the construct only (the side 5 kb 3' of slr2005). The other side may have become integrated at a point near slr2005 creating a small deletion (approx.5 kb) which may not be lethal. Indeed, examination of this region shows that such a deletion would disrupt genes coding for subunits of NADH dehydrogenase which have multiple copies in the genome.

In the case of mutant M4, the sequence flanking one side of the Km\(^{R}\) gene was approx.9 kb 3' of slr2005. The sequence flanking the other side of the Km\(^{R}\) gene was approx.6 kb 5' of slr2005. In this case a 15 kb insertional deletion may have occurred. Again, such a deletion may not be lethal since many of the genes deleted have other copies in the genome.

The lesions in these mutants suggested that the site of disruption causing the inability to perform state transitions was in the region of slr2005. In M6, M11 and M13 the orientation of the flank containing slr2005 sequences suggested that the rest of the construct had inserted 5' rather than 3' to slr2005. This suggested that if a deletion had occurred in these mutants, then genes 5' to slr2005, rather than 3' to slr2005 would have been disrupted. The ORFs slr2005 and sll1926 were therefore chosen for insertional inactivation.

### 3.7 Insertional inactivation of sll1926 and slr2005.

The strategy used for insertional inactivation of sll1926 and slr2005 is illustrated in Figure 3.4. A PCR product containing the sll1926 and slr2005 ORFs was made with SacI and KpnI ends (using primers 1926F and 2005R) and cloned into pBluescript SK(+) to make pBS1.4. The putative coding region of sll1926 was interrupted by cloning a 1.4 kb EagI/HincII Km\(^{R}\) gene from pBSEPkan into the corresponding sites within sll1926 (EagI
Figure 3.4: Diagram of the sll1926 and slr2005 locus showing the positions of the assigned coding regions on the Synechocystis PCC6803 genome (Cyanobase). Restriction enzyme sites used for insertional inactivation of sll1926 and slr2005 and southern analysis are indicated as well as the position and orientation of the PCR primers used for PCR amplification of the genes. The antibiotic resistance genes used for insertional inactivation are shown with the names of the mutants generated beside them.
site at 744,929; *HincII* site at 745,101) to make pBS1926ΔK. The coding region of slr2005 was interrupted by cloning a 1.4 kb *EcoRI/Apal* Km\(^r\) gene from pBSSK into the corresponding sites within slr2005 (*EcoRI* site at 745,766; *Apal* site at 745,870) to make pBS2005ΔK. These constructs were each sequenced with primers T3 and T7 [pBluescript SK(+)]) and pk3 and pk7 (Km\(^r\) gene) to confirm correct insertion of the Km\(^r\) gene into the sll1926 and slr2005 ORFs. These inactivation constructs were then each transformed into wild type *Synechocystis* PCC6803 to generate mutants Δsll1926 and Δslr2005. Transformants were selected by kanamycin resistance.

The extent of segregation of the mutants could be checked by performing PCR on an inoculum of whole cells from the mutants and the wild-type using primers 1926F and 2005R. The wild-type gave a product of 1.4 kb and Δsll1926 and Δslr2005 gave products of 2.6 kb and 2.7 kb respectively. This suggested that the mutants had segregated fully. After 1 month, complete segregation in Δsll1926 and Δslr2005 and correct insertion of the Km\(^r\) gene was confirmed by Southern analysis. Δsll1926 genomic DNA was digested with *NcoI* and Δslr2005 genomic DNA was digested with *BsrXI*. Blots were probed with sll1926 (amplified using primers NF and NR). The single band for Δsll1926 was 1.2 kb larger than the wild-type band (Figure 3.5), and the single band for Δslr2005 was 1.3 kb larger than the wild-type band (Figure 3.6). This confirmed that the mutants had segregated to homoplasmicity and that the Km\(^r\) gene had been inserted correctly. Both Δsll1926 and Δslr2005 were capable of photoautotrophic growth.

### 3.8 Spectroscopic characterisation of Δsll1926 and Δslr2005

#### 3.8.1 77K Fluorescence emission spectra with 600nm (phycocyanin) excitation.

Figure 3.7 shows 77K fluorescence emission spectra with 600nm excitation for the wild type, Δsll1926 and Δslr2005 adapted under either state 1 or state 2 conditions (see also Section 2.3.2). The 600nm excitation is strongly absorbed by phycocyanin (the phycocyanin absorption band peaks at around 620nm). This excitation is therefore designed to be absorbed predominantly by the phycobilisomes.
Figure 3.5: Southern analysis of Δsll1926. Genomic DNA was digested with NcoI. The blot was probed with sll1926.
Figure 3.6: Southern analysis of Δslr2005. Genomic DNA was digested with BstXI. The blot was probed with sl1926.
Figure 3.7: 77K Fluorescence emission spectra with 600nm excitation for a) wild type b) Δsll1926 and c) Δslr2005 adapted under either state 1 or state 2 conditions. Spectra are normalised to the phycocyanin/allophycocyanin (650nm) peak.
The spectra show three main fluorescence emission peaks. There is a broad peak at around 650nm corresponding to a mixture of phycocyanin and allophycocyanin emission. There is a peak at about 680nm originating from the terminal emitters of the phycobilisome as well as PS2 and a peak at 725nm originating from PS1. A shoulder is present at 690nm that corresponds to the PS2 core complex (Su et al. 1992). For the wild type (Figure 3.7a), and Δslr2005 (Figure 3.7c) the ratio of PS2/PS1 fluorescence emission is greater in state 1 than in state 2, reflecting greater relative energy transfer from the phycobilisomes to PS2 in state 1. In Δssl1926 (Figure 3.7b) no significant changes were observed, indicating that the mutant Δssl1926 was not performing state transitions. In addition, the spectrum of Δssl1926 showed no obvious aberrations in phycobilisome-to-photosystem energy transfer. Disruption of such energy transfer pathways can lead to the inability to perform state transitions as well as gross alterations in the 77K fluorescence emission spectra with 600nm excitation (Ashby & Mullineaux 1999a). Furthermore the relative level of PS2 fluorescence emission in the spectrum of Δssl1926 would suggest that this mutant is permanently in state 1.

### 3.8.2 Room temperature fluorescence timecourse measurements

Figure 3.8 shows room temperature fluorescence time course measurements for the wild-type, Δssl1926 and Δslr2005 (see also Section 2.3.2). Fluorescence emission at 685nm (PS2 chlα) was excited by 620nm (phycocyanin absorption) modulated light. Light 1 was used to induce state 1. Modulated excitation (i.e., excitation whose intensity rapidly fluctuates), results in modulated fluorescence emission. This means that it is possible, using a lock-in amplifier, to separate fluorescence emission generated by the excitation beam, from that generated by light 1.

Cultures were adapted to state 2 under the 600nm excitation. Then light 1 was switched on to induce state 1. For the wild-type (Figure 3.8a), and Δslr2005 (Figure 3.8c), when light 1 is switched on, a slow rising phase is observed which can be attributed to increasing absorption cross-section of PS2. This is characteristic of the state 1 transition (Allen et al. 1989, Fork & Satoh 1983). When light 1 is switched off, there is a rapid initial increase in fluorescence. This is because light 1 brings about state 1 by increasing PS1 turnover. When light 1 is switched off therefore there is a decrease in PS1 turnover, leading to reduction of the plastoquinone pool and closure of PS2 reaction centres. This leads to the sudden increase in PS2 fluorescence which is observed. This sudden increase in fluorescence is followed by a phase of decreasing fluorescence as the PS2 absorption cross-section decreases as a result of the transition to state 2 (Allen et al. 1989, Fork &
Figure 3.8: Room temperature fluorescence timecourse measurements for a) wild type, b) Δsll1926 and c) Δslr2005. Excitation was at 620nm and fluorescence measured at 685nm.

a) 

![Graph showing fluorescence timecourse for wild type.]

b) 

![Graph showing fluorescence timecourse for Δsll1926 and Δslr2005.]
Satoh 1983). In Δsll1926 no comparable fluorescence changes were observed (Figure 3.8b). The rapid fluorescence rise observed when light 1 is switched on and the rapid decrease when light 1 is switched off probably result from changes in PS2 reaction centre closure, directly induced by the added light.

This spectroscopic analysis showed that disruption of ORF sll1926 rather than slr2005 was responsible for the inability of the original mutants to perform state transitions.

3.9 Insertional inactivation of sll1927
There are two ORFs immediately downstream of sll1926. The first, sll1927 has homology to the oligopeptide transport ATP-binding protein OppF of Synechococcus elongatus. The second, sll1568 has homology to fibrillin of Capsicum annuum (Kaneko et al. 1996). The possibility that disruption of sll1926 was causing the inability to perform state transitions by a downstream effect on sll1927 or sll1568 was considered. For example sll1926 may be co-transcribed with sll1927 and sll1568, possibly as a part of an operon. To investigate this possibility we generated an insertional inactivation mutant of sll1927. The strategy used for insertional inactivation of sll1927 is illustrated in Figure 3.9.

A PCR product containing the sll1927 ORF was made with SacI and KpnI ends (using primers 1927F and 1927R) and cloned into pBluescript SK(+) to make pBS2. The putative coding region of sll1927 was interrupted by cloning a 1.4 kb HincII Km\textsuperscript{r} gene from pUC4K into the two HincII sites within sll1926 (at 744,380 and 744536) to make pBS1927\textsuperscript{AK}. This construct was sequenced with primers T3 and T7 [pBluescript SK(+)] and pk3 and pk7 (Km\textsuperscript{r} gene) to confirm correct insertion of the Km\textsuperscript{r} gene into the sll1927 ORF. This inactivation construct was then transformed into wild-type Synechocystis PCC6803 to generate mutant Δsll1927. Transformants were selected by kanamycin resistance.

The extent of segregation of the mutant could be checked by performing PCR on an inoculum of whole cells from the mutant and the wild-type using primers 1927F and 1927R. The wild type gave a product of 2.0 kb and Δsll1927 gave a product of 3.2 kb. This suggested that the mutants had segregated fully. After a month, complete segregation in Δsll1927 and correct insertion of the Km\textsuperscript{r} gene was confirmed by Southern analysis (Figure 3.10). Δsll1927 and wild-type genomic DNA were digested with Dral. The blot was probed with sll1926, (amplified using primers NF and NR). The single band for Δsll1927 was 1.2 kb larger than the single wild-type band. This confirmed that the mutant
Figure 3.9: Diagram of the sll1927 locus showing the position of the assigned coding region on the *Synechocystis* PCC6803 genome (Cyanobase). Restriction enzyme sites used for insertional inactivation of sll1927 and southern analysis are indicated as well as the position and orientation of the PCR primers used for PCR amplification of the gene. The antibiotic resistance gene used for insertional inactivation is shown with the name of the mutant generated beside it.
Figure 3.10: Southern analysis of Δsll1927. Genomic DNA was digested with Dral. The blot was probed with sll1926.
had segregated to homoplasmicity and that the Km\textsuperscript{R} gene had been inserted correctly. \textit{\textalpha}sll1927 was capable of photoautotrophic growth.

### 3.10 Spectroscopic characterisation of \textit{\textalpha}sll1927

(see Section 3.8 for a detailed explanation of these measurements)

Figure 3.11 shows 77K fluorescence emission spectra with 600nm excitation for the wild-type and \textit{\textalpha}sll1927 adapted under either state 1 or state 2 conditions. It can be seen that \textit{\textalpha}sll1927 shows the fluorescence changes characteristic of the state transition.

Figure 3.12 shows room temperature fluorescence time course measurements for the wild type and \textit{\textalpha}sll1927. It can be seen that \textit{\textalpha}sll1927 shows the characteristic fluorescence changes of the wild type state transition. These data show that \textit{\textalpha}sll1927 is performing wild type state transitions. It would therefore seem highly unlikely that disruption of sll1926 in \textit{\textalpha}sll1926 is causing the inability to perform state transitions by a downstream effect. The product of sll1926 therefore must have a role in the state transition in \textit{Synechocystis} PCC6803.

### 3.11 Discussion

We have identified an ORF, sll1926, in the cyanobacterium \textit{Synechocystis} PCC6803, that is required for the state transition. 77K Fluorescence emission spectra with 600nm excitation would suggest that an insertional inactivation mutant of sll1926, \textit{\textalpha}sll1926, is permanently in state 1 (Figure 3.7). There may be several reasons why, using this method, we identified mutants with lesions only in sll1926:

1) sll1926 may be the only gene in \textit{Synechocystis} PCC6803 whose disruption gives such a specific and photoautotrophic phenotype.

2) An insufficient number of mutants may have been screened. For the \textit{Sau3A} library, estimates suggested that too low a number of mutants was made and screened to cover all possible mutations.

3) The number and distribution of restriction sites may have been such that lesions in other genes required for the state transition were excluded from mutagenesis. The estimation of the probability of obtaining lesions in the genes of \textit{Synechocystis} PCC6803 was based on the average number of restriction sites in the genome. In reality the number of restriction sites will certainly differ. In addition, certain (especially small) genes may not contain the
Figure 3.11: 77K Fluorescence emission spectra with 600nm excitation for a) wild type and b) Δsll1927 adapted under either state 1 or state 2 conditions. Spectra are normalised to the phycocyanin/allophycocyanin (650nm) peak.
Figure 3.12: Room temperature fluorescence timecourse measurements for a) wild type and b) Δsll1927. Excitation was at 620nm and fluorescence measured at 685nm.
relevant restriction sites. These genes may be excluded from mutagenesis therefore. It should be noted that insertion of the Km^R gene would appear only to be confined to restriction enzyme sites on one side of the random construct (see below). In reality therefore, the estimates of the probabilities of obtaining lesions in every gene of the *Synechocystis* PCC6803 genome (Section 3.4) are probably underestimates.

4) Certain genes are less accessible to disruption. If a gene required for the state transition such as sll1926 is flanked by essential genes whose disruption would be lethal, then the extent of deletion possible around that gene leading to homoplasmic mutants would be greatly restricted compared with such a gene flanked by non-essential genes. Thus, the number of different random constructs capable of creating a homoplasmic lesion in that gene would be less. Such mutants would therefore be represented less in the mutant library. Their isolation in screening therefore may be made difficult or even impossible. For example, sll1926 is in a relatively non-essential region of the genome. To the 5' is sll1927, which is non-essential (see Section 3.9) and to the 3' is slr2005, also non-essential (see Section 3.7). Further 3' of sll1926 are several genes coding for NADH dehydrogenase subunits which have other copies elsewhere in the genome and are therefore likely to be non-essential. Inactivation of the *apcD* gene leads to an inability to perform state transitions and a photoautotrophic growth phenotype (Ashby & Mullineaux 1999a, Zhao et al. 1992). In contrast to sll1926, the *apcD* gene is closely flanked by an ORF which appears to code for the only copy of an s-adenosylmethionine synthetase, a gene which may well be essential (Kaneko et al. 1996). Several mutants disrupted in sll1926 were isolated, and several with seemingly large deletions, but no mutant disrupted in *apcD*. This may be because *apcD* is less accessible to disruption. Thus, other genes may exist whose disruption gives as specific and photoautotrophic a phenotype as sll1926. These genes may, however, be less accessible to disruption, and therefore in too small a proportion in the mutant library to be isolated.

It has been suggested that the recombination events occurring upon insertion of the antibiotic resistance gene into the genome of *Synechocystis* PCC6803 involve homologous recombination (double crossover/gene conversion) between sequences flanking both sides of the antibiotic resistance gene and homologous sequences in the host genome (Labarre et al. 1989). This could lead to an insertion when the flanking sequences are contiguous on the host genome, or an insertional deletion when the flanking sequences are a distance apart on the host genome (Section 3.3). In mutants M6, M11 and M13 such an event did not seem to occur. The sequences flanking the Km^R gene were too far apart on the host genome to represent deletions. Thus homologous recombination between exactly homologous sequences probably occurred at one side of the construct only. The other side of the
construct may have recombined with an area of weak homology or alternatively by some illegitimate recombination event.

In M6, M11 and M13, the flanking sequence undergoing homologous recombination between exactly homologous sequences would appear to be that with slr2005 sequences. Clearly in these mutants at least a small deletion occurred since sll1926 disruption caused their inability to perform state transitions and insertional inactivation of slr2005 leads to a wild-type phenotype (Section 3.8). A consequence of this recombination model would be that insertion of the antibiotic resistance gene into the genome is confined to restriction enzyme sites at one side of the random construct only, the side undergoing recombination with the exactly homologous sequence in the host genome. Insertion of the other side of the random construct by homologous recombination with an area of weak homology, or possibly by some illegitimate recombination event, would be independent of the presence of restriction sites in the host genome.
Chapter 4

CHARACTERISATION OF STATE TRANSITION
MUTANT Δsll1926
CHAPTER 4: CHARACTERISATION OF STATE TRANSITION MUTANT Δsll1926

4.1 Introduction
In Chapter 3 is described the identification of a gene required for the state transition in Synechocystis PCC6803, sll1926. In this chapter, the initial characterisation of the insertional inactivation mutant of this gene, Δsll1926, is presented.

4.2 Sequence analysis of sll1926
The amino acid sequence of sll1926 as assigned by Kaneko et al. corresponds to a polypeptide of 145 amino acids with a molecular weight of 16kDa. A BLAST search of the complete genomic sequence of the filamentous, nitrogen fixing cyanobacterium Anabaena PCC7120 (http://www.kazusa.or.jp/cyano/) revealed an ORF, a region of which had 68% identity to an 85 amino acid region of the sll1926 sequence. A similarity search at the ExPASy molecular biology server (http://www.expasy.ch/) (Appel et al. 1994) using the Bic program (European Bioinformatics Institute) revealed no other significant protein sequence homologues of sll1926 in current databases. A similarity search at the National Centre for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/) using the BLASTN program also revealed no other significant nucleotide sequence homologues. The Anabaena PCC7120 sequence also had no other homologues other than sll1926, at the amino acid or nucleotide level. A motif search (http://www.motif.genome.ad.jp/) revealed no significant sequence motifs in either sequence.

In Figure 4.1 is shown an alignment of the homologous regions of sll1926 and the Anabaena PCC7120 sequences. For the Synechocystis PCC6803 sll1926 sequence, three possible start sites are present within the assignment of Kaneko et al. (1996), at positions 1, 42, and 61. For the Anabaena PCC7120 sequence an in-frame stop codon is present immediately upstream from the start site. This indicates that this methionine must represent the beginning of the ORF. This might suggest that the true start site of sll1926 is at position 61.

A determination of transmembrane segments at the ExPASy molecular biology server using the TMpred program revealed the presence of two likely transmembrane helices in both sll1926 and its homologue in Anabaena PCC7120. These hydrophobic segments can be seen in Figure 4.1. On the basis of the pattern of charged residues, the TMpred program also predicted that both termini are slightly more likely to be on the cytoplasmic side of the membrane than on the luminal/periplasmic side. A secondary structure prediction was carried out at the ExPASy molecular biology server using the Jpred
**Figure 4.1**: An alignment of sll1926 from *Synechocystis* PCC6803 with its homologue in *Anabaena* PCC7120. Boxes enclose conserved residues. Hydrophobic regions are shaded red and hydrophilic regions are shaded blue. Arrows denote putative transmembrane regions. The presence of these helices is supported by a hydropathy analysis and a secondary structure prediction program (see text for details).
program (a consensus method). The results also indicated the presence of the transmembrane helices predicted by the TMpred program. These results would suggest that the product of sll1926 and its homologue in *Anabaena* PCC7120 are membrane-associated. A likely transmembrane topology of the protein is depicted in Figure 4.2. There are several potential sites for phosphorylation: surface exposed serine, threonine and tyrosine residues within the *Synechocystis* PCC6803 as well as the *Anabaena* PCC7120 sequence. Some of these residues are likely to be exposed to the aqueous phase.

4.3 77K Fluorescence emission spectrum with 435nm (chlorophyll) excitation for Δsll1926 (same samples as for spectra with 600nm (phycocyanin) excitation, Figure 3.7).

Figure 4.3 shows 77K fluorescence emission spectra with 435nm excitation for the wild-type and Δsll1926, adapted under either state 1 or state 2 conditions. The 435nm excitation is absorbed strongly by the chla Soret band. The spectra show similar PS2 and PS1 fluorescence emission peaks as the spectra with 600nm excitation (Section 3.8.1), but without contributions from the phycobilisomes (Su *et al.* 1992). In all the spectra state transitions result in a small change in the PS2/PS1 fluorescence ratio.

4.4 Measurements of oxygen evolution and respiratory oxygen uptake

The rates of oxygen evolution under saturating light and oxygen uptake in the dark were measured for wild-type and Δsll1926 liquid cultures, as detailed in Table 4.1 (see also Section 2.3.8). There is no indication that photosynthetic or respiratory electron transport are impaired in Δsll1926. In fact, the saturating rate of oxygen evolution on a per chlorophyll basis appears to be slightly higher in the mutant (Table 4.1). The 77K fluorescence emission spectrum of Δsll1926 with 600nm excitation would suggest that this mutant is permanently in state 1 (Figure 3.7). The higher oxygen evolution rate in Δsll1926 may therefore reflect a change in the balance of linear and cyclic electron flow: a mutant trapped in state 1 would be expected to show a lower proportion of cyclic electron flow around PS1 (Allen 1992).

4.5 Pigment content analysis

For pigment content analysis, cells were grown at 10μE.m⁻².s⁻¹ until late exponential phase in BG11 medium supplemented with 10mM NaHCO₃. The quantities of the various photosynthetic components were established for the wild type and Δsll1926 (Table 4.2) (Section 2.3.4). This showed there to be no apparent impairment in the assembly or
Figure 4.2: Predicted transmembrane topology of the product of sll1926 and its homologue in *Anabaena* PCC7120 (see also Figure 4.1).
Figure 4.3: 77K Fluorescence emission spectra with 435nm excitation for a) wild type and b) Δsll1926 adapted under either state 1 or state 2 conditions. Spectra are normalised to the PS1 (725nm) peak.

a)

b)
Table 4.1: Rates of oxygen evolution under a saturating light source and oxygen uptake in the dark for the mutant Δsll1926 and the wild type. Each rate is an average of three measurements and is presented with the standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Δsll1926</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂ evolution under saturating light (μmol/mg chla/hour)</td>
<td>211 ± 21</td>
<td>270 ± 40</td>
</tr>
<tr>
<td>Respiratory O₂ uptake in the dark (μmol/mg chla/hour)</td>
<td>26 ± 2</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>Pigment Type</td>
<td>Wild type</td>
<td>Δsll1926</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>-----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Chlorophyll / cell</td>
<td>$2.6 \times 10^7$</td>
<td>$3.2 \times 10^7$</td>
</tr>
<tr>
<td>Phycocyanobilin / cell</td>
<td>$1.56 \times 10^7$</td>
<td>$1.89 \times 10^7$</td>
</tr>
<tr>
<td>Phycocyanin / Allophycocyanin</td>
<td>4.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Phycobilisome / cell</td>
<td>33,000</td>
<td>43,000</td>
</tr>
<tr>
<td>PS1 / cell</td>
<td>$152,000 \pm 2000$</td>
<td>$171,000 \pm 2000$</td>
</tr>
<tr>
<td>PS2 / cell</td>
<td>$146,000 \pm 56,000$</td>
<td>$132,000 \pm 42,000$</td>
</tr>
<tr>
<td>PS1 / PS2</td>
<td>1.0 ± 0.4</td>
<td>1.3 ± 0.4</td>
</tr>
</tbody>
</table>

Table 4.2: Pigment contents of Δsll1926 and the wild type (see Section 2.3.4 for details). Each value is from a single measurement. Assays were all performed on the same culture.
function of the reaction centres or phycobilisomes. Low-temperature fluorescence emission spectra also indicate no major changes in light harvesting complexes or reaction centres in Δsll1926 (Figure 3.7).

**4.6 77K Fluorescence emission spectra of intact phycobilisomes**

Phycobilisomes were isolated from Δsll1926 and the wild-type and 77K fluorescence emission spectra recorded with 600nm excitation (Figure 4.4) (Section 2.3.5). For Δsll1926, as for the wild-type, these spectra showed the phycobilisomes to be functionally intact with a large single emission at about 680nm.

**4.7 Polypeptide composition of thylakoid membranes**

Thylakoid membranes were prepared from Δsll1926 and the wild-type as described in Section 2.3.6. These samples were analysed by SDS-PAGE and Coomassie staining (Figure 4.5). No differences in polypeptide composition could be seen between Δsll1926 and the wild-type. This inability to detect the product of sll1926 (assuming it is thylakoid membrane-associated) may be because it is present at very low levels in the thylakoid membrane. Alternatively, a band corresponding to the product of sll1926 may be obscured by other bands. The molecular weight of the product of sll1926, as predicted by Kaneko et al. (1996) is 16kDa. Methionines present within this assignment indicate that the protein may be smaller: 11 or 9kDa. No proteins are visible in the 11 or 9kDa region, and the 16kDa region is obscured by a large protein band which probably corresponds to phycobiliprotein contamination.

**4.8 Northern analysis**

Northern analysis was performed on RNA prepared from wild-type cells (grown at 10μE.m⁻².s⁻¹ to mid-exponential phase). sll1926 (amplified using primers NF and NR) was used as a probe. No sll1926 transcript could be detected. As a control, blots were re-probed using a fragment from the \(psbD\) gene. A strong band corresponding to the major psbD transcript was detected (Figure 4.6). This shows that the sll1926 transcript is too rare to be detected using Northern analysis. Detection of the sll1926 transcript will therefore require a more sensitive method, such as an RNAse protection assay.

**4.9 Regulation of photosystem stoichiometry**

A long-term adaptation mechanism serves to adjust the photosystem ratio in response to prolonged changes in illumination (Fujita 1997). The ability of Δsll1926 to perform this adaptation mechanism was measured. Wild-type and Δsll1926 liquid cultures were grown
Figure 4.4: 77K Fluorescence emission spectra with 600nm excitation of phycobilisomes prepared from Δsll1926 and the wild type. Spectra are normalised to the main 680nm peak.
Figure 4.5: The polypeptide composition of thylakoid membranes prepared from Δssl1926 and the wild type.
Figure 4.6: Northern analysis of total RNA prepared from the wild-type, using a fragment of the \textit{psbD} gene as a probe. Using sll1926 as a probe of the same blot, no bands could be detected.
for two days under either 2μE.m⁻².s⁻¹ yellow light or 100μE.m⁻².s⁻¹ white light. The 2μE.m⁻².s⁻¹ yellow light would be predicted to decrease the PS2/PS1 ratio. The 100μE.m⁻².s⁻¹ white light would be predicted to increase the PS2/PS1 ratio. Absorption spectra of these cultures are shown in Figure 4.7. The peak at 628nm corresponds to phycobilin absorption and that at 680nm to chlorophyll absorption. In both the wild-type and Δss11926, the phycobilin/chlorophyll absorption ratio was greater after 100μE.m⁻².s⁻¹ white light illumination, than 2μE.m⁻².s⁻¹ yellow light illumination. These changes in the absorption spectrum are indicative of changes in photosystem stoichiometry. They therefore show that Δss11926 is not impaired in the long-term regulation of photosystem stoichiometry.

4.10 Discussion
A mutant of the cyanobacterium Synechocystis PCC6803 has been isolated that is specifically unable to perform state transitions. The phenotype is produced by insertional inactivation of an ORF designated sll1926 in the Synechocystis PCC6803 database (Kotani & Tabata 1998). An 85 amino acid stretch of this gene has 68% identity to a region of a gene in the cyanobacterium Anabaena PCC7120. The gene product as assigned by Kaneko et al. (1996) has a predicted molecular weight of 16kDa. Methionines within this assignment indicate that the protein may be smaller: 11 or 9kDa. The protein is predicted to have two transmembrane helices.

Two cyanobacterial mutants unable to perform state transitions have been previously identified. An ndhB-defective mutant in Synechocystis PCC6803 lacks a functional complex I and is permanently in state 1 (Schreiber et al. 1995). The redox state of the plastoquinone pool/cytb₆ seems to be the trigger for the state transition in cyanobacteria (Mullineaux & Allen 1990, Vemotte et al. 1990). The inability of this ndhB-defective mutant to perform state transitions is therefore probably a result of decreased respiratory electron transport and a permanently oxidised plastoquinone pool.

Mutants lacking the phycobilisome core subunit APC-B (apcD gene) are also unable to perform state transitions. This was initially observed in Synechococcus PCC7002 (Zhao et al. 1992). It has since been observed in Synechocystis PCC6803 (Ashby & Mullineaux 1999a). This is probably due to an alteration in energy transfer from the phycobilisomes to the reaction centres.

Δss11926 does not appear to fall into the same category as the ndhB-defective mutant or ΔapcD. There is no impairment or gross alteration in photosynthetic or respiratory electron
Figure 4.7: Long term regulation of photosystem stoichiometry in a) wild type and b) Δsll1926. Spectra are normalised to the phycobilin (628nm) peak.

a)

b)
transport capacity in \( \Delta sll1926 \) which could account for the inability to perform state transitions (Section 4.4). Reaction centres and phycobilisomes are assembled and fully functional (Sections 4.5, 4.6). The 77K fluorescence emission spectrum of \( \Delta sll1926 \) with 600nm excitation shows no obvious perturbations in light harvesting: it resembles that of wild-type cells in state 1 (Figure 3.7).

\( \Delta sll1926 \) was also not impaired in the long-term regulation of photosystem stoichiometry (Figure 4.7). This long-term adaptation mechanism serves to adjust the photosystem ratio in response to prolonged changes in illumination and is triggered by changes in the redox state of cyt\( b/f \) (Fujita 1997). The state transition similarly is triggered by changes in the redox state of plastoquinone/cyt\( b/f \) (Mullineaux & Allen 1990, Vemotte et al. 1990). Indeed, it is possible that these two adaptation mechanisms share common elements involved in the transduction of the initial redox signal. Regulation of photosystem stoichiometry and the state transition have related but quite distinct functions: the former alters PS1 gene expression over hours and the latter alters light harvesting properties in seconds. Thus, if common redox sensing elements existed for both mechanisms, then signal transduction components further downstream would probably be distinct for each adaptation mechanism. Signal transduction would therefore have a forked structure. The finding that \( \Delta sll1926 \) was not impaired in the regulation of photosystem stoichiometry firstly shows that the product of sll1926 is specifically involved in the state transition. Secondly, it supports the finding that the inability of \( \Delta sll1926 \) to perform state transitions is not a consequence of altered photosynthetic or respiratory electron transport. If it were, then it is likely that the regulation of photosystem stoichiometry would be similarly affected.

The lack of information to be gained from sequence analysis makes speculation as to the function of the product of sll1926 difficult. The protein may be a signal transduction factor in the thylakoid membrane, involved at some stage in the events connecting the redox state of cyt\( b/f \)/plastoquinone with changes in excitation energy distribution. Alternatively, the product of sll1926 may not be directly involved in signal transduction. It may have a different, perhaps structural role. The disruption of the state transition in its absence may then be an indirect effect. Given the highly specific phenotype of \( \Delta sll1926 \), the former possibility would seem more likely. The low abundance of the sll1926 transcript (Section 4.8) would also support a signal transduction role for the product of sll1926. As a signal transduction factor, the product of sll1926 may be involved in any one of three stages in the state transition mechanism: (1) the initial redox-sensing mechanism; (2) changes in excitation energy distribution (for example it may be a component of a phycobilisome-
reaction centre supercomplex, and a possible site of a covalent modification during state transitions); or (3) signal transduction between these two stages.

In cyanobacteria, state transitions are accompanied by changes in the 77K fluorescence emission spectra taken with both phyocyanin and chlorophyll excitation. This has been interpreted as evidence for a 'spillover' model for the cyanobacterial state transition (Biggins & Bruce 1989, Murata 1969). In this model, the phycobilisome remains perpetually attached to PS2. The state 1-state 2 transition is brought about by an increase in excitation energy transfer ('spillover') from the PS2 to the PS1 chlorophyll antennae (Figure 1.5). The changes seen in the 77K fluorescence emission spectra with chlorophyll as well as phyocyanin excitation could be explained by a change in 'spillover'.

Δsll1926 showed no change in the 77K fluorescence emission spectrum with phyocyanin excitation, but retained the small change in the spectrum with chlorophyll excitation (Figure 4.3). This provides evidence that the two fluorescence changes do not reflect a single phenomenon (for example 'spillover'). The true origins of these fluorescence changes remain uncertain. There is much evidence to suggest that the phycobilisomes are mobile and can transfer energy directly to PS1 as well as to PS2 (Mullineaux 1994, Mullineaux et al. 1997). A mobile-phycobilisome model for the state transition therefore probably accounts for the changes seen in the 77K fluorescence emission spectra with phyocyanin excitation. The origin of the change seen in the 77K fluorescence emission spectrum with chlorophyll excitation is very unclear. It may be related to a small, photosystem-associated antenna which changes its energy transfer characteristics during the state transition (Mullineaux 1992), or possibly to photosystem oligomerisation (Meunier et al. 1997). There is little evidence for these hypotheses at present however (see Section 9.2.1 for a detailed discussion of this point).

Recently, two transcriptional regulators that appear to be involved in the long-term control of phycobilisome-reaction centre association in *Synechocystis* PCC6803 have been identified (Ashby & Mullineaux 1999a). The genes were designated rpaA and rpaB (for Regulator of Phycobilisome Association). The name rpaC is therefore proposed for sll1926.

No state transition signal transduction components have been identified previously in cyanobacteria. It is possible that sll1926, and its homologue in *Anabaena* PCC7120 represent a new family of regulatory factors required for the state transition in cyanobacteria. It will require more work to determine the role of the product of sll1926 in the state transition. In Chapters 5 and 6 the beginnings of such work is presented.
Chapter 5

HEXAHISTIDINE TAGGING OF RpaC (sll1926)
CHAPTER 5: HEXAHISTIDINE TAGGING OF RpaC (sll1926)

5.1 Introduction
In Chapter 3 the identification of a gene required for the state transition in *Synechocystis* PCC6803 was reported. In Chapter 4, disruption of the state transition in an insertional inactivation mutant of *rpaC* (Δ*rpaC*) was shown to be a specific effect on the regulatory mechanism. The product of *rpaC* may therefore be a signal transduction factor. In this chapter the carboxy-terminal hexahistidine tagging of RpaC is reported.

The ability to detect a particular protein can be an important tool used in gaining an understanding of its function. The ability of antibodies to bind to specific proteins is a property commonly used in this detection. For example, Western blotting entails the electrotransfer of proteins, separated by SDS-PAGE, to a support matrix such as nitrocellulose (Sambrook *et al.* 1989). A specific protein can then be detected by linking the binding of antibody to some detection system; for example a light-emitting reaction catalysed by an enzyme-labelled secondary antibody.

Antibodies can bind to a particular structural feature in a protein, an epitope. As well as raising antibodies to a natural epitope within the protein itself, it is also possible to genetically engineer an epitope onto a protein. This is known as epitope tagging and can be N- or C-terminal. The hexahistidine epitope tag consists of six histidines. This allows for immunological detection of the protein, for example by Western blotting. An advantage of the hexahistidine tag is that it also allows purification of the protein. This purification exploits the ability of metal ions such as Ni^{2+}, immobilised to a matrix, to ligate the imidazole side chains of the adjacent histidines in the hexahistidine tag (Hochuli *et al.* 1987, Sulkowski 1985). This system has been used much in the purification of PS2, for example from *Synechocystis* PCC6803 (Bricker *et al.* 1998, Reifler *et al.* 1999), and *C. reinhardtii* (Sugiura *et al.* 1998). This system can also greatly aid the purification of mutant proteins, and hence facilitate their characterisation (Mitchell & Gennis 1995).

5.2 Hexahistidine tagging of RpaC
Since the position of the start codon in the *rpaC* gene is unclear (see Section 4.2), the C-terminus of RpaC was tagged rather than the N-terminus. The strategy used for the hexahistidine tagging of RpaC is illustrated in Figure 5.1. A 157 bp PCR product (PCR1) was generated with primers P1 and P2. From 5'-3' P1 contained a *Bam*HI site, a stop codon (antisense), six histidines (antisense) and antisense sequences from the C-terminus of *rpaC*. P2 had sense sequences from *rpaC* which contained an *Eag*I site at position 744,967. A second 810 bp PCR product (PCR2) was generated with primers P3 and P4.
1) Generation of hexahistidine tag by PCR

250 bp

2) Generation of pBS6HKmR

3) Following transformation, homologous recombination of pBS6HKmR with host genome generates a hexahistidine tagged version of rpaC linked to antibiotic resistance.

Figure 5.1: Strategy used for the hexahistidine tagging of RpaC (see text for details).
P3 had a *KpnI* end and sequences 810 bp 5' from the *rpaC* C-terminus. P4 had a *BamHI* end and sequences just 5' from the *rpaC* C-terminus. Both PCR products were digested with *BamHI*, gel-purified and ligated (ligation as for standard cloning ligation, see Section 2.2.7). The resulting 1 kb fragment was gel-purified and then digested with *EagI* and *KpnI*, gel-purified again and cloned into pBluescript SK(+) to make pBS6H. A 1.4 kb *KmR* gene from pUC4K was cloned into the *BamHI* site in pBS6H to make pBS6HKmR.

This construct was sequenced with primers T3 and T7 [pBluescript SK(+)]) and pk3 and pk7 (*KmR* gene) to ensure that no errors had been introduced by PCR into the sequences. This construct was then transformed into wild-type *Synechocystis* PCC6803 to generate a hexahistidine tagged version of *rpaC* linked to antibiotic resistance (*6HrpaC*). Transformants were selected by resistance to kanamycin.

The extent of segregation of the mutant could be checked by performing PCR on an inoculum of whole cells from *6HrpaC* and the wild-type using primers P2 and P3. The wild type gave a product of 1.0 kb and *6HrpaC* gave a product of 2.4 kb. This suggested that the mutants had segregated fully. After a month, complete segregation in *6HrpaC* was confirmed by Southern analysis (Figure 5.2). *6HrpaC* and wild-type genomic DNA were digested with *BamHI*. Blots were probed with *rpaC* (sll1926) (amplified using primers NF and NR). The *BamHI* fragment containing *rpaC* is predicted to be 46 kb in the wild-type. Introduction of a *BamHI* site in *6HrpcZ* would reduce the size of this fragment to 9.5 kb. *6HrpaC* had a single band at 9.5 kb. For the wild-type there was hybridisation to a much higher molecular weight region (presumably the 46 kb *BamHI* fragment). This showed that the hexahistidine tagged version of *rpaC*, with the associated *BamHI* site, had segregated to homoplasmicity. *6HrpaC* was capable of photoautotrophic growth.

### 5.3 Spectroscopic characterisation of 6HrpaC

Figure 5.3 shows 77K fluorescence emission spectra with phycocyanin (600nm) excitation for the wild-type and *6HrpaC* adapted under either state 1 or state 2 conditions (see Section 3.8.1 for a description of these spectra). Figure 5.4 shows room temperature fluorescence timecourse measurements for the wild-type and *6HrpaC* (see Section 3.8.2 for a description of these measurements). It is clear from these measurements that *6HrpaC* can perform state transitions similar to the wild-type.

### 5.4 Detection of 6HRpaC by dot blot analysis and purification of 6HRpaC.

Thylakoid and plasma membranes were prepared from *6HrpaC* and the wild-type (Section 2.3.11). In order to attempt to detect RpaC, and as a prelude to Western analysis, these membrane samples were subjected to dot-blot analysis using a hexahistidine antibody (Section 2.3.11). These attempts failed. A soluble hexahistidine tagged protein was used as a positive control. This protein had been successfully purified using Ni^{2+}-NTA affinity
Figure 5.2: Southern analysis of 6HrpaC. Genomic DNA was digested with BamHI. The blot was probed with rpaC (sll1926).
Figure 5.3: 77K Fluorescence emission spectra with 600nm excitation for a) wild type and b) 6HrpaC adapted under either state 1 or state 2 conditions. Spectra are normalised to the phycocyanin/allophycocyanin (650nm) peak.
Figure 5.4: Room temperature fluorescence timecourse measurements for a) wild type and b) 6HrpaC. Excitation was at 620nm and fluorescence measured at 685nm.
chromatography. This protein was also not detected. Attempts were also made to purify RpaC from 6HrpαC whole cells using Ni\(^{2+}\)-NTA affinity chromatography (Section 2.3.11). These attempts also failed.

### 5.5 Discussion

In this chapter the C-terminal hexahistidine tagging of RpaC is reported. Spectroscopic characterisation of 6HrpαC shows that the hexahistidine tag does not affect the ability to perform state transitions (Section 5.3). This indicates that whatever the role of the product of \(rpaC\) in state transitions, C-terminal hexahistidine tagging does not significantly affect it. In many cases, hexahistidine tagging does not interfere with structure or function of proteins, including enzymes, transcription factors and vaccines. Hexahistidine tagging can in some cases lead to phenotypic changes. For example, C-terminal tagging of the D2 protein in \(C.\) reinhardtii led to a decrease in growth rate and a 16% decrease in oxygen evolving capacity (Sugiura \textit{et al.} 1998). It is possible that the incorporation of the hexahistidine tag onto the D2 protein induced a change in the configuration of the reaction centre which led to these changes.

The construction of 6HrpαC provides a useful starting point for further research into the role of the product of \(rpaC\) in the state transition. Western analysis of isolated thylakoid membrane and plasma membrane proteins can be used to confirm and specify the proposed membrane association of the product of \(rpaC\). The use of hexahistidine tagging as a means of detecting a protein can be problematic (A. Smith, personal communication). It has been demonstrated that detection is more effective when a 10 histidine tag is used (S. Buchanan, personal communication). There are several reasons that may account for this. For example, the hexahistidine tag may be inaccessible to antibody. Alternatively, proteolytic cleavage of the hexahistidine tag may occur. The failure to detect 6HRpaC using dot-blot analysis may be a result of such factors. Attempts to purify 6HRpaC failed (Section 5.4). Northern analysis would suggest that the \(rpaC\) transcript is rare (Section 4.8). This might indicate that RpaC is not a bulk membrane component. Purification of 6HRpaC may therefore require a much larger quantity of starting material than was used here (see Section 2.3.11).

The purification of 6HRpaC may lead to structural and biophysical studies. In addition it has been suggested that the purification of histidine-tagged proteins may provide a useful system for studying protein-protein interactions (Hoffman & Roeder 1991). This is because proteins with which a histidine-tagged protein associates or interacts, can become copurified with it. It is therefore possible this system could be used to study associations of the product of \(rpaC\) with membrane protein complexes such as the photosystems, or perhaps even interactions with signal transduction factors.
Chapter 6

ANALYSIS OF PHOSPHOPROTEINS IN $\Delta rpaC$
CHAPTER 6: ANALYSIS OF PHOSPHOPROTEINS IN STATE TRANSITION MUTANT ΔrpaC

6.1 Introduction

In green-plant chloroplasts there is much evidence for the involvement of phosphorylation in the state transition (Allen & Nilsson 1997, Gal et al. 1997). In cyanobacteria the role of phosphorylation in the state transition is far less certain. Allen et al. (1985) observed that in Synechococcus PCC6301 cells grown in the presence of [32P]-orthophosphate there was a light-dependent increase in the phosphorylation of two proteins. These proteins were a 15kDa thylakoid membrane protein and an 18.5kDa soluble protein. A light dependent increase in the phosphorylation of the 15kDa protein was also observed in vitro in isolated thylakoid membranes incubated with [γ-32P]-ATP (Allen et al. 1985). This light treatment was also found to induce changes in the 77K fluorescence emission spectra of cells characteristic of state transitions. It was therefore suggested that phosphorylation of these proteins might have a role in state transitions (Allen 1992, Allen et al. 1985). However, Biggins et al. (1984) studied protein phosphorylation levels in the red alga P. cruentum grown in the presence of [32P]-orthophosphate. Using light of different wavelength to induce state transitions, no difference in the level of protein phosphorylation could be detected between the states.

The differences between the results of Allen et al. (1985) and Biggins et al. (1984) could be a consequence of the different conditions used to induce state transitions. Allen et al. (1985) used light and dark to induce state transitions whereas Biggins et al. (1984) used different wavelengths of light. The changes in phosphorylation observed by the former may therefore be a consequence of light adaptation rather than specifically of the state transition.

The 18.5kDa phosphoprotein identified by Allen and colleagues has since been identified as β-phycocyanin, a component of the phycobilisomes (Harrison 1990). Site-directed mutagenesis has shown that a conserved serine (50) is the site of phosphorylation (Mann & Newman 1999). When mutated to aspartate, the ability to perform state transitions is not affected (Conrad Mullineaux, personal communication). This shows that phosphorylation of β-phycocyanin does not have a role in state transitions.

The sequence of RpaC has potential phosphorylation sites which are likely to be exposed to the aqueous phase (Figure 4.1 and 4.2). Also, the predicted molecular weight of RpaC, as assigned by Kaneko et al. (1996) is 16kDa. This raised the possibility that RpaC is the equivalent in Synechocystis PCC6803 of the 15kDa membrane phosphoprotein identified...
by Allen et al. (1985) in *Synechococcus* PCC6301. To investigate this possibility, *in vitro* $[^\gamma^3P]$-ATP labelling experiments were conducted on a soluble and membrane fraction isolated from ΔrpaC and the wild-type. These experiments were conducted in the laboratory of Dr. N. Mann, University of Warwick, with help from Dr. N. Mann and J. Scanlan.

### 6.2 Results

Membrane and soluble fractions isolated from ΔrpaC and the wild-type were labelled for 10 minutes in the dark with $[^\gamma^3P]$-ATP, in the presence of 10mM duroquinone and 0.5mM dithionite as described in Section 2.3.7. The increased phosphorylation of the 15kDa protein has been correlated with state 2 in thylakoid membranes isolated from *Synechococcus* PCC6301 (Allen et al. 1985). The reducing agents duroquinone and dithionite were therefore included in the incubation to ensure that the plastoquinone pool was reduced and the membranes were in state 2. Following incubation with $[^\gamma^3P]$-ATP, duroquinone and dithionite, samples were analysed by SDS-PAGE followed by autoradiography.

In Figure 6.1 is shown an autoradiograph of this experiment. Several phosphoproteins are present in the membrane as well as the soluble fraction. In the membrane fractions a phosphoprotein of approximately 15kDa is present. In the soluble fractions there is a phosphoprotein of approximately 18.5kDa. These probably correspond to the equivalent in *Synechocystis* PCC6830 of the 15kDa and 18.5kDa phosphoproteins identified in *Synechococcus* PCC6301 by Allen et al. (1985). In the membrane fractions a strongly labelled band is also present at approximately 6kDa. This is probably PsbH, a subunit of PS2 (Race & Gounaris 1993).

No significant differences in the pattern of phosphorylation were observed between ΔrpaC and the wild-type in either the membrane or the soluble fractions.

### 6.3 Discussion

In this chapter is described *in vitro* labelling of a membrane and soluble fraction isolated from ΔrpaC and the wild-type. This was done in order to investigate the possibility that RpaC is a phosphoprotein. It is clear from the results that the product of sll1926 is not any of the phosphoproteins observed, including the 15kDa phosphoprotein presumed to be the equivalent of that identified by Allen et al. (1985) in *Synechococcus* PCC6301.
Figure 6.1: Autoradiograph of membrane and soluble fractions isolated from Δsll1926 and the wild type, [γ-32P]-ATP labelled, and analysed by SDS-PAGE.
It is still possible however that RpaC is a phosphoprotein. The band corresponding to it may simply be obscured by other bands. For example the predicted molecular weight of RpaC, as assigned by Kaneko et al. (1996), is 16kDa. If this protein were a membrane-associated phosphoprotein then a labelled band of this molecular weight may be obscured by the 15kDa phosphoprotein band. Additional methionines within the assignment of Kaneko et al. (1996) indicate that RpaC may be 11 or 9kDa. A phosphoprotein of 9kDa may likewise be obscured by PsbH. Also, the transcript of rpaC seems to be rare (Section 4.8). This might indicate that RpaC is not a bulk component of the membrane. It might be difficult to detect such a rare phosphoprotein using this approach.

Using \textit{in vitro} labelling, many more proteins become labelled than using \textit{in vivo} labelling (Mann 1994). This would suggest that a proportion of the phosphorylations observed using the \textit{in vitro} approach are artefactual and so may have no genuine physiological significance. If RpaC is a phosphoprotein, it might therefore be easier to detect it using an \textit{in vivo} labelling approach. An alternative method of detecting phosphoproteins is to use Western analysis with antibodies to phosphorylated residues such as phospho-serine and phospho-threonine.

The use of state transition mutants such as ΔrpaC in phospholabelling experiments is a potentially useful way of investigating the role of phosphorylation in the cyanobacterial state transition. For example, state transition mutants could be used as controls in experiments such as that of Allen et al. (1985), which attempt to correlate protein phosphorylation with the state transition. If a change in protein phosphorylation is observed in the wild-type but not in a state transition mutant, then that phosphorylation can be correlated directly with the state transition. This approach however is limited since state-transition-related changes in protein phosphorylation may still occur in state transition mutants depending on where in signal transduction the mutant is affected.
Chapter 7

EXAMINATION OF PHYCOBILISOME-PHOTOSYSTEM COUPLING IN STATE TRANSITION MUTANT ΔrpaC
CHAPTER 7: EXAMINATION OF PHYCOCBILISOME-PHOTOSYSTEM COUPLING IN STATE TRANSITION MUTANT ΔrpaC

7.1 Introduction

In green plant chloroplasts there is much evidence to suggest that state transitions involve the movement of an accessory light harvesting system, LHCII, between PS2 and PS1 (Keren & Ohad 1998, Larsson et al. 1986).

In cyanobacteria, the molecular origins of the fluorescence changes seen during state transitions have been a subject of more contention. A mobile model, similar to that in green plant chloroplasts, proposes that the state 1-state 2 transition involves the dissociation of a proportion of phycobilisomes from PS2 and their reassociation with PS1. Much evidence has accumulated in support of such a model. For example, there is efficient and direct excitation energy transfer from the phycobilisomes to PS1 as well as to PS2 (Mullineaux 1994). In addition, phycobilisomes are able to diffuse quite rapidly in the membrane relative to the photosystems (Mullineaux et al. 1997) (see Section 1.9.5).

For the mobile model to occur, there must be a change in the coupling of photosystems to the phycobilisome. This change in coupling might be brought about by a conformational change induced by covalent modification of the phycobilisome or of a photosystem component. In a *Synechococcus* PCC6301 mutant lacking PS2, excitation-energy transfer from the phycobilisomes to PS1 was found to be unaffected by treatments predicted to oxidise and reduce the plastoquinone pool (Mullineaux 1994). This led to the suggestion that in the wild-type, state transitions entail changes in the affinity of phycobilisomes for PS2 alone (Mullineaux 1994). Thus, the state 1-state 2 transition would involve a reduction in the affinity of the phycobilisomes for PS2 and so an increase in the proportion of decoupled phycobilisomes capable of associating with PS1 (Mullineaux 1994).

In this chapter is described the construction and characterisation of ΔrpaC in the PS1* and PS2* backgrounds. On the basis of 77K fluorescence emission spectra, ΔrpaC appears to be in state 1 (Figure 3.7). The construction of ΔrpaC in the PS1* and PS2* backgrounds allows the effect of state, on coupling of the phycobilisomes with both PS2 and PS1 to be examined independently. This therefore provides a useful system for examining how coupling of the phycobilisomes with the photosystems may change during the state transition.
7.2 Generation of ΔrpaC in the PS1\(^-\) and PS2\(^-\) backgrounds

In order to inactivate \(rpaC\) in the PS2\(^-\) and PS1\(^-\) backgrounds, the insertional inactivation construct pBS1926ΔK (see Section 3.7) was transformed into:

1) A chloramphenicol and spectinomycin resistant strain of \(Synechocystis\) PCC6803 with insertional inactivations of the \(psbD1\), \(psbD2\) and \(psbC\) genes. This strain does not assemble PS2 (Vermaas et al. 1990).

2) A chloramphenicol resistant strain of \(Synechocystis\) PCC6803 with insertional inactivation of the \(psaA\) and \(psaB\) genes. This strain does not assemble PS1 (Shen et al. 1993).

Transformants were selected with kanamycin and chloramphenicol on BG11 medium supplemented with glucose. The extent of segregation of the \(rpaC\) inactivation mutants was checked by performing PCR on an inoculum of whole cells using primers 1926F and 2005R. The PS1\(^-\) and PS2\(^-\) strains gave products of 1.4 kb whereas ΔrpaC in the PS1\(^-\) background (ΔrpaCPS1\(^-\)) and ΔrpaC in the PS2\(^-\) background (ΔrpaCPS2\(^-\)) gave products of 2.6 kb. This suggested that the ΔrpaC mutants had segregated fully. After a month, complete segregation of ΔrpaCPS1\(^-\) and ΔrpaCPS2\(^-\) and correct insertion of the \(Km^R\) gene was confirmed by Southern analysis (Figure 7.1). ΔrpaCPS1\(^-\) and ΔrpaCPS2\(^-\) genomic DNA was digested with \(Ncol\). Blots were probed with \(rpaC\) (sll1926, amplified using primers NF and NR). The single bands for ΔrpaCPS1\(^-\) and ΔrpaCPS2\(^-\) were 1.2 kb larger than for the wild-type. This confirmed that the mutants had segregated to homoplasmicity and that the \(Km^R\) gene had been inserted correctly.

7.3 Spectroscopic characterisation of ΔrpaCPS1\(^-\) and ΔrpaCPS2\(^-\)

Figure 7.2 shows 77K fluorescence emission spectra with 600nm excitation for ΔrpaCPS1\(^-\) and PS1\(^-\). Figure 7.3 shows fluorescence emission spectra with 600nm excitation for ΔrpaCPS2\(^-\) and PS2\(^-\). The 600nm excitation is absorbed strongly by phycocyanin. Samples were all dark-adapted prior to freezing. In both PS2\(^-\) and PS1\(^-\) this would be predicted to reduce the plastoquinone pool as in the wild-type.
Figure 7.1: Southern analysis of Δ\textit{rpaCPS1} and Δ\textit{rpaCPS2}. Genomic DNA was digested with \textit{NcoI}. The blot was probed with \textit{rpaC} (sll1926).
Figure 7.2: 77K Fluorescence emission spectra with 600nm excitation for PS1 and \(\Delta rpaCPS1\) (normalised at F660). The spectra depicted are those with F680/F660 ratios closest to the average for that strain. Error bars, for PS1 (thick line) and for \(\Delta rpaCPS1\) (thin line), are standard deviations in F680/F660 for that strain.
Figure 7.3: 77K Fluorescence emission spectra with 600nm excitation for PS2' and ΔrpaCPS2'(normalised at F660). The spectra depicted are those with F680/F660 ratios closest to the average for that strain. Error bars, for PS2' (thick line) and for ΔrpaCPS2' (thin line), are standard deviations in F680/F660 for that strain.
In the spectrum of the PS1\textsuperscript{+} mutant two main peaks are present (Figure 7.2). There is a broad peak at 660nm corresponding to a mixture of phycocyanin and allophycocyanin emission and a large peak at 680nm corresponding to the terminal emitters of the phycobilisome as well as the PS2 chla antennae. There is also a shoulder at 690nm corresponding to the PS2 core complex.

In the spectrum of the PS2\textsuperscript{−} mutant three main peaks are present (Figure 7.3). There is a broad peak at 660nm corresponding to a mixture of phycocyanin and allophycocyanin emission. Again, there is the large peak at 680nm corresponding to the terminal emitters of the phycobilisome, but without the contributions from PS2. There is also a peak at 725nm corresponding to PS1 emission.

In the PS1\textsuperscript{+} and PS2\textsuperscript{−} mutants the ratio of F680/F660 is two-to-threefold greater than in the wild-type (compare Figures 7.2 & 7.3 with Figure 3.7). This is probably due to an increase in the number of non-photosystem-coupled phycobilisomes and hence an increase in fluorescence from their terminal emitters (Bittersmann & Vermaas 1991, Mullineaux 1994). Spectra for PS1\textsuperscript{+} and Δ\textit{rpa}CPS1\textsuperscript{+} and for PS2\textsuperscript{−} and Δ\textit{rpa}CPS2\textsuperscript{−} are depicted together and are normalised in order to facilitate comparison.

In Δ\textit{rpa}CPS1\textsuperscript{−} the ratio of F680/F660 was lower than in PS1\textsuperscript{+}. In Δ\textit{rpa}CPS2\textsuperscript{−} the ratio of F680/F660 was higher than in PS2\textsuperscript{−}. In order to establish whether these observations were significant, the measurements were repeated a number of times in different cultures and an unpaired t-test used to calculate significance (the t-test was performed using Sigma plot for Windows version 5.0) (Tables 7.1 & 7.2). The P values for the F680/F660 ratio were below 0.05 for the PS1\textsuperscript{+} and Δ\textit{rpa}CPS1\textsuperscript{−} (Table 7.1) as well as for the PS2\textsuperscript{−} and Δ\textit{sl1926PS2}\textsuperscript{−} (Table 7.2) data sets. These values are the probabilities that the observed differences occurred by chance. They therefore indicate that the differences observed are significant. The spectra shown in Figures 7.2 & 7.3, have F680/F660 ratios closest to the average value for that strain. The error bars represent the standard deviations in the F680/F660 ratio for that strain.

The differences in F680/F660 between PS1\textsuperscript{+} and Δ\textit{rpa}CPS1\textsuperscript{−} and between PS2\textsuperscript{−} and Δ\textit{rpa}CPS2\textsuperscript{−} could be a result of changes in the coupling of phycobilisomes to photosystems. When the phycobilisomes become functionally decoupled from photosystems an increase in fluorescence from the terminal emitters of the phycobilisome (F680) would be expected (Bittersmann & Vermaas 1991, Mullineaux 1994). Alternatively, they may be unrelated to phycobilisome-photosystem coupling and may be a
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**P VALUE F680/F660: 0.03**

**P VALUE A620/A680: 0.96**

Table 7.1: F680/F660 and A620/A680 ratios for PS1⁻ and ΔarpaCPS1⁻. The P values express the probability that there is no difference between the PS1⁻ and ΔarpaCPS1⁻ F680/F660 and A620/A680 ratios.
<table>
<thead>
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<th>CULTURE NUMBER</th>
<th>PS2&lt;sup&gt;-&lt;/sup&gt; F680/F660</th>
<th>PS2&lt;sup&gt;-&lt;/sup&gt; A620/A680</th>
<th>ΔarpaCPS2&lt;sup&gt;-&lt;/sup&gt; F680/F660</th>
<th>ΔarpaCPS2&lt;sup&gt;-&lt;/sup&gt; A620/A680</th>
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<td>0.91</td>
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R = -0.420

R = 0.134

P VALUE F680/F660: 0.012

P VALUE A620/A680: 0.048

Table 7.2: F680/F660 and A620/A680 ratios for PS2<sup>-</sup> and ΔarpaCPS2<sup>-</sup>. The P values express the probability that there is no difference between the PS2<sup>-</sup> and ΔarpaCPS2<sup>-</sup> F680/F660 and A620/A680 ratios. The Pearson correlation coefficients R, express the degree of correlation between the F680/F660 and A620/A680 values for PS2<sup>-</sup> and ΔarpaCPS2<sup>-</sup>.
direct consequence of differences in the amounts of the phycobilisomes or photosystems. For this reason, for each of the samples for which fluorescence emission spectra were recorded, absorption spectra were also recorded and phycobilin/chlorophyll ($A_{620}/A_{680}$) ratios determined. The $P$ value for the $A_{620}/A_{680}$ ratio was close to one for the PS1' and $\Delta rpaCPS1'$ data set (Table 7.1), indicating no significant difference. For the PS2' and $\Delta rpaCPS2'$ data set the $A_{620}/A_{680}$ ratio was significantly higher in $\Delta rpaCPS2'$ than in PS2' ($p = 0.048$) (Table 7.2). Thus, it was possible that in $\Delta rpaCPS2'$ the elevated $F_{680}/F_{660}$ ratio was a direct consequence of the elevated $A_{620}/A_{680}$ ratio. To investigate this possibility we determined the degree of correlation between the $F_{680}/F_{660}$ and $A_{620}/A_{680}$ values. In Figure 7.4 is shown a scatter plot of $A_{620}/A_{680}$ against $F_{680}/F_{660}$ for the PS2' and $\Delta rpaCPS2'$ data sets. The standard deviations for the points, depicted as error bars, represent typical errors incurred in measurement and were obtained by repeating fluorescence and absorption measurements on a single liquid culture. If $F_{680}/F_{660}$ and $A_{620}/A_{680}$ values were correlated then the points would tend to cluster around a line which describes the relationship between the two values. It can be seen that in this case the points are widely distributed across the plot. This would indicate that $F_{680}/F_{660}$ is not correlated with $A_{620}/A_{680}$. Pearson correlation coefficients were calculated for PS2' and $\Delta rpaCPS2$ using SPSS for Windows statistics software (Table 7.2). When this value is 1 or -1 it indicates a perfect linear correlation. When it is 0 it indicates no correlation. Both values were low, indicating that as predicted from the scatter plot, $F_{680}/F_{660}$ and $A_{620}/A_{680}$ are not correlated. The elevated $F_{680}/F_{660}$ in $\Delta rpaCPS2$', as well as the depressed $F_{680}/F_{660}$ in $\Delta rpaCPS1'$, are probably therefore a consequence of changes in phycobilisome coupling to the photosystems.

### 7.4 Discussion

In this chapter it is reported that the inactivation of $rpaC$ in the PS1' and PS2' backgrounds leads to significant alterations in the 77K fluorescence emission spectra with 600nm excitation. In $\Delta rpaCPS1'$ the $F_{680}/F_{660}$ ratio is depressed compared with PS1'; in $\Delta rpaCPS2'$ it is elevated compared to PS2'. In $\Delta rpaCPS2'$ there was also a significant elevation in the $A_{620}/A_{680}$ ratio compared to PS2'. This indicated an elevation in the phycobilin/chlorophyll ratio and could have been an indirect cause of the elevated $F_{680}/F_{660}$. $A_{620}/A_{680}$ was found however not to correlate with $F_{680}/F_{660}$ in $\Delta rpaCPS2'$ which ruled out this possibility. The differences in $F_{680}/F_{660}$ between
Figure 7.4: Correlation between A620/A680 & F680/F660 for PS2⁻ and ΔrpaCPS2⁻. The error bars show the standard deviations for the points and represent typical errors incurred in measurement.
A/\text{paCPS}1^+\text{ and PS1}^+\text{ as well as between }\Delta\text{paCPS2}^-\text{ and PS2}^-\text{ are probably therefore a direct consequence of alterations in phycobilisome-photosystem coupling.}

In A/\text{paCPS}1^-\text{ the decreased F680/F660 in comparison to PS1}^-\text{ would imply that there is increased coupling of the phycobilisomes to PS2. In }\Delta\text{paCPS2}^-\text{ the elevated F680/F660 in comparison to PS2}^-\text{ would imply that there is decreased coupling of the phycobilisomes to PS1. In the wild-type, 77K fluorescence emission spectra would indicate that }\text{rpaC}\text{ inactivation leads to a permanent state 1 (Figure 3.7). These differences in phycobilisome-photosystem coupling in }\Delta\text{paCPS1}^-\text{ and }\Delta\text{paCPS2}^-\text{ may therefore be due to the creation of a similar state 1 configuration in the PS1}^-\text{ and PS2}^-\text{ mutants: whereas }\Delta\text{paCPS1}^-\text{ and }\Delta\text{paCPS2}^-\text{ would be permanently in a state 1 configuration, PS1}^-\text{ and PS2}^-\text{ would be dark adapted to a state 2 configuration. This is illustrated in Figures 7.5 and 7.6.}

This has several implications. It demonstrates that the state transition involves alterations in the coupling of the phycobilisomes directly with the photosystems. This strongly supports a mobile model for the state transition. It also demonstrates that the state transition involves changes in the affinity of the phycobilisomes for PS1 and PS2. This is contrary to previous work which suggested that the state transition involves a change in the affinity of the phycobilisomes for PS2 alone (Mullineaux 1994).

This alteration in phycobilisome-photosystem affinity may be brought about by a conformational change. This may occur in the phycobilisome and/or in PS2 and PS1. It may be linked to post-translational modification, possibly phosphorylation. In Figure 7.7 are shown two possible models for the state transition based on these results:

a) Changes in the affinity of the phycobilisomes for both photosystems may be brought about by post-translational modification of phycobilisomes. In state 1, phycobilisomes have a high affinity for PS2 and a low affinity for PS1. Phycobilisomes are therefore coupled predominantly to PS2. In state 2, post-translational modification of the phycobilisomes lowers their affinity for PS2 and simultaneously increases their affinity for PS1. The proportion of phycobilisomes coupled to PS2 will therefore be decreased and the proportion of phycobilisomes coupled to PS1 will be increased.

b) Changes in the affinity of the phycobilisomes for the photosystems may be brought about by post-translational modification of both photosystems. Thus in state 1, phycobilisomes have a high affinity for PS2 and a low affinity for PS1. Phycobilisomes are therefore coupled predominantly to PS2. In state 2, post-translational modification of
Figure 7.5: Proposed differences in phycobilisome-PS2 coupling in PS1⁻ and ΔrpaCPS1⁻. ΔrpaCPS1⁻ is permanently in a state 1 configuration whilst PS1⁻ is dark adapted to a state 2 configuration.
Figure 7.6: Proposed differences in phycobilisome-PS1 coupling in PS2\textsuperscript{-} and ΔrpaCPS2\textsuperscript{-}. ΔrpaCPS2\textsuperscript{-} is permanently in a state 1 configuration whilst PS2\textsuperscript{-} is dark adapted to a state 2 configuration.
Figure 7.7: Two models for the state transition (X denotes a post-translational modification):
  (a) state 2 is brought about by post-translational modification of the phycobilisome.
  (b) state 2 is brought about by post-translational modification of both photosystems.
PS2 lowers the affinity of the phycobilisomes for PS2. Post-translational modification of PS1 simultaneously increases the affinity of the phycobilisomes for PS1. The proportion of phycobilisomes coupled to PS2 will therefore be decreased and the proportion of phycobilisomes coupled to PS1 will be increased.

The nature of the phycobilisome-photosystem interaction is unclear. Mullineaux et al. (1997) used a variant of FRAP to measure the mobility of PS2 and the phycobilisomes in the cyanobacterium *Dactylococcopsis salina* (see Section 1.9.5). Whereas no diffusion of PS2 could be detected on the timescale of the measurement, the phycobilisomes were found to diffuse quite rapidly (Mullineaux et al. 1997). It was therefore suggested that the photosystem-phycobilisome interaction is transient: phycobilisomes constantly decouple, diffuse and recouple with photosystems (Mullineaux 1999). The state transition would involve a change in the binding constants for these unstable phycobilisome-photosystem interactions such that the number of phycobilisomes coupled to each photosystem in a steady-state would be altered. The state transition would thus represent a shift in a dynamic, rather than a static equilibrium.

The interpretation of the results reported in this chapter is based on measurements of the F680/F660 ratio. In the PS2- mutant F680 arises exclusively from the terminal emitters of the phycobilisome. In the PS1- mutant however, F680 also has some contributions from PS2. To ascribe more precisely the fluorescence changes at 680nm to the phycobilisome terminal emitters and thus to changes in phycobilisome-photosystem coupling, time-resolved fluorescence measurements will need to be made. These measurements are able to separate fluorescence emission from different pigment pools on the basis of their decay times following an excitation flash.
Chapter 8

THE EFFECT OF \textit{rpaC} INACTIVATION ON CELL GROWTH: THE PHYSIOLOGICAL ROLE OF THE STATE TRANSITION IN CYANOBACTERIA
CHAPTER 8: THE EFFECT OF \( rpaC \) INACTIVATION ON CELL GROWTH: THE PHYSIOLOGICAL ROLE OF THE STATE TRANSITION IN CYANOBACTERIA

8.1 Introduction

The physiological role of the state transition is unclear. It was originally suggested by Murata (1969) that it serves to increase the efficiency of light utilisation in photosynthesis. One of the ways this may happen is through regulation of the ATP/NADPH ratio (discussed in Section 1.10). PS2 is particularly sensitive to oxidative damage, especially on overexcitation (Clarke et al. 1993, Keren & Ohad 1998). It has therefore also been suggested that the state 1-2 transition, which decreases the absorption cross-section of PS2, may have a photoprotective role (Horton & Lee 1985, Mullineaux & Allen 1990). Indeed, there is some evidence that a state 1-2 transition occurs at high light intensities.

Studying the cyanobacterium *Synechococcus* PCC6301, Rouag & Dominy (1994) observed that at high light intensities (>200\( \mu \)E.m\(^{-2} \).s\(^{-1} \)) a partial reversal of the state 1 transition occurred. Under very low light intensity (<5\( \mu \)E.m\(^{-2} \).s\(^{-1} \)) cells were adapted to state 2 and under moderate light intensity (20-60\( \mu \)E.m\(^{-2} \).s\(^{-1} \)) to state 1. The authors therefore suggested that separate sets of regulatory factors controlled the very low-moderate light intensity state transition and the moderate-high light intensity state transition.

In another study, the state transition was measured *in situ* in a cyanobacterial bloom in a shallow estuarine system. State 2 was found to prevail in the near dark conditions of the lower depths. State 1 was found to prevail at moderate depths. Under the high-light conditions near the water surface however, cells were found to adapt to state 2 (Schubert et al. 1995) (see also Section 1.10).

Previously, the only state transition mutants available were those indirectly disrupted in the state transition, for example through electron transport or phycobilisome-to-photosystem energy transfer. \( \Delta rpaC \) is the first state transition mutant which is specifically unable to perform state transitions. This provides a specific system for examining the physiological function of the state transition. By measuring growth rate of \( \Delta rpaC \) under different light regimes, it is possible to identify those regimes which may disadvantage/advantage \( \Delta rpaC \) relative to the wild-type. This may provide information on the physiological function of state transitions. For example, if the state transition has a photoprotective function, growth
of ΔrpaC would be expected to be retarded relative to the wild-type at higher light intensities.

Growth of ΔrpaC has been measured at various constant and variable light regimes. The constant light regimes were designed to test the importance of the state transition for cell growth at different intensities and wavelengths of light. The variable light regimes were designed to test the effect on cell growth of performing continual state transitions.

8.2 Doubling times for ΔrpaC and the wild type under different light regimes.

ΔrpaC and the wild-type were grown under very low white light (2μE·m⁻²·s⁻¹) prior to growth experiments. They were then grown from approx. 1.5 x 10⁷ cells ml⁻¹ in BG11 medium without antibiotic under various light regimes. OD₇₅₀ was measured regularly and doubling times determined as described in Section 2.3.10.

The doubling times for ΔrpaC and the wild-type grown under the various light regimes are shown in Table 8.1. Under high, moderate and low light intensities the doubling times of ΔrpaC and the wild-type were not significantly different. Under very low intensity light however, the ΔrpaC doubling time was significantly longer than that of the wild-type. This very low light effect was wavelength dependent: the doubling time for ΔrpaC was 38% longer than the wild-type under very low yellow light, 28% longer than the wild-type under very low white light and only 8% longer under very low red light. For the variable light regimes, the doubling time of ΔrpaC was significantly longer than the wild-type (22%) under the low red-very low yellow light regime only. These observations were all reproduced several times in different cultures.

8.3 Analysis of the effect of light regime on the state transition

Samples of the wild-type (grown under very low intensity white light) were adapted for 10 minutes under the moderate, low and very low constant light regimes employed in the growth experiments prior to freezing in liquid nitrogen. Table 8.2 shows the PS2/PS1 fluorescence ratios determined from 77K fluorescence emission spectra with phycocyanin excitation for these samples (see Section 3.8.1 for a detailed description of such spectra). The ratio of PS2/PS1 fluorescence was lower for the samples adapted under the very low intensity lights than for those adapted under low and moderate intensity light. This would indicate that the very low intensity lights lead to state 2, and the low and moderate intensity
### CONSTANT LIGHT REGIMES

<table>
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<tr>
<th>STRAIN &amp; ILLUMINATION</th>
<th>DOUBLING TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>High intensity (500μE.m⁻².s⁻¹) white light: W.T.</td>
<td>6.6 ± 0.1</td>
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<tr>
<td>High intensity (500μE.m⁻².s⁻¹) white light: ΔrpaC</td>
<td>7.0 ± 0.2</td>
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<td>Moderate intensity (100μE.m⁻².s⁻¹) white light: W.T.</td>
<td>8.7 ± 1.5</td>
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<tr>
<td>Moderate intensity (100μE.m⁻².s⁻¹) white light: ΔrpaC</td>
<td>10.3 ± 0.4</td>
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<tr>
<td>Low intensity (10μE.m⁻².s⁻¹) white light: W.T.</td>
<td>25.7 ± 2.9</td>
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<tr>
<td>Low intensity (10μE.m⁻².s⁻¹) white light: ΔrpaC</td>
<td>24.6 ± 1.0</td>
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<td>Very low intensity (2μE.m⁻².s⁻¹) white light: W.T.</td>
<td>74.4 ± 0.0</td>
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<td>95.4 ± 7.3</td>
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<td>68.8 ± 0.0</td>
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<td>95.1 ± 3.3</td>
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### VARIABLE LIGHT REGIMES

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<td>10mins Moderate white light-10mins dark: ΔrpaC</td>
<td>11.4 ± 0.0</td>
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<td>10.5 ± 0.3</td>
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<td>10mins Moderate-10mins very low white light: ΔrpaC</td>
<td>12.2 ± 1.2</td>
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<td>10mins Low red-10mins very low yellow light: W.T.</td>
<td>17.3 ± 0.3</td>
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<td>10mins Low red-10mins very low yellow light: ΔrpaC</td>
<td>21.1 ± 0.8</td>
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<tr>
<td>12 hrs Moderate white light-12hrs dark: W.T.</td>
<td>10.0 ± 1.7</td>
</tr>
<tr>
<td>12 hrs Moderate white light-12hrs dark: ΔrpaC</td>
<td>9.8 ± 1.3</td>
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</tbody>
</table>

Table 8.1: Doubling times for ΔrpaC and the wild-type grown under various constant and variable light regimes. Each doubling time was determined for a single culture. Errors represent the accuracy of the non-linear regression used to determine doubling times from measurements of cell number (see Section 2.3.10).
<table>
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<th>ILLUMINATION</th>
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<th>STATE</th>
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<td>2</td>
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<td>Very low intensity yellow light</td>
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<td>2</td>
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<tr>
<td>Very low intensity red light</td>
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<td>2</td>
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Table 8.2: The effect of the constant light regimes employed in the growth experiment (Table 8.1) on state in the wild type.
light to state 1. Under prolonged illumination, long-term adaptation of the photosynthetic apparatus occurs. For example changes in photosystem stoichiometry alter the PS2/PS1 fluorescence ratio (Murakami 1997). These changes may alter the final state of the cells in the growth experiment.

### 8.4 Photoinhibition in ΔrpaC

Photoinhibition was measured in ΔrpaC and the wild-type in the presence and absence of the protein synthesis inhibitor lincomycin as detailed in Section 2.3.9. The protein synthesis inhibitor lincomycin blocks the synthesis of D1 and therefore inhibits repair to PS2 following photoinactivation. The results of this experiment are shown in Figure 8.1. In the absence of lincomycin, no significant decrease in the rate of oxygen evolution was observed for the wild-type or ΔrpaC. In the presence of lincomycin, both the wild-type and ΔrpaC showed an approximately 50% decrease in oxygen evolution rate over a 3 hour period. These results show that ΔrpaC is no more susceptible to photoinhibition than the wild-type. These experiments were conducted in the laboratory of Dr. P. Nixon, with help from Dr. P. Nixon and P. Silva.

### 8.5 Discussion

In order to gain insights into the physiological role of the state transition in cyanobacteria, growth rate of the state transition mutant ΔrpaC has been measured under different constant and variable light regimes.

The doubling time of ΔrpaC was significantly greater than that of the wild-type under very low intensity white light conditions. This would indicate that, as originally suggested by Murata (1969), the state transition serves to increase the efficiency of light utilisation in photosynthesis. This is because under decreasing light intensities, light will become more limiting to cell growth. Any mechanism which increases the efficiency of light utilisation would therefore be expected to become more important for cell growth under these conditions.

Kruse et al. (1999b) isolated a mutant of *C. reinhardtii* specifically disrupted in the state transition. The rate of photoautotrophic growth of this mutant, *stm1*, was found to be considerably retarded under very low white light conditions (5µE.m\(^2\).s\(^{-1}\)). This would suggest that in *C. reinhardtii*, as in *Synechocystis* PCC6803, the state transition serves to increase the efficiency of light utilisation in photosynthesis.
Figure 8.1: Photoinhibition of a) wild type and b) ΔrpaC in the presence and absence of the protein synthesis inhibitor lincomycin (100μg/ml). Each data point is an average of two measurements. The error bars are standard deviations.
b)  

- lincomycin  
- + lincomycin

**OXYGEN EVOLUTION (μmoles O2/mg chla/hour)**

1600 μEm⁻²s⁻¹ light on

1600 μEm⁻²s⁻¹ light off

**TIME (hours)**

0 1 2 3 1 hour recovery (20 μEm⁻²s⁻¹) 16 hour recovery (20 μEm⁻²s⁻¹)
The effect of very low intensity light on ΔrpaC growth rate was found to be wavelength dependent: very low intensity yellow light exacerbated the effect, very low intensity red light ameliorated it. Yellow light is absorbed strongly by the pigments of the phycobilisome. Red light is absorbed strongly by the chlorophyll antennae of the photosystems. The state transition serves to regulate the utilisation of light absorbed by the phycobilisome only. Thus, a possible reason for the effects of yellow and red light on ΔrpaC growth rate is that the utilisation of phycobilisome-absorbed light and hence cell growth under phycobilisome-absorbed light is dependent on the state transition. In contrast, the utilisation of light absorbed by the chlorophyll antennae of the photosystems is independent of the state transition.

A similar effect has been observed in an apcD inactivation mutant of *Synechococcus PCC7002* (Zhao *et al.* 1992). ΔapcD, like ΔrpaC is unable to perform state transitions (Zhao *et al.* 1992). This mutant was found to have doubling times similar to the wild-type under moderate intensity white light conditions. Under yellow illumination however, the doubling time of ΔapcD was significantly greater than the wild type (35%).

Growth rate of ΔrpaC and the wild-type was also measured under various variable light regimes. The purpose of these measurements was to investigate the effect on cell growth of performing continual state transitions. The moderate white light-dark and the moderate white light-very low white light regimes would be predicted to induce state transitions in the wild-type (Section 8.3). Under these regimes the wild-type and ΔrpaC doubling times were not significantly different. This would indicate that performing continual state transitions (every 10 minutes) does not have a significant effect on cell growth. It would also indicate that for the moderate white light-very low white light regime, cell growth under the moderate white light is sufficiently high to mask the retarded growth of ΔrpaC under the very low white light. Under the low red-very low yellow light regime the doubling time of ΔrpaC was significantly greater than the wild type (22%). This is probably because cell growth under low red light is insufficient to mask the retarded growth of ΔrpaC under the very low yellow light. For these variable light regimes, light conditions were switched every 10 minutes. If faster switching times are used, the performing of state transitions in the wild-type may have a significant effect on cell growth. Indeed, a variable light regime may be possible which retards wild-type growth relative to ΔrpaC.
PS2 is particularly sensitive to photo-oxidative damage on overexcitation. It has therefore been suggested that the state 1-state 2 transition, which decreases the absorption cross-section of PS2 may have a photoprotective role (Horton & Lee 1985, Mullineaux & Allen 1990). 77K Fluorescence emission spectra would indicate that ΔrpaC is permanently in state 1 (Figure 3.7). ΔrpaC is therefore a useful system in which to investigate this putative photoprotective role of state transitions. The levels of photoinhibition of ΔrpaC under 1600μE.m⁻².s⁻¹ white light in the presence of the protein synthesis inhibitor lincomycin were similar to those of the wild-type (Figure 8.1). This indicates that ΔrpaC is no more susceptible to photoinhibition than the wild-type. This would suggest that in *Synechocystis* PCC6803 the state 1-2 transition is unlikely to have a photoprotective role. Fleischmann *et al.* (1999) isolated a state transition mutant of *C. reinhardtii* trapped in state 1. Photoautotrophic growth of this mutant, stt7, was found to be unaffected by high light (600μE.m⁻².s⁻¹). This would suggest that in *C. reinhardtii*, as in *Synechocystis* PCC6803, the state transition does not have a role in photoprotection.
Chapter 9

GENERAL DISCUSSION
CHAPTER 9: GENERAL DISCUSSION

9.1 Summary of the main findings of this thesis

1) The gene \( rpaC \) (sll1926) in \( Synechocystis \) PCC6803 is specifically required for the state transition. It may encode a signal transduction factor.

2) The insertional inactivation of \( rpaC \) in the PS1' and PS2' backgrounds leads to changes in the coupling of phycobilisomes to PS2 and PS1 respectively. This demonstrates that the state transition involves alterations in the coupling of the phycobilisomes directly with both photosystems. This strongly supports a mobile phycobilisome model for the state transition.

3) State transitions in cyanobacteria result in changes in the 77K fluorescence emission spectra of cells with phycocyanin as well as with chlorophyll excitation. This has been interpreted as evidence that the principal effect of the state transition is a change in 'spillover' of energy from PS2 to PS1. An insertional inactivation mutant of \( rpaC \) in the wild-type (\( \Delta rpaC \)) shows no change in the spectrum with phycocyanin excitation but retains the change in the spectrum with chlorophyll excitation. This demonstrates that the two fluorescence changes do not reflect a single phenomenon, for example 'spillover'.

4) \( \Delta rpaC \) grows with a similar doubling time to the wild-type at all but very low light intensities (2\( \mu \)E.m\(^{-2}.s^{-1} \)). This suggests that a major role of the state transition is to maximise the efficiency of light utilisation.

9.2 The molecular basis for the state transition

9.2.1 The origin of the change in the 77K fluorescence emission spectrum with chlorophyll excitation
State transitions were first observed by Murata (1969) in the red alga \( P. cruentum \), and by Bonaventura & Myers (1969) in the green alga \( C. pyrenoidosa \). Murata (1969) observed that the 77K fluorescence emission spectra of \( P. cruentum \) with chlorophyll as well as with phycocyanin excitation changed depending on how the cells were pre-illuminated prior to freezing. When cells were pre-illuminated with green light which is absorbed strongly by the phycobilisomes, emission from the PS2 components was lower than if the cells were
pre-illuminated with far-red light which is absorbed strongly by PS1. The lower fluorescence state was termed state 2 and the higher fluorescence state, state 1. A similar phenomenon was later observed in cyanobacteria (Fork & Satoh 1983).

At this time the two fluorescence changes observed were widely believed to reflect a single phenomenon: 'spillover'. This model for the state transition proposed that the phycobilisomes remain perpetually attached to PS2. On illumination with light which is absorbed strongly by PS1, the majority of energy trapped by the phycobilisomes is transferred to PS2. On illumination with light which is absorbed strongly by the phycobilisomes, there is an increase in energy transfer to PS1. This increase in energy delivered to PS1 would be achieved by an increase in energy transfer from the PS2 chlorophyll antenna to the PS1 chlorophyll antenna, or 'spillover'. This spillover model was the simplest explanation for the changes seen in both the 77K fluorescence emission spectra with chlorophyll as well as with phycocyanin excitation.

Since the 'spillover' model was proposed much evidence has accumulated in favour of an alternative mobile model for the state transition (see Chapter 1). In this model, changes in excitation energy transfer from the phycobilisomes to the photosystems are brought about by changes in phycobilisome-photosystem coupling. Thus, the state 1-state 2 transition would entail the detachment of a population of phycobilisomes from PS2 and their reassociation with PS1. There is however evidence to suggest that the state transition is more complex than this pure mobile model. For example, a pure mobile model does not account for the fluorescence change seen in the 77K fluorescence emission spectrum with chlorophyll excitation. Also, a mutant of *Synechococcus* PCC7002 was constructed which is devoid of phycobilisomes and any detectable phycobiliproteins (Bruce et al. 1989). A state transition was still observed in this mutant in the 77K fluorescence emission spectrum with chlorophyll excitation.

In this thesis it is reported that the state transition mutant ΔrpaC only loses the ability to perform state transitions as observed in the 77K fluorescence emission spectrum with phycocyanin excitation. It retains the small fluorescence change observed in the 77K fluorescence emission spectrum with chlorophyll excitation (Section 4.3). This provides evidence that the fluorescence changes observed during the state transition, originally believed to reflect a single phenomenon, reflect at least two phenomena.

The change in the 77K fluorescence emission spectrum with phycocyanin excitation can be ascribed to changes in phycobilisome-photosystem coupling, for which there is much evidence. The origin of the change in the 77K fluorescence emission spectrum with chlorophyll excitation however remains more elusive. It has been suggested that it may be
due to a small, photosystem-associated antenna (Mullineaux 1992). A change in energy transfer from this antenna to one or both photosystems during the state transition could account for this fluorescence change. There is some evidence to suggest that such an antenna may exist (Shen & Vermaas 1994). It has also been suggested that this fluorescence change may originate from changes in photosystem oligomerisation (Meunier et al. 1997). A mutant has been constructed in *Synechococcus* PCC7002 which lacks PsaL (ΔpsaL). This mutant is incapable of forming PS1 trimers and has no impairment in the ability to perform state transitions, as observed in the 77K fluorescence emission spectrum with phycocyanin excitation (Schluchter et al. 1996). It would be interesting to see if ΔpsaL still retained the ability to perform state transitions as observed in the 77K fluorescence emission spectrum with chlorophyll excitation.

Electron microscopy studies of cyanobacterial thylakoid membranes suggest that state transitions involve a major re-organisation of the thylakoid membrane. Freeze-fracture electron micrographs show that PS2 is often organised as long, parallel rows of dimers in the thylakoid membrane (Mörschel & Schatz 1987, Nilsson et al. 1992). Similar studies have shown the phycobilisomes to form similar rows, which associate with the PS2 rows (Giddings et al. 1983). By conducting such ultrastructural studies on *Synechocystis* PCC6714 cells pre-adapted to either state 1 or state 2 conditions, it was observed that in state 2 the proportion of these rows is reduced compared to state 1 (Olive et al. 1986). Such ultrastructural changes have also been observed in *Synechocystis* PCC6803 (Olive et al. 1997). Furthermore, an ndhB-deficient mutant of *Synechocystis* PCC6803 lacks functional complex I and is unable to perform state transitions (Schreiber et al. 1995). This mutant showed no changes in ultrastructure on pre-adaptation to state 1 or state 2 conditions (Olive et al. 1997).

The formation of such PS2 rows in state 1, and their dissociation in state 2, may alter PS2 fluorescence properties in the two states. For example, the close packing of PS2 dimers into rows may result in changes in pigment conformation which alter fluorescence levels. It is possible therefore that these changes in PS2 ultrastructure could account for the change seen in the 77K fluorescence emission spectrum with chlorophyll excitation. This seems plausible, since the changes in PS2 ultrastructure correlated with the state transition in the wild-type are also observed in a mutant entirely devoid of assembled phycobilisomes (Olive et al. 1997). Such mutants still retain the change in the 77K fluorescence emission spectrum with chlorophyll excitation (Olive et al. 1997, Bruce et al. 1989).

There may be several reasons why PS2 forms such rows. It may promote the sharing of excitation energy between PS2 dimers. The resulting energy conducting fibre system
would allow for an efficient energy distribution along the plane of the thylakoid by connecting many PS2 reaction centres (Morschel & Schatz 1987). This would increase the cooperativity between reaction centres (Morschel & Schatz 1987). For example, if one PS2 reaction centre is closed, excitation energy can be transferred to a neighbouring reaction centre and trapped there.

9.2.2 Changes in phycobilisome-photosystem coupling

The insertional inactivation of \textit{rpaC} in the PS1' and PS2' backgrounds was found to result in changes in the coupling of the phycobilisome to PS2 and PS1 respectively. This suggested that the state transition entails changes in the affinity of the phycobilisomes for PS1 as well as PS2 (Chapter 8). Two possible models for the state transition were proposed to account for these findings. In one, conformational changes occurring in both photosystems would alter the affinities of both phycobilisome-photosystem interactions. In the other, a conformational change in the phycobilisome alone would alter the affinities of both phycobilisome-photosystem interactions.

Insertional inactivation of the \textit{apcD} gene leads to the inability to perform state transitions. The \textit{apcD} gene encodes the APC-B subunit, a minor component of the phycobilisome core and one of the terminal emitters of fluorescence. This was originally observed in \textit{Synechococcus} 7002 (Zhao \textit{et al.} 1992). It has since been observed in \textit{Synechocystis} PCC6803 as well (Ashby & Mullineaux 1999a). Insertional inactivation of the \textit{apcF} gene in \textit{Synechocystis} PCC6803 also leads to the inability to perform state transitions (Ashby & Mullineaux 1999a). \textit{apcF} encodes the $\beta^{18.5}$ subunit, another minor component of the phycobilisome core and terminal emitter of fluorescence.

77K Fluorescence emission spectra with phycocyanin excitation would indicate that $\Delta$\textit{apcF} is greatly disrupted in energy transfer from the phycobilisomes to PS2 as well as to PS1 (Ashby & Mullineaux 1999a). In contrast, $\Delta$\textit{apcD} exhibits little disruption in phycobilisome-to-photosystem energy transfer: the 77K fluorescence emission spectrum with phycocyanin excitation resembles that of the wild-type in state 1 (Ashby & Mullineaux 1999a). This would suggest that in $\Delta$\textit{apcD}, the inability to perform state transitions is a direct effect on the state transition. In contrast, for $\Delta$\textit{apcF}, it seems likely that the inability to observe a state transition is due to the severe disruption of phycobilisome-to-photosystem energy transfer.

It has been suggested that the state transition may involve a conformational change in the phycobilisome (see above and Chapter 8). It is possible that the inability of $\Delta$\textit{apcD} to
perform state transitions is a result of disruption to such a conformational change. This may be an indirect effect. Alternatively, one can speculate that the APC-B subunit may have a direct role in inducing this conformational change. For example, the APC-B subunit may be the target of a covalent modification which alters the conformation of the phycobilisome and thus triggers the state transition.

In summary, the findings of this thesis support the notion that the changes in excitation energy distribution occurring during the state transition involve two distinct events:
1) A change in the coupling of the phycobilisomes to both photosystems.
2) Possibly a change in the ultrastructural organisation of PS2.

9.3 The state transition and signal transduction
In this thesis, a gene specifically required for the state transition has been identified in the cyanobacterium *Synechocystis* PCC6803. This gene, *rpaC* may encode a signal transduction factor required for this process. It is important when considering the molecular basis for state transition signal transduction in cyanobacteria, to place this in the context of the evolution of oxygenic photosynthetic organisms, and in particular the evolution of light harvesting systems.

Oxygenic photosynthetic organisms can be roughly divided into two groups: those organisms which contain chlorophyll *b*-based accessory light harvesting systems, and those which contain phycobilin-based accessory light harvesting systems. Among the prokaryotes the cyanobacteria have phycobilin-based accessory light harvesting systems whereas the prochlorophytes have chlorophyll *b*-based accessory light harvesting systems. Among the eukaryotes, organisms containing chlorophyll *b*-based accessory light harvesting systems include the Chlorophyta (green algae). The Rhodophyta (red algae) and Glaucocystophyta in contrast have phycobilin-based accessory light harvesting systems.

There is much evidence to suggest that chloroplasts arose from a single cyanobacterium-like ancestor (Douglas 1994, Douglas 1998, Wolfe *et al.* 1994). The prochlorophytes were originally considered to be ancestors of chlorophyte chloroplasts (Lewin 1976, Lewin & Withers 1975). However, these organisms have since been shown to be diverged members of the cyanobacteria rather than the specific ancestors of chloroplasts (Palenik & Haselkorn 1992, Urbach *et al.* 1992).

Recently, a phylogenetic analysis was performed on the chlorophyll *b* synthesis genes (chlorophyll *a* oxygenase) from several prochlorophytes and chlorophytes (Tomitani *et al.* 1999). This analysis suggested that the chlorophyll *a* oxygenase genes of prochlorophytes and chlorophytes have a common evolutionary origin. The authors therefore proposed that
all oxygenic photosynthetic organisms were descended from a single common ancestor (Figure 9.1). This ancestor would have contained chlorophyll $b$ and phycobilin-based accessory light harvesting systems and would have given rise independently to the prokaryotic and eukaryotic oxygenic photosynthetic organisms. Loss of the chlorophyll $b$-based accessory light harvesting system would have given rise to the cyanobacteria and loss of the phycobilin-based accessory light harvesting system would have given rise to the prochlorophytes. Following endosymbiosis of this ancestor by a non-photosynthetic eukaryote, similar events would have given rise to the phycobilin containing eukaryotes and to the chlorophyll $b$ (LHC) containing eukaryotes.

The prochlorophyte *Prochlorococcus marinus* contains a type of phycoerythrin in addition to chlorophyll $b$ (Hess *et al.* 1996). It is possible that this organism derived from an ancestral form without major pigmentary changes. The same may be true for the rhodophyte *P. cruentum* which has, in addition to phycobilisomes, a chlorophyll $b$-based accessory light harvesting complex which is immunologically related to the chlorophyll $a\beta$ complexes of chlorophytes and higher plants (Wolfe *et al.* 1994).

The state transition occurs both in photosynthetic organisms containing chlorophyll $b$-based accessory light harvesting systems and phycobilin-based accessory light harvesting systems. It is interesting to speculate about the evolution of the state transition in the light of evolutionary models such as that of Tomitani *et al.* (1999). For example, state transition signal transduction systems specific to chlorophyll $b$-based light harvesting and phycobilin-based light harvesting may each have existed in the hypothetical common ancestor of oxygenic photosynthetic organisms. In descendants with only chlorophyll $b$ or only phycobilin-based accessory light harvesting systems one of the signal transduction systems may have become obsolete and lost. In chlorophytes as well as in cyanobacteria the state transition would seem to be triggered by the redox state of cyt $b$ (Mullineaux *et al.* 1990, Vener *et al.* 1998, Vemotte *et al.* 1990). Thus, signal transduction elements involved in redox sensing may be more conserved between chlorophyll $b$ and phycobilin-containing organisms than signal transduction elements involved in changes in light harvesting. The identification of the genes encoding state transition signal transduction factors from chlorophyll $b$ as well as phycobilin-containing organisms may allow the evolution of signal transduction to be traced. This will permit these hypotheses to be tested.

The complete genomic sequence of the prochlorophyte *P. marinus* (MED 4) is now available (http://bbrp.llnl.gov/igi/microbial/prochloro_homepage.html). Also, the genome sequence of the higher plant *A. thaliana* is now 82% complete (http://www.arabidopsis.org/agi.html). There are no *rpaC* homologues within these sequences. There is however a *rpaC* homologue in the complete genome sequence of the
Figure 9.1: Hypothetical scheme for the evolution of oxygenic photosynthetic prokaryotes & eukaryotes [reproduced in simplified form from Figure 3, Tomitani et al. (1999)].

Glaucocystophyta  Rhodophyta  Chlorophyta

EUKARYOTES

Prokaryotes

Prochlorophytes  Cyanobacteria

Common ancestor of Cyanobacteria, Prochlorofytes and Chloroplasts

Shaded: phycobilin-based accessory light harvesting system
Hatched: chlorophyll b-based accessory light harvesting system
Black: Photosystem
cyanobacterium *Anabaena* PCC7120 (Section 4.2). This might indicate that *rpaC* is specific to phycobilisome-containing organisms.

As discussed in Section 9.2, the state transition in phycobilisome-containing organisms appears to involve two distinct phenomena:
1) a change in the 77K fluorescence emission spectrum with phycocyanin excitation. This is probably related to changes in the coupling of the phycobilisomes to both photosystems.
2) a change in the 77K fluorescence emission spectrum with chlorophyll excitation. This may be related to a change in the ultrastructural organisation of PS2.

*ΔrpaC* is affected in the 77K fluorescence emission spectrum with phycocyanin excitation only. This would indicate that RpaC is specifically involved in the phycobilisome-photosystem coupling phenomenon of state transitions. Given this, in addition to the apparent presence of *rpaC* in phycobilisome containing organisms only, it would seem likely that RpaC specifically has some role in regulating the interaction of phycobilisomes with photosystems. RpaC may be part of a phycobilisome-photosystem supercomplex and a target for covalent modification which changes the affinity of the phycobilisome-photosystem interaction.

### 9.4 Further research

#### 9.4.1 Mutagenesis

A primary area for future research is the identification of other genes required for the state transition in *Synechocystis* PCC6803. This will help lead towards an understanding of signal transduction at the molecular level. In Section 3.3, the advantages of random cartridge mutagenesis are discussed. It is clear from the identification of the lesions in state transition mutants that random cartridge mutagenesis also has some disadvantages. The insertion, and insertion with deletion of random constructs into the genome, can clearly involve recombination events between sequences which are not exactly homologous (see Section 3.6). This can make the identification of the site of a particular lesion in the genome difficult. In addition, it is possible that certain genes are less accessible than others to disruption using random cartridge mutagenesis. This means that certain mutants may be in too small a proportion in the mutant library to be screened and selected (this point is discussed in detail in Section 3.11).

For these reasons, alternative methods of mutagenesis may be more suitable for the generation of the mutant library. For example, transposon mutagenesis may be used. Transposon Tn5 was found to integrate into a single site within the *Synechocystis* PCC6803 genome (see Section 3.2). This behaviour however is likely to be specific to a particular transposon sequence. Other transposons may integrate randomly. Also, *in vitro*
Transposon mutagenesis has been used to successfully generate random transposon mutants in *Synechocystis* PCC6803 (A. Grossman, personal communication). Such a technique involves the incubation of genomic DNA in the presence of a transposon and a transposase. After transposon cointegration, the genomic DNA is transformed into the cyanobacterium and transposon mutants selected using antibiotic resistance.

An alternative, but less reliable, method of identifying genes involved in the state transition, would be to isolate pseudorevertants of Δ*rpaC* (Vermaas 1998). Random mutations could be generated in Δ*rpaC*, for example by chemical mutagenesis (Golden 1988). Mutants able to perform state transitions (fully or partially), may then be isolated using a fluorescence video imaging system. The site of the mutation in these pseudorevertants could then be determined. A method is available for the identification of the sites of point mutations in *Synechocystis* PCC6803 (Vermaas 1998). This may identify other genes whose modification compensates for the loss of *rpaC* function.

With the existence of complete genomic sequences for organisms such as *Synechocystis* PCC6803 and *Anabaena* PCC7120, it is possible to construct insertional inactivation mutants of likely regulatory genes. For example, insertional inactivation mutants of all 43 potential histidine kinase genes in *Synechocystis* PCC6803 have been constructed (Murata 1999). The screening of such mutants for the inability to perform state transitions, as well as other light acclimation mechanisms, may be a useful means of identifying signal transduction factors. So far, 14 insertional inactivation mutants of potential histidine kinase genes from *Synechocystis* PCC6803 have been screened for the inability to perform state transitions. They all perform state transitions (Conrad Mullineaux, personal communication).

A method for the identification of residues within RpaC which are important for the state transition would be to use localised random mutagenesis of the *rpaC* gene (Vermaas 1998). Random mutants could be screened for the inability to perform state transitions using a fluorescence video imaging system. The identities of the mutated residues in state transition mutants may then identify residues within RpaC which are important for the state transition. A disadvantage of such a method is that it would be very difficult to separate mutations which affect residues functionally important in the state transition, from mutations which affect residues important in RpaC assembly, or structure.

Another area for future research is the role of the cytbf complex in the state transition. In higher plants and green algae, cytbf is the trigger for state transitions (Gal *et al.* 1997, Vener *et al.* 1997). In cyanobacteria, there is some evidence that cytbf is also the trigger for
state transitions (Mullineaux & Allen 1990, Vernotte et al. 1990). In higher plants and green algae, structural models have been proposed for how cytbf in a particular redox state may interact with signal transduction factors and trigger the state transition (Vener et al. 1998, Zito et al. 1999). The structural basis for the role of cytbf in state transitions however remains unknown.

Localised random mutagenesis of the cytbf genes in *Synechocystis* PCC6803 provides a good system by which to investigate this cytbf function. There are eight genes in the *Synechocystis* PCC6803 genome which code for the subunits of the cytbf complex (Kaneko et al. 1996). Random mutations could be generated in these genes. For example, a mutator strain of *E. coli* defective in DNA repair (XL1-Red competent cells, available from Stratagene) can be used for this purpose. Random cytbf mutants could then be screened for the inability to perform state transitions, using a fluorescence video imaging system. The identification of the mutations in state transition mutants would then identify cytbf residues required for the state transition. The structural and biophysical characterisation of mutant cytbf proteins could then be used to determine the structural origin of the mutant phenotypes.

The advantage of using a cyanobacterium such as *Synechocystis* PCC6803 for such a study is that the cytbf complex is essential for cell viability. This means that cytbf state transition mutants affected in, for example, electron transport function are automatically selected against and therefore remain heteroplasmic. A large proportion of homoplasmic state transition mutants will therefore be affected directly in signal transduction. In such mutants, interactions of cytbf with other proteins such as signal transduction factors may be disrupted. With the accumulation of more detailed structural information for cytbf (Mosser et al. 1997), such studies may lead to an understanding, at the molecular level, of how cytbf triggers state transitions.

**9.4.2 Identifying homologues of rpaC**

The presence of a homologue of rpaC in *Anabaena* PCC7120 indicates that certain parts of the RpaC sequence are conserved (Figure 4.1). These regions may have functional significance. PCR primers which incorporate these regions, may be used to amplify rpaC from other cyanobacteria. Such knowledge of conserved regions may also be used to guide site-directed mutagenesis strategies. In addition, the identification of rpaC in other organisms may allow insights into the evolution of the state transition (see Section 9.3).
9.4.3 The expression pattern of rpaC

ΔrpaC was found to have a doubling time significantly longer than the wild type (approx. 30%), under very low intensity light (2μE.m⁻².s⁻¹). It would therefore appear that the state transition is more important for cell growth under these conditions (Chapter 8). It is possible that the expression pattern of rpaC reflects this increased requirement for the state transition under very low light intensities. Thus, rpaC expression may be increased under very low light intensities in comparison to higher light intensities.

9.4.4 Regulatory networks

There is evidence to suggest that bacterial signal transduction pathways interact with one another, a phenomenon known as "crosstalk" (Schneider et al. 1991). This has led to the idea of "neural" signal transduction networks in bacteria (Hellingwerf et al. 1995, Hellingwerf et al. 1998). An individual signal transduction pathway can produce a given output signal for a given input signal. The interaction between individual signal transduction systems leads to the possibility that they may form a complex, interacting, regulatory network. Such regulatory networks, like the neural networks of the human brain, would have the ability to modify output signals in response to changing input signals. In other words they would have a primitive learning ability.

The adaptation of the photosynthetic apparatus to changing light conditions may represent a regulatory network (see Section 1.7.6). It is possible therefore that certain state transition mutants may also be affected in other light acclimation mechanisms such as the regulation of photosystem stoichiometry (Fujita 1997), the long term regulation of phycobilisome-photosystem coupling (Ashby & Mullineaux 1999c) etc. The isolation of mutants affected in more than one light acclimation mechanism may be one way of mapping such regulatory networks (Ashby 1999).

9.4.5 The origin of the change in the 77K fluorescence emission spectrum with chlorophyll excitation

As discussed in Section 9.2.1, ΔrpaC is unable to perform state transitions as observed in the 77K fluorescence emission spectrum with phycocyanin excitation only: it retains the change in the spectrum with chlorophyll excitation. This mutant therefore provides a useful system for investigating the origin of the change in the 77K fluorescence emission spectrum with chlorophyll excitation.

For example, this fluorescence change may be related to changes in PS2 ultrastructure (see Section 9.2.1). By conducting electron microscopic ultrastructural studies of ΔrpaC
thylakoids prepared after adaptation of cells under state 1 or state 2 conditions it may be possible to determine whether changes in PS2 ultrastructure still occur in this mutant.

It has also been suggested that the change in the 77K fluorescence emission spectrum with chlorophyll excitation may be due to a photosystem-associated antenna (Mullineaux 1992). A change in energy transfer from this antenna to one or both photosystems during the state transition could account for the fluorescence change. Flash-induced absorbance changes at 700nm can be used to determine the absorption cross-section of PS1 in state 1 and state 2 (Mullineaux 1992, Mullineaux 1994) (Section 1.9.4). Using this technique with ΔrpaC, it may be possible to detect a change in PS1 absorption cross-section resulting from a change in energy transfer from such an antenna. In ΔrpaC such measurements would not be complicated by changes in phycobilisome-to-photosystem energy transfer.
REFERENCES
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