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Characterisation of the slr1212 genomic region of the freshwater cyanobacterium *Synechocystis* sp. PCC 6803

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

University of Warwick, Department of Biological Sciences

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

Acknowledgements

Declaration

This thesis is my own work unless otherwise acknowledged and at no other time has been submitted for another degree.

....

James May

I certify this statement to be correct.

....

David Scanlan

Abstract

Synechocystis sp. PCC 6803 is a unicellular, freshwater cyanobacterium. Its dependence upon light to support its photoautotrophic lifestyle increases the importance of environmental sensing mechanisms to be able to maximise light-harvesting whilst avoiding the harmful effects of light-mediated cell damage. The sequencing of the *Synechocystis* sp. PCC 6803 genome in 1996 now allows the identification of genes that encode putative proteins with roles in sensing the environment.

Two such open reading frames, slr1212 and slr1213, were identified from the genome following computer analysis of the protein sequences. These two proteins encode a putative two-component signal transduction system with a role in sensing the environment. Slr1212 possesses homology to (i) the binding domain of ethylene receptors of higher plants, (ii) PAS/PAC domains, potentially involved in ligand binding and protein dimerisation, (iii) GAF domains, which contain the chromophore binding region of plant phytochromes, and (iv) histidine kinases of two-component signal transduction systems. Slr1213 possesses homology to characterised response regulators, and contains a helix-turn-helix DNA binding motif.

This study set out to characterise a physiological role for these enigmatic proteins by analysing interposon mutants. Single and double interposon mutants were generated in these open reading frames. Growth of these mutants was unaffected in different light qualities, but Slr1212 was shown to be involved in the acclimation of the *Synechocystis* sp. PCC 6803 cells to high light irradiance as analysed by 77K fluorescence spectroscopy, which also indicated possible structural alterations in PSI reaction centres of ORF slr1212 mutants.

Using laser photoacoustic spectroscopy, it was shown that *Synechocystis* sp. PCC 6803 could release ethylene following incubation with ACC suggesting a possible ethylene biosynthetic route, though genome analysis revealed no obvious homologues of ACC oxidase, an enzyme required for conversion of ACC to ethylene in vascular plants. It is hypothesised that an ethylene signalling mechanism may be present that regulates cell responses to non-specific stress.

Site-directed mutagenesis of Slr1213 that caused constitutive activation of the protein had a lethal effect in *Synechocystis* sp. PCC 6803 cells. The mutation consisted of a substitution of the conserved aspartate residue with glutamate, thus mimicking the phosphorylated state of the protein.

In summary, Slr1212 has a role in the acclimation of cells to high light irradiance and binds ethylene, and may act in conjunction with Slr1213 to modulate these responses.

Abbreviations

1-MCP	1-methylcyclopropene
aa	Amino acid
ACC	1-aminocyclopropane-1-carboxylic acid
AdoMet	S-adenosyl-L-methionine
ADP	Adenosine diphosphate
APS	Ammonium persulfate
ATCC	American type culture collection
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BLAST	Basic local alignment search tool
Bp	Base pair
BSA	Bovine serum albumin
CCA	Complementary chromatic adaptation
cGMP	Cyclic guanosine monophosphate
Cfu	Colony forming units
CPM	Counts per minute
dH_2O	Distilled water
DIG	Digoxigenin
DMPC	Dimethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded DNA
EDTA	Ethylenediamine tetraacetic disodium salt
EGTA	[Ethylene-bis(oxyethylenenitrilo)]tetraacetic acid
ExPASy	Expert protein analysis system
FAD	Flavin adenine mononucleotide
F/R	Forward/reverse
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HPt	Histidine-containing phosphotransfer module
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
IR	Infra-red
Kan	Kanamycin

Kb	Kilobase (1000 bp)
kDa	Kilodalton
KMBA	2-oxo-methyl-thiobutyric acid
LB	Luria-Burtani
MACC	N-malonyl-ACC
MBq	Mega becquerels
MCS	Multiple cloning site
MIC	Minimum inhibitory concentration
OD	Optical density
ORFs	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PAS	PER/ARNT/SIM
PAC	PAS associated
PCC	Pasteur culture collection
PCR	Polymerase chain reaction
ppm	Parts per million
PSI	Photosystem I
PSII	Photosystem II
PSORT	Prediction of protein localisation sites (version 6.4 (www))
rRNA	Ribosomal RNA
RPM	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sm	Streptomycin
Spc	Spectinomycin
TBE	Tris base-boric acid-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
Tris	Tris[hydroxymethyl]methylamine
UV	Ultraviolet radiation
$\mu mol\ m^{-2} s^{-1}$	Micromoles of quanta per meter squared per second
W	Watt
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Chapter 1

Introduction

1. Introduction

The cyanobacteria, sometimes termed the 'blue-green algae', are a varied and well distributed group of prokaryotic photosynthetic organisms which differ from other bacteria in possessing chlorophyll *a* and in carrying out oxygenic photosynthesis. Cyanobacteria are obligate photoautotrophs, and consequently are found only in habitats where light is available. Such habitats range from tropical to polar regions, and cyanobacteria may be found in fresh, brackish, marine and hypersaline waters, hot springs, soils, muds, sediments and salt marshes. Others can live in intimate association with protozoa, fungi, and green plants (Curtis, 1992).

Around 150 genera and over 1000 species have been described based on observations of field materials (Rippka *et al.*, 1979). Generally, cyanobacteria are unicellular or filamentous, and no strains have flagella at any stage although many are capable of gliding motility (Hoiczyk, 2000). It is believed that cyanobacteria were important for the generation of oxygen in the atmosphere, having dominated the Earth's biota during the middle to late Precambrian era (about 2500-2570 million years ago) (Tandeau de Marsac and Houmard, 1993). Cyanobacteria contribute significantly to marine primary productivity and make a continuing contribution to the equilibrium of the Earth's atmosphere by producing oxygen and removing carbon dioxide. Many strains are also capable of nitrogen fixation (Bryant, 1994).

Given the diversity of the environments to which cyanobacteria have adapted, there is an innate requirement for mechanisms by which cells can sense and rapidly adapt to environmental fluctuations. Unexpected fluctuations in nutrient supplies can occur at any time and survival would depend on the ability to quickly switch to the metabolism of a different substrate. Eukaryotic unicellular organisms share this requirement to be able to rapidly sense and adapt, though more complex multicellular organisms are restricted to a more constant set of metabolic pathways and do not have the same need to respond to external circumstances. In such sensing pathways, a stimulus must be transformed into a biochemical change which can be passed through the cell. Flexibility is therefore a very important trait in the bacterial world. However, it would be disadvantageous to produce the enzymes required to metabolise a particular substrate if that substrate was not present, and so economy is also important.

The adaptive responses of bacteria can range from rapid transient changes to long term global reorganisations of gene expression and cell morphology (Stock *et al.*, 1989). The ability of cyanobacteria to detect and respond to alterations in their environment is of crucial importance. Due to their photoautotrophic lifestyle, a dependency exists for chemical and physical factors such as inorganic nutrients, light and temperature. Variations in these factors represent the primary environmental stimuli for adaptation (Mann, 2000).

The sun represents the primary source of energy on Earth, and in conjunction with the evolution of the photosynthetic machinery, a range of responses to light have developed, allowing organisms to respond in subtle and even pre-emptive ways to changes in the ambient light environment. Light plays two roles in the life of organisms: firstly, light supplies the organisms that have adapted to a phototrophic mode of life with energy for maintenance and growth, and secondly, light acts as a source of information. However, the use of light by a phototrophic organism has inherent associated risks. Upon absorption of a photon of light, the surroundings of a light-absorbing chromophore of a photoactive protein heats up 200°C in a nanosecond (Hellingwerf *et al.*, 1998). Light, through the interaction of high energy electrons generated by light absorption with water and/or molecular oxygen, can also lead to the generation of high energy, highly reactive molecular species. Species such as singlet oxygen and hydroxyl radicals are highly reactive, readily diffusible and long-lived (Hodgson and Berry, 1998). Furthermore, UV-radiation can cause damaging photochemical reactions in nucleic acids (Hellingwerf *et al.*, 1998). Therefore, sensitive sensory mechanisms are essential to ensure that an organism can maximise its use of photosynthetically available light, whilst avoiding the potentially harmful effects. The light environment may fluctuate in terms of irradiance, duration, direction and spectral quality. Inorganic nutrient availability also influences the rate and extent of growth. Interactions between metabolism, photosynthesis, nutrient availability and the switch to dark metabolism represent areas where adaptive responses are likely to operate in cyanobacteria (Mann, 2000).

Bacteria may respond to many other stimuli in addition to light and nutrient availability, both internal and external to the cell. Other sensory inputs include osmolarity (Hall and Silhavy, 1981), temperature (Suzuki *et al.*, 2000), toxic

substances (Bartsevich and Shestakov, 1995), oxygen (Armitage, 1997), carbon dioxide (Stretton and Goodman, 1998), and population density (Greenberg, 2000; Parsek and Greenberg, 2000). Traditionally regulatory mechanisms were all thought to act at the transcriptional level, and the lactose regulatory system of *E. coli* (Lewin, 1994) provides a paradigm for this type of control mechanism. Further transcriptional control mechanisms include the regulation of alternative sigma factors, anti-termination and integration host factor (Mann, 2000). However, more recent research has demonstrated that sensory mechanisms can effect changes at any level of the genotype to phenotype pathway. Regulatory mechanisms can occur at the translational level through the control of translation initiation and mRNA stability (such as the *lacZYA* genes). Post-translational modifications also represent a very important level of regulation for adaptive responses. Such modifications can directly affect the biological activity of a protein and include methylation, phosphorylation and ADP-ribosylation (see Haag and Koch-Nolte (1998) for example). These regulatory mechanisms frequently overlap to provide an exquisite level of control over an adaptive response to a fluctuation in an environmental stimulus (Stock *et al.*, 1989).

1.1. *Synechocystis* sp. PCC 6803

The study of adaptive responses in cyanobacteria has been greatly facilitated in recent years by the sequencing of the genome of the unicellular, freshwater cyanobacterium, *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996*a,b*). This information is readily accessible via the internet (http://www.kazusa.or.jp/cyano/cyano.html) (Nakamura *et al.*, 1998). The availability of this information represents a huge step forwards for research into the mechanisms of photosynthesis and sensing of the environmental cues intricately involved. A prokaryotic organism is more amenable to genetic manipulation and due to the ability of *Synechocystis* sp. PCC 6803 to grow both photoautotrophically and photoheterotrophically, it is particularly suited to the study of the mechanism of photosynthesis. The selection of *Synechocystis* sp. PCC 6803 for sequencing reflects its amenability to genetic modifications, and its natural competence.

From the 3.57 Mb genome, 3,168 potential protein coding genes were identified. Of these, 1,426 (45%) had no apparent similarity to any known genes in

databases. It was found that the identified open reading frames (ORFs) covered 87% of the total genome length, indicating that the genome has a very compact arrangement of protein and RNA coding regions (Kaneko *et al.*, 1996*a,b*).

Information from the genetic sequence of *Synechocystis* sp. PCC 6803 can be used to identify ORFs that potentially encode proteins involved in the sensing of environmental stimuli, and signal transduction mechanisms. Screening of CyanoBase (the on-line genome database) with protein sequences from other organisms allows the identification of similar sensing mechanisms, or excludes a role for them in *Synechocystis* sp. PCC 6803.

1.2. ORFs slr1212 and slr1213

An open reading frame (ORF) designated slr1212 was identified from CyanoBase as potentially encoding a protein involved in sensing an environmental stimulus and acting as a signal transducer. The 2535 base pair (bp) ORF encodes an 844 amino acid polypeptide, with a predicted molecular weight of 97.3 kDa. Comparison of the predicted protein sequence with those in databases reveals an intriguing multi-domain protein (see Figure 1-1). It was found that Slr1212 contains domains homologous to the binding regions of ethylene receptors of higher plants (e.g. ETR1 of *Arabidopsis thaliana* (Chang *et al.*, 1993)), multiple PAS/PAC domains (Taylor and Zhulin, 1999), a GAF domain (Ho *et al.*, 2000) and a histidine kinase domain (Loomis *et al.*, 1997), indicative of a sensor protein of a so-called 'two-component' signal transduction system (Stock *et al.*, 2000). There are predicted to be five transmembrane helices, all located within the N-terminal 595 amino acids (analysed with TMpred). The transcriptional start codon of ORF slr1213 is located 50 bp downstream of ORF slr1212, and intriguingly the predicted protein exhibits homology to response regulator proteins of two-component signal transduction systems. These ORFs might therefore together form a two-component signal-transduction system in *Synechocystis* sp. PCC 6803.

Figure 1-1 shows the structural elements of the predicted Slr1212 protein.

Figure 1-1 :- The structural elements of the Slr1212 protein are indicated as follows: hydrophobic region related to ETR1 (red rectangle, residues 37-148), PAS domains (blue ellipse's, residues 197-266 and 323-400), PAC domains (green ellipse's, residues 270-322 and 402-455), GAF motif (turquoise triangle, residues 438-621), and histidine protein kinase core (pink hexagon, residues 655-844).

The predicted protein encoded by ORF slr1213 shows greatest similarity to a sensory transduction regulatory protein from *Methanobacterium thermoautotrophicum* (Genbank accession no. AE000931). The predicted protein has a CheY-type histidine receiving module of two-component signal transduction systems, and an AraC-type helix-turn-helix DNA binding motif. This domain organisation is indicative of a response regulator protein.

ORFs slr1212 and slr1213 are clustered together with several other ORFs highlighted in Figure 1-2. ORF slr1211 shares homology with proteins from a variety of organisms involved with cobalamin biosynthesis (Crouzet *et al.*, 1991), whilst ORF slr1214 shows greatest homology to PatA from *Anabaena* sp. PCC 7120, a protein possessing a CheY-type histidine receiving module of two-component signal transduction systems.

This study set out to determine a physiological role for the Slr1212 and Slr1213 proteins in *Synechocystis* sp. PCC 6803, and therefore the mode of action of two-component signal transduction systems will be discussed in subsequent sections.

1.7 cm = 1 kb

slr1211 - 3279 bp
Similar to :-
cobN of *Pseudomonas aeruginosa* and *denitrificans*
Cobalamin biosynthetic protein of *Streptomyces coelicolor*

slr1212 - 2535 bp
Main features :-
Ethylene binding domain of higher plant ethylene receptors
Repeat PAS/PAC domains
GAF domain
Histidine kinase domain

slr1213 - 736 bp
Similar to :-
Sensory transduction regulatory protein of
Methanobacterium thermoautotrophicum
Main features :-
Histidine receiving module
Helix-turn-helix DNA binding motif

slr1214 - 1119 bp
Similar to :-
patA of *Anabaena* sp. PCC 7120
Main features :-
Histidine receiving module

Figure 1-2 :- Organisation of ORFs around slr1212 in the *Synechocystis* sp. PCC 6803 genome, highlighting similar proteins and main features of the predicted proteins encoded by the surrounding ORFs.

1.3.   Two-component signal transduction

In the last several years it has become apparent that much of the sensory information perceived by prokaryotic organisms is processed by related families of proteins that comprise the two-component signal transduction systems. These systems have been discovered in a wide variety of organisms such as prokaryotes (including cyanobacteria) (Kotani and Tabata, 1998; Stock *et al.*, 1989), and eukaryotes (Chang and Meyerowitz, 1995; Ota and Varshavsky, 1993) and are a common way of regulating adaptive responses. It is noteworthy that the abundance of such signalling mechanisms in different classes of organism differs substantially. Two-component signal transduction systems account for the majority of signalling pathways in bacteria, and are quite rare in eukaryotes, in which serine/threonine and tyrosine phosphorylation pathways predominate (Stock *et al.*, 2000). The completion of numerous genome sequencing projects has allowed an assessment of the occurrence of such sensing pathways. In *E. coli*, 30 histidine protein kinases and 32 response regulators have been identified (Mizuno, 1997), whilst none have been identified in *Mycoplasma genitalium* (Fraser *et al.*, 1995). In *Synechocystis* sp. PCC 6803 80 two-component proteins have been identified, accounting for ~2.5% of the genome (Mizuno, 1998). The widespread nature of this type of mechanism implies that it is a powerful device for a plethora of adaptive responses in prokaryotic organisms.

At the most basic level, such a system comprises two components: a histidine protein kinase and an associated response regulator. Histidine protein kinases act as sensors, and respond to an alteration in a specific environmental stimulus. The sensor domain is located towards the N-terminus of the histidine protein kinase, and environmental stimuli are detected either directly or indirectly. The sensor domains of this group of proteins share little similarity, which supports the hypothesis that these domains each evolved separately to detect a specific ligand/stimulus (Stock *et al.*, 2000). However, to date the mechanism by which the environmental signal is transmitted to the histidine kinase domain remains elusive. The signalling activity of these proteins is modulated by the sensor domain. Histidine protein kinases undergo ATP-dependent autophosphorylation at a highly conserved histidine residue within the kinase core, a reaction that is readily reversible. This phosphorylation step is a bimolecular reaction between homodimers, in which one histidine protein kinase catalyses

phosphorylation of the conserved histidine residue in the second monomer (Surette *et al.*, 1996; Swanson *et al.*, 1993). This phosphoryl-group is transferred from the phospho-histidine protein kinase by the cognate response regulator to a conserved aspartate residue within its regulatory domain. Signalling mechanisms mediated by two-component signal transduction systems are therefore controlled by the ability of the histidine protein kinase to regulate the phosphorylation state of the response regulator. A further level of control is exhibited by many histidine protein kinases proteins, which possess a phosphatase activity which can catalyse the removal of the phosphoryl group from the conserved aspartate residue of their cognate response regulator (Igo *et al.*, 1989). Typically, histidine protein kinases have membrane-spanning regions associated with the sensor domain, whilst the transmitter domain extends into the cytoplasm.

Response regulators tend to be cytoplasmic, and consist of at least one domain. This domain contains the conserved aspartate residue that receives the phosphoryl-group from the histidine kinase protein. Often these proteins have an output domain located towards the C-terminus, which commonly consists of a DNA-binding region which functions to activate and/or repress transcription of specific genes (Stock *et al.*, 2000). In this manner, the appropriate response can be regulated according to external circumstance.

The transmitter and receiver domains of histidine protein kinases and response regulators can be used with a variety of input and output domains, and can be arranged in a variety of configurations. EnvZ of *E. coli* exemplifies an orthodox two-component signal transduction system, comprising an osmosensor (EnvZ) with two transmembrane regions in the N-terminus sensing domain, and a C-terminus containing a histidine kinase core. Other sensors are known to have multiple transmembrane regions in the sensing domain including FixL, involved with the control of expression of genes involved with nitrogen fixation in response to oxygen availability in *Rhizobium meliloti* (Lois *et al.*, 1993). A more complicated configuration, under the umbrella name 'hybrid kinases', involves proteins containing multiple histidine kinase and response regulator domains. Thus, hybrid kinases carry out a multistep phosphotransfer reaction on the same protein. This allows a more sophisticated level of control, with more inputs and checks integrated

into the pathway. An example of this is the ArcB protein of *E. coli* (Ishige *et al.*, 1994), which has a sensor domain, a histidine kinase domain, a response regulator-type domain, and a second histidine-containing domain, often referred to as a histidine-containing phosphotransfer module (HPt). In prokaryotes, HPts are almost exclusively found in hybrid kinases, whereas in eukaryotes they tend to exist as separate proteins. Such domains contain a histidine residue that can be phosphorylated, but do not exhibit kinase or phosphatase activity (Tsuzuki *et al.*, 1995). Hybrid kinases tend to be more common in eukaryotic organisms, as typified by the ETR1 receptor of *Arabidopsis thaliana* (Chang and Meyerowitz, 1995).

The transmitter and receiver domains of histidine protein kinases and response regulators form two protein 'superfamilies'. At the amino acid level, these proteins exhibit some highly conserved features, and these are therefore indicative of proteins involved with two-component signal transduction.

The conserved features of histidine protein kinases are found within the approximately 240 amino acid residue transmitter region, and on average any two homologous kinase domains share ~25% sequence identity with a few specific residues conserved in all members of the family (Stock *et al.*, 1995). It has been observed that the conserved histidine kinase domain can be subdivided into two sub-domains, with a variable connecting region (Parkinson and Kofoid, 1992). The first sub-domain contains the conserved histidine residue within a conserved 'H box' motif. The second sub-domain usually contains four highly conserved sequences termed the N, D, F, and G boxes, and whilst these are conserved, the spacing between these motifs can vary. The ATP-binding site of the histidine protein kinases consists of the conserved residues from the N, D, F, and G boxes. Typically, the N box motif is located approximately 110 amino acids C-terminal of the phosphorylated histidine. The N box contains an absolutely conserved asparagine, surrounded by other fairly well conserved residues. The D/F box contains a conserved DxGxG motif, often followed by a conserved phenylalanine residue. The G box, located 20-50 amino acids further towards the C-terminus, is a glycine rich sequence containing a conserved GxG motif.

In transmembrane histidine protein kinases, the sensor domain is connected to the histidine kinase domain by a transmembrane helix and a cytoplasmic linker. This

region is not currently well understood, although it does seem to be critical for correct signal transduction (Stock *et al.*, 2000). Figure 1-3 highlights the conserved features of histidine protein kinases. In the alignment of Slr1212 with other characterised proteins, it can be seen that the H, N and D/F boxes are conserved. However, the GxG motif in the conserved G box is replaced instead with SxG. The conserved residues from the N, D/F, and G boxes form the ATP-binding site of histidine protein kinases (Stock *et al.*, 2000). Despite lacking one of the conserved residues involved with histidine kinase activity, given that Slr1212 contains the majority of the conserved features of histidine protein kinases it is possible that this protein will function as part of a two-component signal transduction system. Interestingly, Bleecker (1999) concluded that the Slr1212 protein does not contain a histidine kinase module, apparently based upon the analysis of the predicted protein sequence as described here.

Response regulators are the terminal components of the classical two-component signal transduction system. They catalyse the transfer of a phosphoryl group from the phospho-histidine of the histidine protein kinase to a conserved aspartate residue within their own regulatory domain (Stock *et al.*, 2000). When modulated by phosphorylation, they effect the appropriate adaptive response. For example, OmpR binds DNA more efficiently when phosphorylated (Alex and Simon, 1994). In this manner, a signal (physical e.g. temperature, or chemical e.g. metabolite abundance) can be converted into a biochemical signal. Response regulators share a conserved receiver domain approximately 125 amino acids in size, generally extending from the N-terminus of the protein. Any two response regulators tend to share 20-30% sequence identity, and residues corresponding to aspartate-13, aspartate-57 (the site of phosphorylation), and lysine-109 of the classic *E. coli* response regulator CheY are conserved in most response regulators (Stock *et al.*, 1989). Response regulators have a conserved N-terminal receiver domain, and a variable C-terminal effector domain. Often the response regulator is a transcription factor with a DNA-binding effector domain. In such cases, the DNA-binding domains can be sub-divided into three families, typified by OmpR, NarL and NtrC (Stock *et al.*, 2000). However, there are several examples of response regulators which do not possess DNA-binding activity. For example, CheB of *E.coli* demethylates the chemotaxis-response

```
DctB    410  G V A H E I N Q P V A T I R A Y A D N A R T F L D R G Q T A P A G - - - E N L E S I A A L T E R I G S I T E E L K T F A R K G R G S A E P T
FixL    282  T L A H E L N Q P L S A I A N Y S H G C T R L L R D M D D A V A T R I R E A L E E V A S Q S L R A G Q I I K H L R E F V T K G E T E K A P E
PhoR    210  N V S H E L R T P L T V L Q G Y L E - - - - - M M D E Q P L E G A V R E K A L H T M R E Q T Q R M E G L V K Q L L T L S - - - K I E A A P T
EnvZ    240  G V S H D L R T P L T R I R - - - - - - - - - L A T E M M S E Q D - - - G Y L A E S I N K D I E C N A I I E Q F I D Y L R - - T G Q E M P M
Slr1212 655  E I H H R V K N N L Q I M S S - - - - - - - - L L Y L Q F S K A S P A I Q Q L S E E Y Q N R I Q S M A L I H E Q L Y R S E - - - - D L A N I

                                H box


DctB    477  G L K D V I E G A V M L L R S R F A G R M - - - - D T L D I D L P P D E - L Q V M G N R I R L E Q V L I N L L Q N A L E A V A P K A G E G R
FixL    352  D I R K L V E E S A A L A L V G S R E Q G - - - - V R T V F E Y L P G A - E M V L V D R I Q V Q Q V L I N L M R N A I E A M R H V D R R E L
PhoR    272  Q L L N E K V D V P M M L R V V E R E A Q T L S Q K K Q T F T F E I D N G L K V S G N E D Q L R S V I S N L V Y N A V N H T P - - E G T H I
EnvZ    296  E M A D L N A V L G E V I A A E S G Y E R - - - - - E I E T A L Y P G S - I E V K M H P L S I K R A V A N M V V N A A R Y G N - - - - - G W
Slr1212 713  D F S Q Y L K N L T H N I C Q S Y G C N T - - - - D S I K I K L L V E Q V K V P L E Q S I P L G L I I Q E L V S N A L K H A F P - T T E G E

                                                                                                              N box


DctB    542  V E I R T S T D A G M V T V T V A D N G P G I P T E I R K G L F T P F N T S K E S - - - - - - G L G L G L V I S K D I V G D Y G G R M D V A
FixL    417  T I R T M P A D P G E V A V V E D T G G G I P E E V A G Q L F K P F V T T K A S - - - - - - G M G I G L S I S K R I V E A H G G E M T V S
PhoR    340  T V R W Q R V P H G - A E F S V E D N G P G I A P E H I P R L T E R F Y R V D K A R S R Q T G G S G L G L A I V K H A V N H H E S R L N I E
EnvZ    355  I K V S S G T E P N R A W F Q V E D D G P G I A P E Q R K H L F Q P F V R G D S A R - - T I S G T G L G L A I V Q R I V D N H N G M L E L G
Slr1212 778  I S I K F T S M N S H Y S L Q V W D N G V G I S R D I D L E N T D - - - - - - - - - - - - - - - - S L G M Q L I Y S L T E Q L Q G E L H Y E

                                D/F box                                                                      G box


DctB    606  S - D S G G T R F I V Q L R K A -
FixL    481  K N E A G G A T F R F T L P A Y L
PhoR    409  S T V G K G T R F S F V I P E R L
EnvZ    423  T S E R G G L S I R A W L P V P V
Slr1212 832  Y V G G A Q F G L E F S L - - - -
```

Figure 1-3 :- CLUSTAL W sequence alignment of the conserved regions of histidine protein kinases. Residues that are conserved in all sequences are highlighted in red, identical residues in blue, and similar residues in gray. Sequences are from the following sources: DctB (*Rhizobium leguminosarum*; Ronson *et al.*, 1984); FixL (*Rhizobium meliloti*; David *et al.*, 1988); PhoR (*E. coli*; Wanner, 1987); EnvZ (*E. coli*; Csonka, 1989); Slr1212 (*Synechocystis* sp. PCC 6803; Rodriguez *et al.*, 1999).

proteins, and phosphorylation of the CheB receiver module enhances this activity (Lupas and Stock, 1989). Other response regulators can lack an effector domain altogether, such as CheY which consists only of a receiver domain. CheY associates in an intermolecular fashion with FliM, a component of the flagellar motor (Welch *et al.*, 1993). Figure 1-4 highlights the conserved features of response regulators. The alignment of the Slr1213 protein sequence with other response regulators shows that all of the conserved features characteristic of these proteins are present. Magnesium (Mg^{2+}) ions are required for phosphoryl transfer and dephosphorylation, and characterisation of the structure of Mg^{2+}-bound CheY revealed that coordination involved aspartate-57, aspartate-12, the backbone oxygen of aspartate-57 and three water molecules (Stock *et al.*, 2000). Threonine-87 and lysine-109 are implicated in the phosphorylation-induced conformational change (Stock *et al.*, 2000). It therefore seems likely that Slr1213 acts as a response regulator in a two-component signal transduction system. Given the close physical proximity of ORFs slr1212 and slr1213, they may act together to modulate cellular adaptation to particular environmental stimuli.

The overall mechanism of signal transduction by histidine protein kinases and response regulators is shown in Figure 1-5.
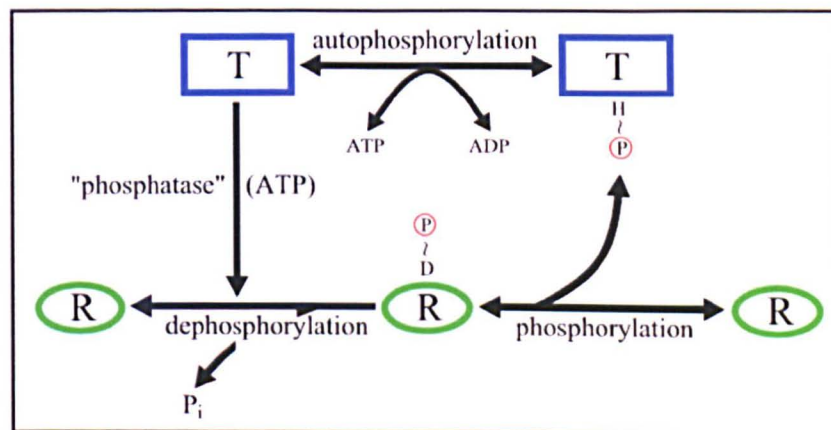
Figure 1-5 :- Diagrammatical representation of the phosphorylation activities of histidine protein kinases and response regulators in two-component signal transduction systems (taken from Parkinson and Kofoid (1992)). T represents transmitter, and R represents regulator.

Thus, the main levels of regulation in two-component signal transduction systems are

CheY   1   MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQAGGYGFVISDWNMP-NMDGLELL
DctD   1   -MSAAPSVFLIDDDRDLRKAMQQTLELAGFT-VSSFASATEALAELSADFAGIVISDIRMP-GMDGLALF
Spo0F   1   --MMNEKILIVDDQYGIRILLNEVFNKEGYQ-TFQAANGLQALDIVTKERPDLVLLDMKIP-GMDGIEIL
OmpR   1   -MQENYKILVVDDDMRLRALLERYLTEQGFQ-VRSVANAEQMDRLLTRESFHLMVLDLMLP-GEDGLSIC
Slr1213 1   ---MVTKILIVEDERLVAQHIAQLLKSDGYEICVIASDGATALKKIAEFYPDLVLLDIRIKGEIDGIEVA

                      D13   D54

CheY   70   KTIRADGAMSALPVLMVTAEAKKENIIAAAQAGASGYVVKPFTAATLEEKLNKIFEKLGM-----------
DctD   68   GKVLALDPD--LPMILVTGHGDIPMAVQAIQDGAYDFIAKPFAADRLVQSARRAEEKRRLVMENRSLRRA
Spo0F   67   KRMKVIDEN--IRVIIMTAYGELDMIQESKELGALTHFAKPFDIDEIRDAVKKYLPLKSN-----------
OmpR   68   RRLRSQSNP--MPIIMVTAKGEEVDRIVGLEIGADDYIPKPFNPRELLARIRAVLRRQANELPGAPSQEE
Slr1213 68   ERIKSLYSI---PIVYLTAFSDGETLERAQKTNPQGYVIKPFRREQLLSTVAIAIANHQQQRKPEEDTLS

                     T87   K109

CheY   - - - -
DctD   136 AEA-
Spo0F   - - - -
OmpR   136 AVI-
Slr1213 135 TSTG

Figure 1-4 :- CLUSTAL W sequence alignment of the conserved regions of response regulators. Residues that are conserved in all sequences are highlighted in red, identical residues in blue, and similar residues in gray. Sequences are from the following sources: CheY (*E. coli* ; Macnab, 1987); DctD (*Rhizobium leguminosarum*; Ronson *et al.,* 1984); Spo0F (*Bacillus subtilis*; Losick *et al.*, 1986); OmpR (*E. coli*; Csonka, 1989); Slr1213 (*Synechocystis* sp. PCC 6803; Nakamura *et al.*, 1998).

that histidine protein kinases regulate the level of response regulator phosphorylation through autophosphorylation and phosphatase activities. However, not all histidine protein kinases possess phosphatase activity, and thus, regulation occurs only via autophosphorylation (Borkovich *et al.*, 1989). Many response regulators also have autophosphatase activity, and this can decrease the lifetime of the phosphoprotein (Stock *et al.*, 2000). Some two-component systems also have specific modes of associated regulation. For example, some histidine kinases can phosphorylate more than one histidine residue, and competition for phosphoryl groups can influence the activation of different branches of the signalling pathway. The transfer of phosphoryl groups from the histidine-to-aspartate residues can also be regulated. In the hybrid histidine protein kinase VirA of *Agrobacterium tumefaciens*, the aspartate-containing domain modulates the phosphotransfer role of the histidine kinase domain through physical interactions with the autophosphorylation site (Stock *et al.*, 2000).

1.3.1. Two-component signal transduction in cyanobacteria

The prevalence of histidine protein kinases and response regulator homologues in the genome of *Synechocystis* sp. PCC6803 suggests a dependence on such mechanisms to sense its environment and adapt accordingly. A number of two-component signal transduction systems have now been characterised from different species of cyanobacteria. These include the phytochrome-like proteins *phy* (Hughes *et al.*, 1997) and *plpA* (Wilde *et al.*, 1997) of *Synechocystis* sp. PCC 6803, complementary chromatic adaptation (CCA) signalling proteins of *Fremyella diplosiphon*, *rcaC* (Chiang *et al.*, 1992), *rcaE* (Kehoe and Grossman, 1996), and *rcaF* (Kehoe and Grossman, 1997), and the phosphate sensing system of *Synechococcus* WH 7803, *phoB/phoR* (Watson *et al.*, 1996) (for a more detailed review of current knowledge concerning two-component signal transduction systems in cyanobacteria, see Mann (2000)). Little is currently understood concerning cross-talk between signalling pathways, or the physiological function of many two-component signalling proteins identified from the *Synechocystis* sp. PCC 6803 genome sequencing project. Insights into the function of these proteins will only be gained through analysis of the protein sequence and comparison with proteins of known physiological function, followed by experimental investigation.

1.3.2. Slr1212 and Slr1213

The predicted protein sequences of Slr1212 and Slr1213 suggests that these proteins interact together as a two-component signal transduction system, as described in section 1.3. Slr1212 codes for an orthodox histidine protein kinase (as opposed to a hybrid kinase such as ArcB) that would act in combination with Slr1213, which upon activation could effect an appropriate response to the stimulus sensed by Slr1212 by regulating the transcription of a set of genes.

As discussed in section 1.2, the predicted Slr1212 protein shares regions of similarity with ETR1 of *Arabidopsis thaliana*, PAS/PAC domains, GAF motifs, and histidine kinases. The region of similarity between the predicted Slr1212 protein and ETR1 (and the other members of the *Arabidopsis thaliana* ethylene receptor family, ERS, ERS2, ETR2 and EIN4) is within the hydrophobic ethylene-binding region (Schaller and Bleecker, 1995) (see Figure 1-6). Four residues have been identified in ETR1 of *Arabidopsis thaliana* as being critical for ethylene binding (A31, I62, C65, and A102), and it can be seen that the first three of these are conserved in Slr1212. In place of the fourth, alanine-102, is another hydrophobic amino acid, valine. Over a 94 amino acid section, the two proteins share 36.2% identity. This evidence strongly suggested that Slr1212 of *Synechocystis* sp. PCC 6803 may have a functional ethylene-binding domain (see section 1.4).

During the course of this study, ethylene-binding by Slr1212 was demonstrated by Rodriguez *et al.*, (1999). Using a whole-cell assay, cells were incubated with ^{14}C-ethylene, and subsequently aired. Ethylene was then collected in a mercuric perchlorate trap and subject to scintillation counting. It was shown that Δslr1212 cells lost the ethylene-binding activity inherent to wild-type cells.

Taken together, this evidence suggests that ethylene receptors may have evolved from a cyanobacterial ancestor. This hypothesis has been strengthened further by the identification of proteins from the unfinished genome sequences of *Anabaena* sp. PCC 7120 (http://www.kazusa.or.jp/cyano/*Anabaena*/) and *Nostoc punctiforme* A T C C 2 9 1 3 3 (http://spider.jgi-psf.org/JGI_microbial/html/*Nostoc*/*Nostoc*_homepage.html) that may contain ethylene-binding domains (May, this work, unpublished). These regions are very similar (an N-terminal 193 amino acid region has 50.8% sequence identity between Slr1212 and C192-2 of *Nostoc punctiforme*), and implies that these proteins may also

```
ETR1     1   ------------------------------MEVCNCIEPQ--WPADELLMKYQYISDFFIAIAYFSIPLE
ERS1     1   ------------------------------MESCDCFETH--VNQDDLLVKYQYISDALIALAYFSIPLE
ETR2     1   MVKEIASWLLILSMVVFVSPVL--AINGGGYPRCNCEDEGNSFWSTENILETQRVSDFLIAVAYFSIPIE
EIN4     1   MLRSLGLGLLLFALLALVS------GDNDYVSCNCDDEG--FLSVHTILECQRVSDLLIAIAYFSIPLE
ERS2     1   MLKTLLVQWLVFFFFFLIGSVVTAAEDDGSLSLCNCDDED-SLFSYETILNSQKVGDFLIAIAYFSIPIE
Slr1212  1   -----------MAITAFTLG-----------DFFQANSYIPHGHCYLWQTPLVVWLHVSADFFTAIAYYSIPLT
                                                                    A31V
```

```
ETR1    39   LIYFVKKSAVFP-YRWVLVQFGAFIVLCGATHLINLWTFT-THSRTVALVMTTAKVLTAVVSCATALMLV
ERS1    39   LIYFVQKSAFFP-YKWVLMQFGAFIILCGATHFINLWMFF-MHSKAVAIVMTIAKVSCAVVSCATALMLV
ETR2    69   LLYFVSCSNVP--FKWVLFEFIAFIVLCGMTHLLHGWTYS-AHPFRLMMAFIVFKMLTALVSCATAITLI
EIN4    62   LLYFISFSNVP--FKWVLVQFIAFIVLCGMTHLLNAWTYYGPHSFQLMLWLTIFKFLTALVSCATAITLL
ERS2    70   LVYFVSRTNVPSPYNWVVCEFIAFIVLCGMTHLLAGFTYG-PHWPWVMTAVIVFKMLTGIVSFLTALSLV
Slr1212 52   LLYFLRKRQDIP-FPNIIFLESTFILCCGTSHFFDIITLW----YPIYWISGTVKASMAIVSIITVFELI
                               I62F C65Y                                      A102T
```

```
ETR1   107   HIIPDLLSVKTRELFLKNKAAELDREMGLIRTQEETGRHVRMLT
ERS1   107   HIIPDLLSVKNRELFLKKKADELDREMGLILTQEETGRHVRMLT
ETR2   136   TLIPLLLKVKVREFM----------------------------
EIN4   130   TLIPLLLKWKVRELYLKQNVL-----------------------
ERS2   139   TLLPLLLKAKVR--------------------------------
Slr1212 117  QIVPNALNLKSPTELATLN-LALNQEIKERQTAEI---------
```

Figure 1-6 :- CLUSTAL W sequence alignment of the N-terminal ethylene binding region of the *Arabidopsis thaliana* ethylene receptor family and *Synechocystis* sp. PCC 6803 Slr1212. The four substitutions which cause dominant ethylene insensitivity in *Arabidopsis thaliana etr1* are highlighted. Three of the four critical amino acid residues are conserved in Slr1212. Residues that are conserved in all sequences are highlighted in red, identical residues in blue, and similar residues in gray.

be capable of binding ethylene (see Figure 1-7). It can be seen that the four mutant alleles of *Arabidopsis thaliana* ETR1 (see section 1.4) are conserved in the *Anabaena* and *Nostoc* proteins. No proteins with putative ethylene-binding regions were identified from the genome sequences of marine *Synechococcus* or *Prochlorococcus*. All of the identified proteins have histidine kinase domains and, like ETR1 of *Arabidopsis thaliana*, C360-2, 566, and 647 all have histidine receiving modules. The proteins 566, 598, and C360-2 also have PAS/PAC domains (598 has five PAC domains and four PAS domains). This family of multi-modular proteins, related to Slr1212 of *Synechocystis* sp. PCC6803 and ETR1 of *Arabidopsis thaliana* by virtue of similarity to the N-terminal ethylene-binding domain, would therefore seem to be highly conserved. Of the cyanobacterial proteins only Slr1212 has a GAF domain, which may indicate a dual sensing role in the perception of ethylene and light.

Insights into what the stimulus that Slr1212 might sense is gained through examination of the modular structure of the protein. The following sections discuss how the different domains present in Slr1212 may influence the sensory role this protein is proposed to have.

1.4. Ethylene signalling

The simple hydrocarbon ethylene was the first chemically identified endogenous regulator of plant growth and development (Bleecker, 1999). It is one of five naturally occurring plant hormones, the others being auxin, cytokinins, abscisic acid, and gibberellin (Kende and Zeevaart, 1997). Ethylene regulates a surprisingly wide variety of developmental and stress responses including leaf and flower senescence, seed germination, fruit ripening, and pathogen and wound responses (Chang, 1996). Such processes are dependent upon a plants ability to both produce ethylene, and to perceive it as a signal. There is a requirement for high-affinity ethylene-binding receptors because it has been shown that ethylene can have dramatic effects on plant growth at concentrations as low as 0.1 parts per million (ppm) in air (Zarembinski and Theologis, 1994)

Slr1212 1 - - M A I T A F T L G D F F Q A N S Y I P H G H C Y L W Q T P L V W L H V S A D F F T A I A Y Y S I P L T L L Y F L R K R Q D I P F P N I I
C192-2   1 - - - - M L E F I H - N I F A D N L F I P H G H C Y L W Q P G L V W L H L L S D F L I A V A Y Y S I P I T L I Y F V R K R Q D L P F K W I F
C360-2   1 - - - - M S E L W T - Y F F N S T P F I P H G H C Y L W Q T D L V W L H I I S D G L I A L A Y Y S I P A T L F Y F V R K R Q D L P F Y W I F
566   1 M S E I I S E F W T - N F F T S G S F I P H G H C Y L W Q T N L V W L H I L S D A F I A L A Y Y S I P A T L F Y F V R K R Q D L P F D W I F
598   1 - - - - M L E F W K - S L F A Y K Q F I P H G H C Y L W K P E L V G L H I V S D S L I A L A Y Y S I P I A L V Y F V R K R Q D F P F N W I F
647   1 - - - M M Q T F F N - Y P F Q S N G F I P H G H C Y L W Q T G L V W L H I I S D A T I A L A Y Y S I P F L L I Y F I S K R K D V P F N G V F

A31

Slr1212 69 F L F S T F I L C C G T S H F F D I I T L W Y P I Y W I S G T V K A S M A I V S I I T V F E L I Q I V P N A L N I K S P T E L A T L N L A L
C192-2   66 L L F G A F I I S C A T S H V M E I W T L W H P T Y W L S G F M K A I T A F I S I Y T A I V L V P I V P Q L L A L P S P A Q L E A A N N Q L
C360-2   66 L L F S G F I V A C G T T H I M E V W T L W Y P T Y W V S G F L K A I T A I I S V F T A L T L I P L V P K A L L L P S S A Q L E R A N K D L
566   70 L L F S A F I V A C G T T H L I E I W T L W H P T Y W V S G F V K A V T A M I S V I T A V Q L V S L V P Q A L A L P S P A Q I E Q A N Q E L
598   66 L L F A T F I I T C G T T H L M D I W T L W Y P T Y W L S G C I K A I T A I I S L Y T A S E L I P L I P K A L A L P S H A Q L E I A N Q E L
647   67 L L F G A F I A C G T G H L M D I W T L W Y P D Y W I A G G L K A L T A I I S I Y T A F A L F Y L M P Q A L A L P S P D Q L E A I N R I L

I62 C65   **A102**

Slr1212 139 N Q E I K E R Q T A E I - - -
C192-2   136 K I E I I E R K T A E A A L L
C360-2   136 Q N E I G E R L K V E A E L R
566   140 Q T Q I A E R L R I E - - - -
598   136 E R E I S I R K Q A E L A L Q
647   137 S T E I V E R K R I E K E L -

Figure 1-7 :- CLUSTAL W sequence alignment of the N-terminal ethylene binding region of *Synechocystis* sp. PCC 6803 Slr1212 with two ORFs identified from the sequence of *Anabaena* sp. PCC 7120 (C192-2 and C360-2) and three ORFs identified from the sequence available for *Nostoc punctiforme* (566, 598, and 647). Residues that are conserved in all sequences are highlighted in red, identical residues in blue, and similar residues in gray. The residues critical for ethylene binding by ETR1 of *Arabidopsis thaliana* are marked.

1.4.1. Ethylene perception

The study of the biochemical and genetic processes underlying ethylene signalling in higher plants has been greatly facilitated by the triple response screen, which has allowed the identification of a wide range of ethylene-response mutants. First identified by Neljubow (1901), the 'triple response' refers to a set of morphological alterations to etiolated seedlings that normally occur in the presence of ethylene. In *Arabidopsis thaliana*, the triple response consists of radial swelling of the hypocotyl, retention and accentuation of the apical hook, and inhibition of hypocotyl and root elongation (Alonso *et al.*, 1999). This alteration in growth pattern facilitates penetration of the soil, whilst protecting the delicate shoot apex (Harpham *et al.*, 1991). Mutants insensitive to ethylene lack these responses.

Using this screen, a dominant mutant was identified that was insensitive to ethylene. The *ETR1* gene was cloned by chromosome walking, and was found to encode a protein with similarity to components of prokaryotic two-component signal transduction systems (Chang *et al.*, 1993). Four mutant alleles were identified, each containing a missense mutation towards the novel N-terminus (A31V, I62F, C65Y and A102T) (Fluhr and Mattoo, 1996). Early evidence showed that the ethylene-binding in *etr1-1* (C65Y) plants was approximately 20% that of wild-type *Arabidopsis thaliana* (Bleecker *et al.*, 1988). The indication of this evidence therefore was that the ETR1 protein may act as an ethylene receptor. This was later demonstrated when the ETR1 protein was expressed in transgenic yeast, which then showed saturable binding of ethylene. Following expression of a mutant ETR1 (*etr1-1*) protein, the yeast was unable to bind ethylene. Furthermore, by expressing truncated ETR1 proteins in transgenic yeast, it was shown that the ethylene-binding domain was located in the novel N-terminus of the protein (Schaller and Bleecker, 1995). The fact that ethylene-binding in *Arabidopsis thaliana etr1-1* plants was not reduced to zero relative to wild-type plants, but was in transgenic yeast expressing the *etr1-1* gene, implied the presence of other ethylene receptors in *Arabidopsis thaliana*.

The carboxy-terminus of *Arabidopsis thaliana* ETR1 contains a histidine kinase domain and a response regulator domain, common to two-component signal transduction systems (see section 1.3). This modular structure is similar to that of

many bacterial histidine kinases, incorporating a sensing domain at the N-terminus and signalling domains at the C-terminus. It therefore seemed likely that an early step in ethylene signal transduction could involve the transfer of a phosphoryl group as in two-component signal transduction systems (Chang *et al.*, 1993). Immunological analysis demonstrated that the ETR1 protein exists as a membrane-associated, disulphide-linked dimer (Schaller *et al.*, 1995). Acting in this manner in a signalling pathway was consistent with the hypothesis that signal transduction may involve a phosphotransfer reaction common to two-component signal transduction systems, as histidine kinases also function as dimers (see section 1.3). It has since been demonstrated that the ETR1 protein of *Arabidopsis thaliana* does have histidine kinase activity, and that ethylene signalling pathways may be regulated by changes in the histidine kinase activity of the protein (Gamble *et al.*, 1998).

Using the triple response screen or heterologous hybridisation other ethylene receptors were subsequently identified. These included ERS1 (ethylene response sensor) which was identified by cross-hybridisation with ETR1, and also possesses a histidine kinase domain (but not a receiver domain) (Hua *et al.*, 1995), and ETR2, identified using the triple response screen (Sakai *et al.*, 1998). Using *ETR2* as a probe two further related genes were isolated, *ERS2* and *EIN4* (Hua *et al.*, 1998). All of the identified proteins were found to share homology in the N-terminal region shown to be responsible for ethylene-binding (Schaller and Bleecker, 1995).

The ethylene receptor family of *Arabidopsis thaliana* can be separated into two distinct subfamilies on the basis of structural differences. The first family, containing ETR1 and ERS1, have three hydrophobic domains at the N-terminus and a histidine kinase domain. The second family, which consists of ETR2, ERS2, and EIN4, is characterised by proteins which have an extra hydrophobic domain at the N-terminus and have degenerate histidine kinase domains that lack at least one of the catalytic domains generally accepted to be necessary for function (ETR2 contains only the G box, EIN4 contains only the H box, and ERS2 lacks all of the critical residues for histidine kinase activity) (Bleecker, 1999). ETR1, ETR2 and EIN4 are all hybrid histidine kinases, containing a receiver domain in addition to a histidine kinase domain. All of the *Arabidopsis thaliana* ethylene receptors also contain GAF domains (as does Slr1212). Slr1212 does not quite fit into either family, having

five predicted transmembrane helices located N-terminally, with the majority of the residues present considered necessary for histidine kinase activity. Interestingly given the divergence of the ethylene receptor family in *Arabidopsis thaliana*, some lacking conserved residues necessary for histidine kinase activity, it has been hypothesised that histidine kinase activity is not required for responses typically associated with ethylene signal transduction (Gamble *et al.*, 1998).

1.4.2. Ethylene signal transduction

As discussed, the identification of a putative two-component signal transduction pair from *Synechocystis* sp. PCC 6803 that potentially senses ethylene raises the possibility that an ethylene signalling pathway exists in cyanobacteria. Given that the ethylene receptor family of *Arabidopsis thaliana* encodes proteins all of which contain remnants of two-component signalling pathways, knowledge to date concerning the signal transduction pathway in *Arabidopsis thaliana* is discussed here.

The triple response screen has proved to be a very powerful tool in the identification of mutants defective in the ethylene signalling pathway. Different classes of mutants have been isolated using this approach: those that fail to display the triple response in the presence of exogenous ethylene (insensitive) e.g. *etr1* (ethylene response 1), *ein2* (ethylene insensitive 2), *ein3*, *ein4*, *ein5*, *ein6*, *ein7*, and *ain1* (ACC insensitive 1), and those that constitutively display the triple response in the absence of ethylene e.g. *ctr1* (constitutive ethylene response 1), *eto1* (ethylene overproduction 1), *eto2*, and *eto3* (Theologis, 1998).

Ethylene receptors appear to act when not bound to ethylene. A lack of ethylene receptor activity leads to a constitutive ethylene response, that is to say, when ethylene is absent, the receptors are in the 'on' state (Theologis, 1998). How ETR1 transduces this signal is not currently understood. ETR1 has been shown to possess autophosphorylation activity at a histidine residue in its conserved histidine kinase core *in vitro* (Gamble *et al.*, 1998). However, phosphotransfer to the receiver domain has not yet been demonstrated (Chang and Shockey, 1999).

Using the triple response approach ethylene insensitive (Bleecker *et al.*, 1988) and constitutively active mutants were readily identified. Mutations at the

CTR1 loci caused a constitutive ethylene response phenotype, indicating that CTR1 acts as a negative regulator of response pathways (Kieber *et al.*, 1993). Epistasis (double mutant) studies have shown that CTR1 acts downstream of ETR1 in the signalling pathway (Hua and Meyerowitz, 1998). The regulatory domain of CTR1 has been shown to associate with the cytoplasmic portions of ETR1 and ERS1 in yeast *in vitro* (Clark *et al.*, 1998). CTR1 is a serine/threonine protein kinase, related to mammalian RAF kinases that initiate MAP-kinase cascades, which in turn often regulate transcription factors (Bleecker, 1999). Double *CTR1/EIN3* mutants have an ethylene-insensitive phenotype, indicating that EIN3 acts downstream of CTR1. The *EIN3* family of transcription factors are required for ethylene signalling (Chao *et al.*, 1997). EIN2 is required for CTR1 signalling to the nucleus. *EIN2* encodes a novel integral membrane protein with similarity to the Nramp family of metal-ion transporters, but has no detectable metal transport activity (Chang and Shockey, 1999). Genetic studies place EIN3 downstream of EIN2 in the signalling cascade (Bleecker and Kende, 2000).

Ethylene signalling in plants produces significant alterations in gene expression. A group of DNA-binding proteins identified as regulating ethylene-mediated transcription includes the ethylene-responsive element binding protein family (Solano *et al.*, 1998). It is now known that a member of this family, *ERF1* is a target for up-regulation by EIN3 (Solano *et al.*, 1998). *ERF1* is rapidly expressed in response to ethylene and overexpression confers a subset of constitutive ethylene responses (Bleecker and Kende, 2000). Current knowledge is summarised in Figure 1-8.

Thus, current knowledge does not fit with a two-component signal transduction system as is hypothesised to exist in cyanobacteria. However, a possibility remains that there is an as yet unidentified response regulator which could also interact with the ethylene receptor family. This seems unlikely however, given the extensive screening of mutant libraries with the triple response screen.

1.4.3. Ethylene-binding and copper

It had long been hypothesised that ethylene-binding by receptors would be mediated via coordination to a metal group. This was based on a positive correlation between
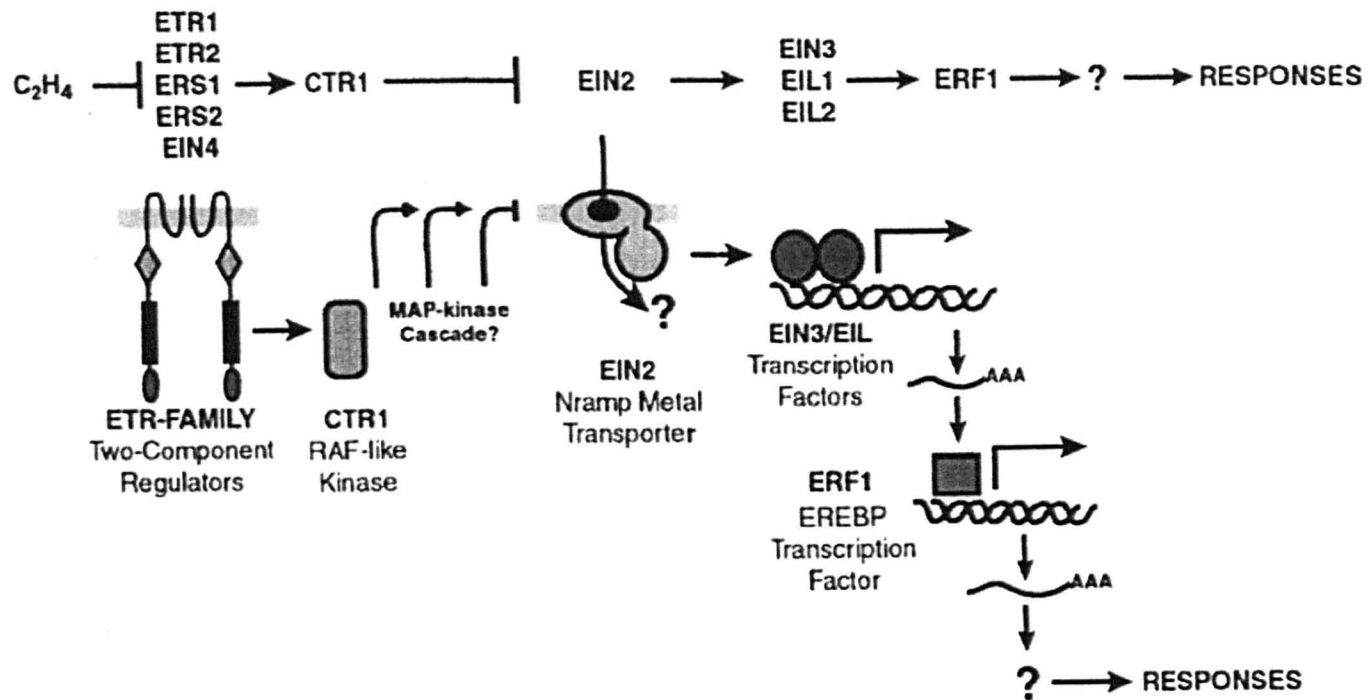
Figure 1-8 :- Summary of the ethylene signal transduction pathway in *Arabidopsis thaliana*. Ethylene negatively regulates membrane-associated receptors. The histidine-kinase domains of these receptors interact with CTR1, which in turn negatively regulates EIN2. EIN2 positively signals to the EIN3 family of transcriptional regulators in the cell nucleus. A target of EIN3, ERF1, activates trancription of a subset of genes involved in a series of ethylene responses (taken from Bleecker, 2000).

the effectiveness of ethylene and related agonists (such as CO and acetylene) to bind these receptors and their capacity to interact with silver complexes, prompting the hypothesis that the ethylene-binding site contains a transition metal (Burg and Burg, 1967). Using an assay based on that first developed by Sisler (1979), it has now been demonstrated that ethylene-binding by *Arabidopsis thaliana* ETR1 is mediated by a copper ion (Rodriguez *et al.*, 1999). It was seen that the addition of copper sulphate enhanced ethylene-binding by ETR1 10-20 fold, and that the missense *etr1-1* mutant allele (C65Y) caused complete loss of ethylene-binding activity. This residue was also found to be essential for copper binding. *In vitro* mutagenesis, topological analysis and sequence comparison between *Arabidopsis* ETR1 and Slr1212 were used to formulate a model in which the Cu(I) cofactor is embedded in a hydrophobic, electron-rich pocket formed by three membrane spanning alpha helices. Ligands for copper binding were provided by the cysteine-65 and histidine-69 residues in the second alpha helix (Esch *et al.*, 1998). It is assumed that ethylene-binding alters the coordination chemistry of the copper that causes an alteration in the conformation of the binding site, which is then propagated to the signalling portion of the ETR1 dimer (Bleecker, 1999).

The role of copper in ethylene perception has been further implicated following the identification of *RAN1* in *Arabidopsis thaliana*. This gene was shown to encode a P-type ATPase copper transporter, and could complement a copper transport mutant in yeast. It has been hypothesised that RAN1 acts to deliver copper to ethylene receptors at the post-golgi (Hirayama *et al.*, 1999).

It has also been reported that Δslr1212 mutants are more resistant to copper toxicity than wild-type *Synechocystis* sp. PCC 6803 and that this indicates a possible role for slr1212 in copper scavenging. It was suggested that Slr1212 functions as a copper sensor that fortuitously has the correct chemistry to bind ethylene, and that the N-terminal ethylene-binding region was recruited in evolution to serve as the input domain in plant ethylene receptors (Esch *et al.*, 1998).

1.4.4. Ethylene biosynthetic pathways

It has already been demonstrated that the Slr1212 protein of *Synechocystis* sp. PCC 6803 is capable of binding ethylene (Rodriguez *et al.*, 1999). The discovery of the presence of ORFs potentially encoding other proteins with ethylene-binding

domains from *Nostoc punctiforme* and *Anabaena* sp. PCC 7120 (see section 1.3.2) implies that there may be a conserved ethylene signalling mechanism in cyanobacteria. Furthermore, the existence of the ethylene receptor family of *Arabidopsis thaliana* which all contain modules unique to two-component signalling pathways could imply a common ancestor for these proteins in the cyanobacterial lineage. The study of any ethylene signalling pathways in these genetically amenable prokaryotes may allow the study of a model signalling system which can then be applied to further study in higher plants. It has been reported elsewhere that *Synechocystis* sp. PCC 6803 produces no detectable ethylene and has no known responses to applied ethylene (Bleecker, 1999). Furthermore, it has also been reported that *Synechococcus* sp. PCC 7942 R2-SPc does not produce detectable levels of ethylene (Fukuda *et al.*, 1994).

Although the implication from the data concerning ethylene-binding by Slr1212 is that there may be an ethylene signalling system in cyanobacteria, such a pathway would also probably require a biosynthetic mechanism. Furthermore, a role for ethylene in regulating cell physiology in some manner would need to be characterised. This is more difficult than the analysis first carried out in higher plants to isolate mutants defective in the ethylene signalling pathway, where the triple response screen was applied to scan large libraries of random mutants to isolate those plants insensitive to ethylene. In *Synechocystis* sp. PCC 6803 however, no role for ethylene has currently been assigned and so such an approach is of no value.

1.4.4.1. Ethylene biosynthesis in higher plants

In plants, the biosynthesis of ethylene is regulated by a wide variety of environmental and developmental stimuli including wounding, flooding, temperature stress, pathogenic attack and pollination (Fluhr, 1998). The biosynthetic pathway of ethylene in higher plants has been well characterised. It is formed by the Yang cycle via *S*-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1977). AdoMet synthetase catalyses the formation of AdoMet from L-methionine. The conversion of AdoMet to the cyclic nonprotein amino acid ACC, and of ACC to ethylene is catalysed by the enzymes ACC synthase and ACC oxidase respectively (Kende, 1993). ACC synthase can also lead to the

formation of 5'-methylthioadenosine, which can be used to synthesise a new methionine molecule via a modified methionine cycle, and it is in this manner that a high rate of ethylene biosynthesis can be maintained even when the pool of free methionine is small (Bleecker and Kende, 2000).

The activity of ACC synthase has been found to be the rate-limiting step in ethylene synthesis (Zarembinski and Theologis, 1994), and is enhanced by factors which promote ethylene formation (Yang and Hoffman, 1984). It is a cytosolic enzyme and represents the first committed step in the biosynthetic pathway. The enzyme is encoded by multi-gene families in all plant species so far studied (Bleecker and Kende, 2000). The emerging picture following the cloning of this gene family from many plant species is that each gene is differentially regulated by a subset of inducers (Liang *et al.*, 1992; Zarembinski and Theologis, 1994). Sequential expression of two ACC synthase genes was observed in response to biotic and abiotic stresses in potato (Schlagnhaufer *et al.*, 1997).

ACC can be converted to ethylene, carbon dioxide and hydrogen cyanide by ACC oxidase, or to N-malonyl-ACC (MACC) by malonyl transferase. The conversion of ACC to MACC could be a regulatory step in ethylene biosynthesis by inactivating ACC (Yang and Hoffman, 1984). It has also been demonstrated that ACC oxidase genes are differentially expressed in flowers (Barry *et al.*, 1996). Furthermore, it has been shown that distinct signal transduction pathways can influence the expression of an ACC oxidase gene of melon following wounding and ethylene treatment (Bouquin *et al.*, 1997).

Ethylene production by many plants is light-dependent. In a study of *Tillandsia usneoides* L., it was observed that ethylene production was strictly light-dependent and no endogenous rhythm could be detected (Bessler *et al.*, 1998). In contrast, a recent study with *Arabidopsis thaliana* showed that ethylene was produced in a circadian manner under constant conditions (Millar and Thain, 2000). Circadian production of ethylene has also been demonstrated in *Sorghum bicolor* L. Moench (Finlayson *et al.*, 1999).

Ethylene biosynthesis is therefore regulated mainly through transcriptional control of the genes encoding the biosynthetic enzymes, potentially mediated by different signal transduction and gene regulatory mechanisms. ACC synthase and ACC oxidase homologues have not however been identified in the annotated genome sequence of *Synechocystis* sp. PCC 6803. Analysis of the genome carried out in this study, using BLAST searches with the ACC synthase and ACC oxidase protein sequences from *Arabidopsis thaliana* appear to confirm this absence (homologues were also not identified in *Anabaena* sp. PCC 7120 or *Nostoc punctiforme* ATCC 29133).

1.4.4.2.   Ethylene biosynthesis by microbes

The formation of ethylene by microorganisms is a widely recognised phenomenon. Indeed, it has been hypothesised that perhaps all species of bacteria and fungi may be capable of ethylene production (Primrose, 1979). The threshold limit of ethylene sensitivity for plants varies dependant upon species, but generally the limit is 10 ppb, half-maximal effect at 0.1 ppm, and maximal effect at 1 to 10 ppm (Elsgaard, 1998). Therefore, even low levels of ethylene production by microorganisms may impact upon plant development.

The first report of ethylene production by a microbe was by Gane (1934) with bakers yeast. The first observation of ethylene production by bacteria was in banana fruits infected with *Pseudomonas solanacearum* (Freebairn and Buddenhagen, 1964). A hypothesis that microbial ethylene production could cause symptoms of plant disease was formed (Primrose, 1979). Little is still known about the role of microbially produced ethylene in plant pathogenicity, although it has been shown that increased levels of ethylene production by diseased plant tissue infected with *Pseudomonas syringae* was produced by the bacteria (Weingart and Volksch, 1997).

Two main pathways via which ethylene biosynthesis may proceed in microorganisms have been described. The first pathway, carried out by a group of organisms referred to as methionine-dependent ethylene-producing microorganisms including *Escherichia coli* (Ince and Knowles, 1985; Primrose, 1976), proceeds via L-methionine and 2-oxo-methyl-thiobutyric acid (KMBA) (Ince and Knowles, 1986),

converted by a NADH:Fe(III)EDTA oxidoreductase (Fukuda *et al.*, 1989). The last step of this reaction occurs nonenzymatically (Weingart and Volksch, 1997). The second pathway, carried out by 2-oxoglutarate-dependent ethylene-producing organisms such as *Penicillium digitatum* (Fukuda *et al.*, 1989), proceeds via 2-oxoglutarate which is converted to ethylene by an ethylene-forming enzyme. This enzyme has been cloned from several bacterial species (Fukuda *et al.*, 1992), though again no homologue can be found in the *Synechocystis* sp. PCC 6803 genome (or in the genomes of *Anabaena* sp. PCC 7120 or *Nostoc punctiforme*). Most reported microorganisms produce ethylene via KMBA, with the notable exceptions of *Penicillium digitatum*, *Penicillium cyclopium* (Fukuda *et al.*, 1989) and *Pseudomonas syringae* (Nagahama *et al.*, 1991).

Despite much speculation regarding the role of ethylene in the microbial lifestyle, the significance of its production is not well understood. One hypothesis is that the formation of KMBA may allow the recovery of nitrogen from methionine (Ince and Knowles, 1985). Another hypothesis is that KMBA formation from methionine could allow the cell to quench excess oxygen radicals produced in aerobic conditions (ethylene is not formed in anaerobic conditions by *E. coli*) (Mansouri and Bunch, 1989).

More recently a bacterial isolate from compost, *Bacillus* sp. ALK-7, was shown to be able to produce ethylene from the plant ethylene precursor ACC. Production was also efficiently induced by L-methionine, and it was assumed that ACC was derived from L-methionine by a bacterial ACC synthase (Bae and Kim, 1997).

Furthermore, a study with *Hapalosiphon*, a branching nitrogen-fixing cyanobacterium, has demonstrated the ability to produce ethylene when cultivated on synthetic media or on the surface of soil. It was found that ethylene production proceeded at a constant rate when cells were illuminated, but activity was lost in a dark environment. It was also observed that the addition of methionine and ACC substantially increased the release of ethylene by this organism, suggesting the presence of a plant-like biosynthetic pathway (Huang and Chow, 1984). Another study with the green alga *Chlorella pyrenoidosa* Pringch 82T found that ethylene production was regulated by red/far-red light, presumably mediated by a

phytochrome-like photoreceptor and was also affected by the addition of exogenous cytokinins, linuron and kinetin (Kreslavsky *et al.*, 1997).

1.5. Light Sensing

The modular protein structure of Slr1212 that has been determined from the genome sequence of *Synechocystis* sp. PCC6803 has implied that this protein may have a role in sensing the light environment as well as ethylene signalling, acting in a two-component signalling pathway. This is based on BLAST searching which has revealed the presence of a GAF domain towards the centre of the primary sequence, and multiple PAS/PAC domains.

1.5.1. PAS/PAC domains

Often, cytosolic sensing domains are integrated into histidine protein kinases. A common example are PAS domains, characterised by two imperfect repeats. PAS is an acronym named after the proteins in which these imperfect repeat sequences were first recognised: the *Drosophila* period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and *Drosophila* single-minded protein (SIM) (Taylor and Zhulin, 1999). It has also been reported that similar 40-45 amino acid regions (PAS-associated (PAC) motifs) are often located carboxy-terminal of PAS sequences and are likely to contribute to the PAS structural domain (Ponting and Aravind, 1997). These versatile, approximately 100 amino acid, signalling modules can monitor changes in light, redox potential, oxygen, small ligands, and overall energy level of a cell (Taylor and Zhulin, 1999). The parameter monitored is dependent upon the associated cofactor (Stock *et al.*, 2000). For example, the PAS domain of FixL associates with a heme group and binds oxygen, and this mediates the kinase activity of the protein (Miyatake *et al.*, 1999). Bacterial photoactive yellow protein associates with *p*-hydroxycinnamic acid (Borgstahl *et al.*, 1995), and flavin adenine mononucleotide (FAD) associates with the bacterial redox potential sensor NifL (Soderback *et al.*, 1998).

PAS/PAC domains have been reported in proteins from diverse organisms including mammals, insects, plants, fungi and cyanobacteria, and are usually paired with a repeat domain and have an involvement with protein-protein interactions

(Zhulin *et al.*, 1997). However, despite their widespread nature PAS domains are not present in all organisms. The analysis of bacterial genomes has identified PAS/PAC homologues in *E. coli*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, and *Bacillus subtilis*, but not in *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, or *Helicobacter pylori* (Ponting and Aravind, 1997). Indeed, out of eleven microbial genomes analysed five contained no PAS domains (Taylor and Zhulin, 1999). The genome of *Synechocystis* sp. PCC6803 encodes 61 PAS/PAC domains, the largest number yet identified in one organism. This may be a reflection of the complexity of the organism, containing photosynthetic and respiratory electron transport pathways. A positive correlation has been identified between the total number of PAS domains an organism possesses and the abundance of the respiratory and photosynthetic electron transport-associated proteins (Zhulin and Taylor, 1998). This is highlighted by animal parasites, which have the lowest incidence of electron transport proteins, and contain no PAS domains, and *Synechocystis* sp. PCC 6803 which contains 61 PAS/PAC domains, whose survival is aided through sensing light, redox potential, and oxygen (Taylor and Zhulin, 1999).

PAS domains are also important in photoreceptors and clock proteins. Photoactive yellow protein was originally identified in *Ectothiorhodospira halophila* (Baca *et al.*, 1994). It consists of a single PAS domain linked via a thioester linkage at a cysteine residue to a 4-hydroxycinnamyl chromophore. Plant phytochromes attach to a linear tetrapyrrole chromophore, also at a conserved cysteine residue. This receptor region is separated from two PAS domains by a hinge region (Quail *et al.*, 1995). It has been suggested that the PAS domains may transduce the light signal to regulate the kinase activity (Taylor and Zhulin, 1999) (phytochromes have a histidine kinase-like domain which has serine/threonine kinase activity (Yeh and Lagarias, 1998)). PAS domains have also been identified in protein components of the circadian clock mechanisms from mice, flies, and fungi (Millar, 1997).

Many PAS proteins mediate signal transduction through protein-protein interactions, and the PAS core probably engages in PAS-PAS interactions (Huang *et al.*, 1993). In PAS containing proteins such as the dioxin receptor and ARNT, the basic helix-loop-helix (bHLH) is sufficient for heterodimerisation to occur. However, the PAS domain is essential to confer dimerisation specificity to the dioxin receptor (Pongratz *et al.*, 1998). A possible role for PAS domains in the dimerisation

of plant phytochrome has also been suggested (Edgerton and Jones, 1993). However, it has also been suggested that the role of PAS-mediated dimerisation in signal transduction may have been overemphasised, based on the evidence that the PAS domains of FixL and HERG are monomeric (Taylor and Zhulin, 1999).

The majority of PAS domains identified in prokaryotes occur in members of the histidine protein kinase family (Zhulin and Taylor, 1998). An example is found with the FixL/FixJ oxygen sensing two-component pair of *Sinorhizobium meliloti.* In this pathway FixL acts as an oxygen sensor, and dissociation of oxygen from the PAS domain causes altered conformation and increased autophosphorylation activity of the transmitter domain (Gong *et al.*, 1998). Numerous examples exist (Zhulin *et al.*, 1997), stressing the importance of PAS domains in two-component signal transduction systems.

The fact that Slr1212 is one of a group of proteins identified from the *Synechocystis* sp. PCC 6803 genome containing PAS/PAC domains provides interesting insights into a possible physiological role for this protein. Taken in combination with the fact that there is also a GAF motif present in the predicted protein, this could imply a role in sensing the light environment (see section 1.5.2).

1.5.2. GAF domains

GAF (named after their presence was identified in cGMP-regulated cyclic nucleotide phosphodiesterases, adenyl cyclases, and FhlA, a bacterial response regulator) domains are homologous domains identified in plant and cyanobacterial phytochromes, and vertebrate and invertebrate 3', 5' cyclic guanosine monophosphate (cGMP)-stimulated phosphodiesterases (Aravind and Ponting, 1997). Over ninety such domains have been identified in current databases, and one of the characterised functions is that of cGMP binding (Charbonneau *et al.*, 1990). GAF domains are structurally similar to PAS domains, and both have been identified in phototransducing proteins of diverse genera (Ho *et al.*, 2000).

Phytochromes, a red/far red light sensing protein family, constitute one of the largest families of proteins which contain GAF domains (Ho *et al.*, 2000). Light is sensed via a linear tetrapyrrole covalently linked to a conserved cysteine residue within the GAF domain (Quail, 1997). Most GAF domains however lack this conserved

cysteine residue required for chromophore attachment (Ho *et al.*, 2000), although this residue appears to be conserved in Slr1212 (it is not present in ETR1 of *Arabidopsis thaliana*) (Bleecker, 1999). It is therefore pertinent to consider a role for the Slr1212 protein in both ethylene and light signalling mechanisms. The lack of a conserved cysteine residue in other GAF-containing proteins does not preclude the possibility of a role in light sensing. It has been shown with BphP of *Deinococcus radiodurans*, a phytochrome-like protein, that the chromophore attaches through a Schiff's base to a histidine residue (Davis *et al.*, 1999). The presence of Slr1211 immediately upstream of Slr1212 (see section 1.2), potentially encoding for a protein involved with tetrapyrrole biosynthesis, may also suggest the involvement of Slr1212 with light sensing. Slr1211 is a homologue of CobN of *Pseudomonas denitrificans*, a protein involved with the biosynthesis of the tetrapyrrole cobalamin. The tetrapyrroles are a group of compounds whose molecules have four rings of the pyrrole type, and as discussed in section 1.5.4, some are crucial for phytochrome function.

The photosynthetic lifestyle of *Synechocystis* sp. PCC 6803 implies that light sensing mechanisms will be of crucial importance to maximise survival and growth. Phytochrome signalling in higher plants is therefore summarised here, and the evolution of this group of light sensors is discussed.

1.5.3. Plant light sensors

Plants must have the ability to rapidly sense and adapt to an altering light environment. Fluctuations in day length, spectral quality, light intensity and direction all represent factors for adaptation. Sensing mechanisms allow photosynthetic organisms to maximise light-harvesting, whilst preventing light-induced cellular damage, of particular importance in plants as they are not motile. In order to carry out this function, higher plants have evolved at least two distinct families of phototransducing proteins.

Phytochromes regulate responses to the spectral quality of light as determined through measurement of the ratio of red to far-red light. Blue/UV-A photoreceptors (cryptochromes) detect the quantity of blue light. Also, responses to UV-B (280-315 nm) light have been observed, although a specific UV-B receptor has not yet been

isolated (Ballare, 1999). A flavoprotein, phototropin (encoded by *NPH1*), with the properties of a photoreceptor has also recently been identified, and regulates phototropism (Briggs and Huala, 1999; Huala *et al.*, 1997). Together, these photoreceptors allow plants to sense and respond to alterations in the quality, quantity, and spatial patterns of the light environment (Nagy and Schäfer, 1999).

1.5.4. Phytochrome signalling

Members of the bacterial, plant, and animal kingdoms sense light through the use of photoreceptors. In every case, such a receptor is associated with an organic chromophore, and upon absorption of a photon the chromophore undergoes a simple *cis*- to *trans* isomerisation around a double bond (Essen and Oesterhelt, 1998). This causes conformational alterations which can cause activation or inactivation of signalling pathways.

Phytochrome is a ubiquitous light-sensing protein in higher plants. These sensors are sensitive to alterations in the ratios of red and far-red in the incident light. The quantity of red light in daylight is determined by many environmental factors including canopy position, cloud cover and time of day (Jones and Edgerton, 1994).

When bound to a heme-derived linear tetrapyrrole chromophore, these receptors are photointerconvertible between an active Pfr (far-red absorbing) and inactive Pr (red absorbing) form (Quail *et al.*, 1995), and are synthesised in the Pr form (Batschauer, 1998). Red light causes the conversion of phytochrome to the active far-red absorbing form. Far-red light converts Pfr to inactive Pr. Phytochrome photoconversion is accompanied by alterations in the absorbance maxima of purified protein; Pr absorbs maximally at 666 nm and Pfr at 730 nm (Quail, 1997). In daylight, it can take several minutes for an equilibrium to be reached between Pr and Pfr. Radiation below approximately 700 nm causes the photoconversion of both Pfr and Pr, and in daylight a ratio of around 60% Pfr/P (P is total phytochrome) is established (Smith, 2000). Many morphological alterations are mediated by phytochrome including seed germination and leaflet movement (Lagarias *et al.*, 1995).

Phytochrome was first discovered in the 1950s and has been the subject of intense scrutiny since. Thus far, in all of the plant species studied, phytochrome has been found to be encoded by a small family of divergent genes (Ballare, 1999). In *Arabidopsis thaliana*, this gene family consists of at least five genes: *PhyA*, *PhyB*, *PhyC*, *PhyD*, and *PhyE* (Clack *et al.*, 1994). An additional *PhyF* has been identified in tomato (Herdman *et al.*, 2000). PhyA tends to be the most abundant phytochrome in etiolated plant tissue, whilst PhyB-E are less abundant (Whitelam and Devlin, 1997).

All plant phytochrome proteins are similar in structure. Phytochromes are homodimers of approximately 124 kDa (Whitelam and Devlin, 1997). The chromophore, an open-chain linear tetrapyrrole molecule (phytochromobilin in plants), is covalently linked to a conserved cysteine residue within the GAF domain (Quail, 1997). Each phytochrome monomer separately attaches to a single chromophore molecule, and upon *cis-trans* isomerisation of the chromophore alterations in the protein conformation occur which generate the Pr and Pfr forms of phytochrome (Sharma, 2001). Phytochromes consist of two structural domains, a globular N-terminal domain and a linear C-terminal domain (Smith, 2000). Chromophore attachment and spectral integrity are associated with the N-terminal domain, whilst dimerisation sites are located in the C-terminus (Quail *et al.*, 1995).

Evidence has shown that whilst different members of the phytochrome family have some distinct roles, there is a degree of inherent redundancy. Current knowledge suggests that PhyB is the major phytochrome species regulating responses to red/far-red light. The functioning of PhyD and PhyE can only be observed in a PhyB deficient background, illustrating the redundancy that exists in the phytochrome family (Sharma, 2001).

1.5.5. Phytochrome action

Many investigators believed that phytochrome acts to modulate gene transcription of a subset of genes in response to an altering ambient light environment. A recent study identified a number of PhyA˙ regulated genes in *Arabidopsis thaliana* using a fluorescent differential display screen. Indeed, through the analysis of a PhyA/PhyB

double mutant, overlapping roles for these proteins in regulating gene expression were identified (Kuno *et al.*, 2000).

The mode of action of phytochrome has been the subject of intense study, and only recently is this becoming understood. It has long been hypothesised that phytochrome may act via protein kinase activity, as the C-terminal domain of phytochrome has homology with histidine kinases of two component systems (Schneider-Poetsch *et al.*, 1991). Further support for this hypothesis was gained following the sequencing of the *Synechocystis* sp. PCC 6803 genome, which revealed the presence of several ORFs encoding putative phytochrome-like proteins (Kaneko *et al.*, 1996*a,b*).

One of these ORFs, slr0473 (*cph1*), shares 30% identity with the N-terminal region of plant phytochrome including the chromophore attachment region (Batschauer, 1998), and possesses a histidine kinase domain situated C-terminally.

Following overexpression of *cph1* in *E. coli* it was demonstrated that purified apoprotein could autocatalytically associate with phycocyanobilin, and that this holoprotein was red/far-red photoreversible (Hughes *et al.*, 1997).

A subsequent study demonstrated that Cph1 was a light-regulated histidine kinase, and that this autophosphorylation activity was greater in the Pr form (i.e. following exposure to far-red light). This is in contrast to plant phytochrome, in which Pfr is regarded as being the active form. A downstream gene, *rcp1* (*response regulator for cyanobacterial phytochrome*), was identified as encoding a putative response regulator in the signalling system, and was shown to be transphosphorylated by Cph1 in a light dependent manner (Yeh *et al.*, 1997). This data suggests that Cph1 is a functional red/far-red light photoreceptor in *Synechocystis* sp. PCC 6803, and that it acts in a two-component signalling manner in conjunction with Rcp1.

Therefore, through the analysis of plant phytochrome sequences and biochemical studies with cyanobacterial phytochrome, it was hypothesised that plant phytochrome would also act via a protein kinase signalling cascade (despite lacking the ATP binding site common to serine/threonine kinases). This issue was finally resolved when oat phytochrome was overexpressed in yeast. It was shown that the protein could be phosphorylated in a light-dependent manner, and this phosphorylated protein was shown to be acid stable and base labile, a feature

characteristic of phosphoserine or phosphothreonine. Furthermore, it was shown that following phytochrome autophosphorylation, it was able to transphosphorylate Rcp1 of *Synechocystis*, albeit on a serine residue rather than the conserved aspartate (Yeh and Lagarias, 1998).

The identification of a protein target phosphorylated by PhyA, PKS1, lends further support to the idea that phytochrome action is kinase mediated (Fankhauser *et al.*, 1999).

The use of yeast two-hybrid screen has now identified several proteins that interact directly with phytochrome. One of the best characterised is PIF3 (Ni *et al.*, 1998). PIF3 is a putative bHLH transcription factor, and is located in the cell nucleus. It has been demonstrated that PIF3 associates with full length phytochrome B *in vitro* in a light–dependent manner. Whilst Pfr bound efficiently to PIF3, this was reversed by exposure to far-red irradiation (Ni *et al.*, 1999). Study of a mutant with altered red-light responses has found that this *poc1* mutant overexpresses PIF3, indicating a direct role of PIF3 in phytochrome-mediated responses (Halliday *et al.*, 1999). This seems likely to represent a signalling mechanism in higher plants by which phytochrome can directly mediate gene expression.

Traditional thought was that phytochrome was located in the cell cytosol. However, phytochrome interaction with PIF3 in the nucleus would require nuclear localisation. It has now been demonstrated that phytochrome can undergo light-dependent translocation to the nucleus from the cytosol. Translocation of PhyB upon red irradiation, and PhyA upon far-red irradiation might represent general mechanisms for phytochrome action (Frohnmeyer, 1999).

1.5.6. Prokaryotic phytochromes

In addition to *cph1* of *Synechocystis* sp. PCC 6803, several other phytochrome-like proteins have been identified in prokaryotic organisms. RcaE, a gene involved in the regulation of CCA, from *Fremyella diplosiphon* was one of the first identified. CCA is a key process in many cyanobacteria for responding to a changing light environment. First described nearly a century ago, the genetics are only now becoming understood. CCA is the mechanism by which cells alter the relative ratios of their light-harvesting pigments in order to be able to absorb more light for

photosynthesis. Hence, the cells colour becomes complementary to that of the light of their environment (Allen and Matthijs, 1997; Kehoe and Grossman, 1996). Thus, in red light cells synthesise more phycocyanin, and in green light they synthesise more phycoerythrin. The gene *rcaE* was found to complement a mutant of *F. diplosiphon* that is unable to chromatically adapt. The gene encodes a deduced protein with a domain similar to the chromophore attachment domain of plant phytochromes (though lacking the conserved cysteine residue). The protein also shows similarity to plant ethylene receptors and two-component histidine kinases (Kehoe and Grossman, 1996). Interestingly, this putative photoreceptor must be able to sense the change between red and green light in order to regulate CCA, rather than the red and far-red alterations sensed by phytochrome in higher plants.

A phytochrome-like protein has been identified in the purple photosynthetic bacterium *Rhodospirillum centenum* (Jiang *et al.*, 1999), and remarkably, phytochrome-like proteins (*bphP* – bacterial *phytochrome photoreceptor* genes) have also been identified in two non-photosynthetic prokaryotes, *Deinococcus radiodurans*, and *Pseudomonas aeruginosa* (Hughes and Lamparter, 1999). Despite lacking the conserved cysteine, BphP of *Deinococcus* has been shown to assemble with phycocyanobilin at histidine-381, rather than the cysteine residue usually located at position 380 (Hughes and Lamparter, 1999), and the holoprotein is photoreversible (Davis *et al.*, 1999).

Despite *Synechocystis* sp. PCC 6803 being unable to chromatically adapt (containing only phycocyanin and allophycocyanin, but not phycoerythrin, as its primary light-harvesting pigments), the genome sequence has revealed the presence of seven ORFs potentially encoding phytochrome-like proteins. *cph1* (slr0473), and *cph2* (sll0821), *plpA* (sll1124), sll1473, slr1393, slr1969 and *etr1* (slr1212) (Fiedler *et al.*, 2000). Several of these ORFs encode proteins that share similarity with histidine protein kinases. A possibility is that the spectral quality of light may therefore affect other cellular responses through two-component signal transduction systems, such as photosystem stoichiometry.

1.5.7. Phytochrome evolution

The discovery of an array of phytochrome-like proteins in prokaryotic organisms raises the possibility of a common ancestor for this family of photoreceptors. Indeed,

a recent phylogenetic analysis suggested that plant phytochromes are ancestral to cyanobacterial phytochromes, whilst Ppr of *Rhodospirillum* was the most distant (Jiang *et al.*, 1999).

A study of the bilin lyase domain from a host of phytochrome and phytochrome-like proteins was carried out to identify a 130-180 amino acid domain required for covalent attachment of chromophores. This was then verified by showing that Cph2 of *Synechocystis* sp. PCC 6803 could associate with phycocyanobilin within the identified region (Park *et al.*, 2000; Wu and Lagarias, 2000). Slr1212 was shown to contain all of the features required for chromophore attachment, although to date chromophore association with this protein has not been demonstrated.

A phylogenetic analysis of the chromophore attachment region of phytochrome-like proteins from eleven cyanobacterial strains, and several eukaryotic proteins revealed that Slr1212 (along with RcaE from *F. diplosiphon*, and *plpA* from *Synechocystis* sp. PCC 6803) is only distantly related to phytochromes of higher plants (Herdman *et al.*, 2000).

Taking all the evidence presented so far together, there is a strong indication that the Slr1212 protein of *Synechocystis* sp. PCC 6803 has a role in ethylene and/or light signal transduction mechanisms.

1.6. Project Aims

The sequencing of the genome of the freshwater, unicellular cyanobacterium *Synechocystis* sp. PCC 6803 has given investigators intriguing insights into environmental sensing and the regulation of many important components involved with cellular metabolism. The ability to readily generate gene knockouts in this organism allows investigators to take a directed approach in evaluating the role of many interesting genes in the perception of environmental stimuli, and the manner in which these proteins may regulate cellular responses to an altering ambient environment.

This project focuses on two open reading frames identified from the genome of *Synechocystis* sp. PCC 6803, slr1212 and slr1213. ORF slr1212 putatively encodes a

protein involved with the sensing of either ethylene, a hormone involved with many critical processes in higher plants, and/or light quality. The protein appears to be a histidine kinase, and perhaps interacts with Slr1213, the ORF for which is located immediately downstream, a putative response regulator protein with a helix-turn-helix DNA-binding motif.

Through the generation of single Δslr1212 and Δslr1213, and double Δslr1212 Δslr1213 mutants, the aim of this study was to characterise a physiological role for this proposed two-component signal transduction system in either ethylene signalling mechanisms or light sensing.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. *E. coli* strains

The strains of *E. coli* used in this study are described in Table 2-1.

Strain	Genotype	Source	Reference
MC1061	F- *araD139* Δ*(ara-leu)7696 galE15 galK16Δ(lac)X74 rpsL* (Str^r) *hsdR2* (r_k^- m_k^+) *mcrA mcrB1*	ATCC	Wertman *et al.* (1986)
XL1-BLUE	F'::Tn*10 proA$^+$B$^+$ lacI^q* Δ*(lacZ)M15/recA1 endA1 gyrA96*(Nal^r) *thi hsdR17* ($r_k^-m_k^+$) *supE44 relA1 lac*	Stratagene, USA	Bullock *et al.* (1987)
TOP 10F'	F' {*lacI^q* Tn*10* (Tet^r)} *mcrA* Δ*(mrr-hsdRMS-mcrBC)* φ80*lacZΔM15* Δ*lacX74 recA1 deoR araD139* Δ*(ara-leu)7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen, Netherlands	
Epicurian coli® XL1-Blue supercompetent cells	*RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^{q}Z*ΔM15 Tn*10* (Tet^r)]^c	Stratagene, USA	

Table 2-1:- Genotype and sources of the *E. coli* strains used in this study.

2.2. Cyanobacterial strains

The cyanobacterial strains used in this study are described in Table 2-2.

Strain	Source	Reference
Synechocystis sp. PCC 6803	Pasteur culture collection	(Rippka *et al.*, 1979)
Synechocystis sp. PCC 6803 FJ	Françoise Joset, University of Marseille	
Synechocystis sp. PCC 6803 glucose-tolerant	Conrad Mullineaux, UCL	
Gloeotrichia	Warwick cyanobacterial culture collection	
Nostoc sp. PCC 7118	Pasteur Culture Collection	(Rippka *et al.*, 1979)

Table 2-2 :- Cyanobacterial strains used in this study.

2.3. Bacterial growth media

All of the cyanobacteria used in this study were cultured in BG-11 medium as shown in Table 2-3 (Rippka *et al.*, 1979) with shaking at 30°C. Continuous illumination from a white fluorescent light was provided at a fluence rate of 30 μmol m^{-2} s^{-1}.

Constituent	Amount (g l^{-1})
NaNO$_3$	1.5
K$_2$HPO$_4$·3H$_2$O	0.04
MgSO$_4$·7H$_2$O	0.075
CaCl$_2$·2H$_2$O	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA·(Na$_2$Mg)	0.001
Na$_2$CO$_3$	0.02
Trace metal mix	1 ml
Distilled water	To 1l

Trace metals	Amount (g l^{-1})
H$_3$BO$_3$	2.86
MnCl$_2$·4H$_2$O	1.81
ZnSO$_4$·7H$_2$O	0.222
Na$_2$MoO$_4$·2H$_2$O	0.39
CuSO$_4$·5H$_2$O	0.079
Co(NO$_3$)$_2$·6H$_2$O	0.049

Table 2-3 :- BG-11 medium.

Usually 100 ml or 1 litre culture volumes were used. BG-11 medium was autoclaved at 121°C for 15 minutes before use. One litre cultures were supplemented with 0.1% (w/v) sodium bicarbonate, and bubbled with 1% (v/v) CO$_2$ in air.

To make BG-11 solid medium containing 1.5% (w/v) agar, a 2x BG-11 solution and 2x agar solution were autoclaved separately, before mixing at 65°C, and pouring.

Cultures were monitored for contamination using agar plates supplemented with 2% (w/v) glucose and 0.15% (w/v) yeast extract. Cultures were spotted onto a plate, which was incubated in the dark at 30°C for 7 days. If no growth was seen after this time, it was assumed that the culture was axenic.

Both kanamycin and spectinomycin were used at a final concentration of 25 μg ml^{-1}.

E. coli strains were cultured in LB medium with shaking, or on solid LB medium (1.5% w/v agar) at 37°C as described in Sambrook *et al.* (1989). Overnight cultures for cloning were inoculated from single colonies on a solid agar plate stored at 4°C

with the appropriate antibiotics added. Kanamycin was used at a final concentration of 50 μg μl^{-1}, and ampicillin at 100 μg μl^{-1}.

2.4. Maintenance of bacterial stocks

Cyanobacterial strains were maintained in photoautotrophic conditions on 1.5% (w/v) agar slopes, containing appropriate antibiotics. Following growth at 30°C, slopes could be kept in dim light for up to 4 months.

Frozen stocks were maintained in 5% (v/v) DMSO in liquid nitrogen.

Stocks of *E. coli* strains were maintained at −70°C as described in Sambrook *et al.* (1989). A fresh overnight culture (850 μl) was mixed with 150 μl glycerol in a cryo-vial by vortexing. This was frozen in liquid nitrogen and transferred to a −70°C freezer.

2.5. Growth curves of *Synechocystis* sp. PCC 6803

All cultures for use in growth curves were sub-cultured from exponentially growing cultures in white light conditions to an OD_{750} of 0.035, and light scattering at 750 nm was measured at least once daily. Light filters were obtained from Rosco UK. Data points are represented as means ± the standard deviation of the mean calculated from a minimum of triplicate data.

To calculate the generation time (the time required for a bacterial population to double) the following method was used (Pelczar *et al.*, 1993).

$$n = 3.3 \; (\log_{10} N - \log_{10} N_0) \qquad \text{Where } n = \text{number of generations}$$

$$N = \text{final population}$$

$$N_0 = \text{initial population}$$

$$g = \frac{t}{n} \qquad \text{Where } g = \text{generation time}$$

$$t = \text{time interval (hours)}$$

2.6. Cell counts

Cell counting was carried out using a Weber Scientific counting chamber. A dilution of between 1:5 and 1:20 was typically made, dependent upon the cell concentration.

2.7. Chlorophyll determination

Chlorophyll *a* concentrations were determined from the absorbance of methanol extracts at 665 nm (Porra *et al.*, 1989).

2.8. Sequence analysis

Synechocystis sp. PCC 6803 sequences are available via CyanoBase, the genome database, at http://www.kazusa.or.jp/cyano/cyano.html. *Anabaena* sp. PCC 7120 sequence was accessed at http://www.kazusa.or.jp/cyano/*Anabaena/*. The *Nostoc punctiforme* ATCC 29133 sequence is also available via the internet at http://spider.jgi-psf.org/JGI_microbial/html/*Nostoc*_homepage.html. Basic local alignment search tool (BLAST) searches were carried out at the NCBI homepage (http://www.ncbi.nlm.nih.gov/). Protein sequence analyses were carried out using the suite of tools available through the ExPASy (Expert Protein Analysis System) gateway (http://www.expasy.ch/). Sequences were aligned using CLUSTAL W (v1.81) (http://www2.ebi.ac.uk/clustalw). Alignment shading was carried out using Boxshade v3.31 C.

2.9. General molecular biological techniques

2.9.1. Plasmid list

Plasmid	Features	Source
pCR®2.1-TOPO®	PCR cloning vector	Invitrogen, Netherlands
pBR322	Cloning vector	New England Biolabs, USA
pHP45Ω	Carries Spc^R/Sm^R cassette	Prentki and Krisch, (1984)
pUIDK1	Carries Kan^R cassette	Bardonnet and Blanco, (1992)
p36	Etr1 forward/reverse (F/R) PCR product cloned into pCR®2.1-TOPO®	This study
p1b	Spc^R/Sm^R cassette inserted into Etr1 F/R PCR product of p36	This study
pJIM5	LumQ F/R PCR product cloned into pCR®2.1-TOPO®	This study
pJIM6	LumQ F/R PCR product from pJIM5 *Eco*RI cloned into pBR322	This study
pJIM7	Kan^R cassette inserted into LumQ F/R PCR product of pJIM6	This study
pMUT1	LumQ Del1 F/R PCR product cloned into pCR®2.1-TOPO®	This study
pMUT2	LumQ Del1 F/R PCR product from pMUT1 *Eco*RI cloned into pBR322	This study
pMUT3	Kan^R cassette inserted into LumQ Del1 F/R PCR product of pMUT2	This study
SD pMUT1	pMUT1 containing site-directed mutations of ORF slr1213 to cause a D54E substitution and insert a unique *Sna*BI restriction site	This study
SD pMUT1^R	Spc^R/Sm^R cassette inserted between ORFs slr1213 and slr1214 of SD pMUT1	This study

2.9.2. Restriction endonuclease digestion of DNA

DNA in solution was digested using restriction enzymes with the supplied buffer (Roche Molecular Biochemicals, Germany or New England Biolabs, USA). Generally, the total volume of a reaction was 20-50 µl, containing 5-15 units of enzyme, 0.5 mg ml^{-1} DNase-free RNase A (prepared according to Sambrook *et al.* (1989)) and 4 mM spermidine. Reactions were incubated at 37°C (unless instructed otherwise by the manufacturer) for 2 hours, and visualised by agarose gel electrophoresis (see section 2.9.3).

2.9.3. Agarose gel electrophoresis of DNA

Gel electrophoresis was used to separate and/or isolate DNA fragments, essentially according to Sambrook *et al.* (1989). Samples were prepared in 1x loading buffer (0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol FF, 2.5% (w/v) Ficoll (type 400)). Lambda DNA (GibcoBRL, USA) digested with *Pst*I or a 1 kb DNA ladder (GibcoBRL, USA) were used as markers. Typically, 0.7–2% (w/v) agarose (ultrapure agarose, GibcoBRL, USA) gels containing 1x TBE (0.9 M Tris.HCl, 0.9 M boric acid, 20 mM EDTA pH 8) and 0.5 μg ml^{-1} ethidium bromide were run at 80 volts in 1x TBE for one hour and visualised and photographed using short wave UV. If any fragments were to be purified from the gel for cloning, these were removed using a scalpel under long-wave UV light to prevent DNA nicking, before visualisation of the gel with short-wave UV.

2.9.4. DNA purification from agarose gel slices

The purification of DNA fragments from agarose gels was carried out using a GeneClean® II kit (Bio 101, USA) according to the manufacturer's recommendations. Before DNA fragments were used in cloning strategies, an aliquot of the recovered sample was visualised on an agarose gel.

2.9.5. Phenol/chloroform/isoamyl alcohol extraction of DNA

Phenol/chloroform/isoamyl alcohol extraction of DNA samples was carried out essentially as described by Sambrook *et al.* (1989). An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA sample and mixed to an emulsion. The sample was centrifuged at 13,000 rpm in a MSE micro centrifuge for 2 minutes and the upper aqueous layer transferred to a fresh tube without disturbing the interface. This was repeated and then an equal volume of chloroform/isoamyl (24:1) alcohol was added to the sample and mixed well. Following centrifugation, DNA was recovered from the upper aqueous layer by ethanol precipitation (see section 2.9.6).

2.9.6. Ethanol precipitation of DNA

DNA was precipitated from aqueous samples by the addition of 0.1 volumes 3 M sodium acetate pH 4.8 and 2 volumes 100% (v/v) ethanol. Samples were mixed by inversion and chilled at –20°C for 2-3 hours. The sample was then centrifuged at full speed in a MSE micro centrifuge for 20 minutes and the supernatant discarded. The pellet was washed with 70% (v/v) ethanol, and recovered by centrifugation. The supernatant was completely removed by aspiration and the pellet dried in a vacuum dryer. The dry pellet was resuspended in 50 μl of 10 mM Tris.HCl pH 8.

2.9.7. Spectrophotometric quantification of DNA, RNA and oligonucleotides

The method used was that of Sambrook *et al.* (1989) for determining the concentration of relatively pure samples of nucleic acids. A suitable dilution of the sample was made in distilled H_2O (typically 1:50 or 1:100), and the A_{260} and A_{280} determined using a Pharmacia LKB Ultrospec III spectrophotometer. Quartz cuvettes were used to reduce background absorbance to a minimum.

Nucleic acid concentrations were determined from spectrophotometric measurements. Thus, an A_{260} of 1 signifies a concentration of 50 μg ml^{-1} for dsDNA, 40 μg ml^{-1} for ssDNA or RNA and 20 μg ml^{-1} for oligonucleotides. Sample purity was determined by the A_{260}/A_{280} ratio. A ratio of 1.8-2.0 indicates a pure sample, whilst lower values signify the presence of protein contamination.

2.9.8. Alkaline phosphatase treatment of DNA

To prevent the re-ligation of vector DNA in cloning strategies, DNA was treated with calf intestinal alkaline phosphatase (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. This treatment catalyses the removal of the 5′ phosphate from DNA, preventing vector re-ligation.

2.9.9. Ligation of DNA fragments

Usually an insert to vector ratio of between 5:1 and 3:1 was used as in most cases this ensured that the molar concentration of ends of the insert was comparable with that of the vector. In practice, a range of ratios was employed. Vector DNA was treated with alkaline phosphatase to prevent self-ligation (see section 2.9.8). The insert and vector were mixed with 4 units DNA ligase (GibcoBRL, USA), 1x ligation buffer (10x supplied with the enzyme), 2 mM ATP and distilled H_2O to a total volume of 20 μl. Reactions were incubated overnight at 15°C.

2.10. DNA extraction

2.10.1. Preparation of chromosomal DNA from *Synechocystis* sp. PCC 6803

Chromosomal DNA extraction was based on the method of Lind *et al.* (1985), as modified by Scanlan *et al.* (1990).

25-50 ml of exponentially growing cells (OD_{750} 0.4-0.8) were harvested by centrifugation at 6,000 rpm in a Hettich EBA12 centrifuge using the centrifuge-specific 6x120g rotor for 10 minutes at room temperature. The cell pellet was resuspended in 0.5 ml 0.25 M Tris.HCl pH 8, 25% (w/v) sucrose containing freshly added 10 mg ml^{-1} lysozyme (Sigma, UK) and transferred to a 2 ml Eppendorf. This was incubated at 37°C for 1 hour. Sarkosyl (16 μl) and 20 μl 5 mg ml^{-1} proteinase K were added followed by incubation at 65°C overnight. The DNA was then extracted with phenol/chloroform/isoamyl alcohol (see section 2.9.5). The DNA was precipitated by the addition of 0.4 volumes 7.5 M ammonium acetate and 1 volume isopropanol. Precipitation was allowed to proceed for 10 minutes at room temperature, and the DNA was pelleted by centrifugation for 20 minutes at 13,000 rpm in a MSE micro centrifuge. The supernatant was discarded and the pellet washed with 1 ml 70% (w/v) ethanol. Following a final centrifugation step, the DNA pellet was vacuum dried, resuspended in 50 μl 10 mM Tris.HCl pH 8, and analysed spectrophotometrically (see section 2.9.7) and by agarose gel electrophoresis (see section 2.9.3).

2.10.2. Preparation of plasmid DNA from *E. coli*

2.10.2.1. Alkaline lysis plasmid mini-prep

The small-scale preparation of plasmid DNA was carried out using the alkaline lysis mini-prep technique similar to that in Sambrook *et al.* (1989). A fresh overnight culture (1.5 ml) was harvested by centrifugation. The pellet was resuspended in 150 μl ice-cold GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8, 10 mg ml^{-1} lysozyme (Sigma, UK, freshly added)), and incubated on ice for 5 minutes. To this, 200 μl freshly prepared 0.2 M NaOH, 1% (w/v) SDS was added, mixed by inversion and incubated on ice for 5 minutes. Ice-cold potassium acetate pH 4.8 (150 μl) (3M potassium acetate, 11.5% (w/v) glacial acetic acid) was then added, and the tubes gently vortexed, followed by a further incubation on ice for 5 minutes. The cell debris was harvested by centrifugation, and the DNA-containing supernatant transferred to a fresh tube. The solution was then phenol extracted (see section 2.9.4) and the DNA ethanol precipitated (see section 2.9.6). The DNA pellet was resuspended in 40 μl 10 mM Tris.HCl pH8.

The QIAprep® spin miniprep kit (Qiagen, UK) was also used according to manufacturer's instructions.

2.10.2.2. Large scale plasmid prep

The large-scale preparation of plasmid DNA was carried out using the QIAGEN plasmid midi kit (Qiagen, UK) according to manufacturers' instructions.

2.11. Bacterial transformations

2.11.1. Calcium chloride transformation of *E. coli* with plasmid DNA

An overnight culture of the strain to be transformed was prepared as described in section 2.3. This overnight culture (2 ml) was then used to inoculate 50 ml of LB medium which was incubated at 37°C with shaking until the OD$_{550}$ reached approximately 0.5. The culture was then chilled on ice for 10 minutes. Aliquots (20

ml) were centrifuged in pre-chilled universals in a 'Wifug' Labor-M50 bench top centrifuge at 4500 rpm for 10 minutes at 4°C to pellet the cells. Each cell pellet was resuspended in 10 ml ice-cold 100 mM $CaCl_2$ (Sigma, UK) and incubated on ice for 20 minutes. The cells were then pelleted again, and both cell pellets resuspended in a total of 1 ml ice-cold 100 mM $CaCl_2$.

For each transformation, 100 µl of cells were transferred to a chilled 1.5 ml Eppendorf, along with 50 ng of the DNA to be transformed. The cells were placed on ice for 1 hour followed by a two minute heatshock at 42°C, before being placed back on ice for 5 minutes. LB broth (0.5 ml) was added to each tube before it was incubated at 37°C with shaking (300 rpm) for one hour to allow cells to express the newly acquired antibiotic resistance gene. Cells were then spread using glass beads onto selective plates in 50, 100 and 150 µl volumes, and then incubated overnight at 37°C. If the selection used was ampicillin, the transformants were picked after 12-14 hours, as satellite colonies developed around the true transformants if the plates were left for longer.

2.11.2. Transformation of *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803 is naturally transformable (Barten and Lill, 1995), and the following method was used for transformation.

A late exponential phase culture (OD_{750} 0.7-1.2) was harvested by centrifugation in a MSE Hi-spin 21 centrifuge with the 8x50 ml fixed-angle rotor at 10,000 rpm for 10 minutes. Cells were washed twice with BG-11 medium and concentrated to a density of 1 x 10^9 cells ml^{-1}. Cells (150 µl) were transferred to a 0.5 ml Eppendorf, to which 5-10 µg plasmid DNA was added. Cells were then incubated in 30 µmol m^{-2} s^{-1} white light at 30°C for 30 minutes, and then spread on to thick BG-11 agar plates (50 ml volume). The plates were incubated overnight for 16-18 hours at 30°C with white light illumination at 30 µmol m^{-2} s^{-1}, until green confluent growth was observed. The appropriate antibiotic was then added underneath the agar using a pipette (spectinomycin and kanamycin were used at a final concentration of 25 µg ml^{-1}), and plates incubated for 10-14 days until colonies became visible.

Colonies were then segregated by repeated patching on selective plates. Segregation was confirmed by PCR and Southern blotting analysis.

2.12. Polymerase chain reaction

In general, the polymerase chain reaction (PCR) was performed as described in standard protocols (Sambrook *et al.*, 1989). Oligonucleotides for use in PCR, RT-PCR, DNA sequencing and probe synthesis for hybridisations were synthesised by GibcoBRL, USA. Reactions were carried out in a MJ Research PTC-200 peltier thermal cycler. Briefly, reaction mixes (1x PCR buffer (GibcoBRL, USA), 2.5 mM $MgCl_2$, 10 pmol each primer, 25 μM dNTPs (Promega, USA), 250 pg-1 ng template DNA) were kept at 94°C for 5 minutes and then held at 80°C in a hot start reaction as 0.2 μl (1 unit) of *Taq* DNA polymerase (GibcoBRL, USA) was added. Reactions were then subjected to a programmed cycle, consisting of a 94°C denaturation step (1 min), a primer annealing step (1 min) and a 72°C primer extension step (1 min), which was repeated 29 times, followed by a final extension of 7 minutes. The primer annealing temperature was typically 5°C below the lowest primer melting temperature.

PCR primers used in this study are detailed in Table 2-4.

Primer	T_m (°C)	Sequence (5′ to 3′)	Target Site*
Etr1 F	54	AACTAAAGGTACGTCCATG	-17 to +2 of ORF slr1212
Etr1 R	54	GGAGCTAGCTAAAGTGAAA	+2543 to +2525 of ORF slr1212
CpcB F	60	AAGCTGATGCTCGCGGCG	+32 to +49 of ORF sll1577
CpcB R	60	AGCTACGGAAGCACCGGG	+384 to +367 of ORF sll1577
LumQ F	58	GGCATTGATTCATGAGCAGT	+2088 to +2107 of ORF slr1212
LumQ R	58	TTCCAGTAGTATGGCTTTGG	+609 to +590 of ORF slr1213
107 F	70	GGACGGGTGAGTAACGCGTGR	
1313 R	70	CTTCACGTAGGCGAGTTGCAGC	

* Position of the target site is given with respect to the relevant ORF in CyanoBase.

Table 2-4 :- Sequence and melting temperatures of PCR primers used in this study.

Primers designed for the PCR amplification of the 16S rDNA gene of oxygenic phototrophs (107 F and 1313 R) were as described by West *et al.* (2001). Primers CpcB F/CpcB R were provided by Julie Scanlan for the amplification of the *cpcB* (phycocyanin B subunit) gene from *Synechocystis* sp. PCC 6803.

2.12.1. Oligonucleotide melting temperature calculations

This formula was used to calculate the melting temperature of all oligonucleotides up to 20 bp in length (Thein and Wallace, 1986).

$$T_m = 2°C \cdot (A+T) + 4°C \cdot (G+C)$$

The melting temperature of the oligonucleotides used for site-directed mutagenesis was calculated using the following formula (QuikChange™ product manual, Stratagene, USA).

$$T_m = 81.5 + 0.41(\%GC) - 675/N - \%mismatch$$

Where N = primer length in bp.

2.12.2. PCR amplification using boiled cells as template

For the amplification of DNA directly from a cell template, a small amount of cell biomass was transferred to a 0.5 ml Eppendorf containing 100 µl distilled H_2O. The cells were heated to 96°C for 15 minutes, and 5 µl used directly in a PCR reaction as template.

2.12.3. Cloning PCR products

PCR products were cloned using the TOPO™ TA cloning® kit (Invitrogen, Netherlands) according to the manufacturer's instructions.

2.12.4. Addition of 3′ A overhangs to blunt-ended PCR products

Cloning PCR products using the Invitrogen® TOPO™ TA cloning® kit (see section 2.12.3) requires 3′-A overhangs, normally added by *Taq* DNA polymerase which has a nontemplate-dependent terminal transferase activity. The linearised vector supplied with the kit has a single, overhanging deoxythymidine residue, which allows the efficient insertion and ligation of PCR products amplified with *Taq* DNA

polymerase. However, it is often difficult to clone products amplified with proofreading enzymes such as *Pwo* DNA polymerase, which remove the 3′ A-overhangs necessary for this cloning. To clone such products, the following protocol was used to add 3′ adenines.

Following amplification with *Pwo* polymerase, 1 unit of *Taq* polymerase (GibcoBRL, USA) was added to each tube and mixed well. Reactions were incubated for 10 minutes at 72°C, and the polymerase removed by a phenol-chloroform extraction. The DNA was subsequently ethanol precipitated and resuspended in sterile distilled water to use in cloning experiments.

2.12.5. PCR amplification of ORF slr1213 for site-directed mutagenesis

For the site-directed mutagenesis of ORF slr1213, a proof reading DNA polymerase enzyme was used to ensure no errors were incorporated into the PCR product. *Pwo* DNA polymerase (Roche Molecular Biochemicals, Germany) was used according to manufacturer's instructions. The primer sequences used are detailed in Table 2-5.

Primer	T_m (°C)	Sequence (5′ to 3′)	Target Site*
LumQ Del1 F	56	AAGTTACGGCTGTAATACTG	+2181 to +2200 of ORF slr1212
LumQ Del1 R	58	GGATTGGATACCGTTAGTCT	+92 to +73 of ORF slr1214

* Position of the target site is given with respect to the relevant ORF in CyanoBase.

Table 2-5:- Primer sequences used for the amplification of ORF slr1213 for site-directed mutagenesis.

Reaction mixes were kept at 94°C for 2 minutes and then subjected to a programmed cycle, consisting of a 94°C denaturation step (15 seconds), 52°C primer annealing (30 seconds) and 72°C primer extension (1 min), which was repeated 10 times. For the next 20 cycles, the length of the primer extension step was increased by 5 seconds per cycle. There was a final extension step of 7 minutes.

2.13. Oligonucleotide directed mutagenesis using the QuikChange™ site-directed mutagenesis kit

The QuikChange™ site-directed mutagenesis kit (Stratagene, USA) was used according to the manufacturer's directions. The primers used in the site-directed mutagenesis of ORF slr1213 are shown in Table 2-6.

Primer	T_m (°C)	Sequence (5′ to 3′)	Target Site*
LumQ MutF	78	GATCTAGTTTTACTAGAAATACGTATTAAAGGAGAGATCGACGGG	+145 to +189 of ORF slr1213
LumQ MutR	78	CCCGTCGATCTCTCCTTTAATACGTATTTCTAGTAAAACTAGATC	+189 to +145 of ORF slr1213

* Position of the target site is given with respect to the relevant ORF in CyanoBase.

Table 2-6:- Sequence of primers used for the site-directed mutagenesis of ORF slr1213.

The primers were designed according to the manufacturer's instructions, and accordingly were 45 bp in length, had a Tm of around 78°C, had at least 10-15 bp either side of the desired mismatches, a GC content of close to 40%, and were PAGE (polyacrylamide gel electrophoresis) purified.

2.14. DNA sequence analysis

2.14.1. Preparation of template for automated sequencing

Double stranded DNA plasmid vector (250 ng) or PCR product (50 ng) was mixed with 4 μl sequencing mix (BigDye dye terminator, Perkin Elmer Applied Bioscience, UK), 1.6 pmol primer and sterile distilled water to a total volume of 10 μl. *Taq* cycle sequencing was carried out on a MJ Research PTC-200 peltier thermal cycler. Samples were held for 5 minutes at 95°C, before 35 cycles as follows: 95°C for 30 seconds, 55°C for 20 seconds, 60°C for 4 minutes. DNA was precipitated by the addition of 90 μl 0.52 mM $MgCl_2$ in ethanol, and stored overnight at −20°C. The pellet was collected by centrifugation at 13,000 rpm in a MSE micro centrifuge, washed in 70% (v/v) ethanol and vacuum dried.

The primers used in this study for sequencing are shown in Table 2-7.

Primer	T_m (°C)	Sequence (5′ to 3′)	Target Site*
pMUT1 2F SD	60	TGACATGGGAGGAAATGCTC	+1140 to +1121 of ORF slr1213
pMUT1 3F SD	60	AGGTCTAATCCTTGGCAAGG	+900 to +881 of ORF slr1213
pMUT1 4F SD	60	ATTGATAGGGGGAACATCCC	+562 to +543 of ORF slr1213
pMUT1 5F SD	60	GCTTAATCACATAGCCCTGG	+313 to +294 of ORF slr1213
pMUT1 6F SD	60	AATTGGGCAATGTGTTGGGC	+62 to +43 of ORF slr1213
pMUT1 7F SD	60	CTGGAGTTGTTCAGTTAGGC	+2475 to +2456 of ORF slr1212
pMUT1 3R SD	64	GTGATCGCCAGTGATGGAGC	+91 to +110 of ORF slr1213
9JPM	60	CCAACTAGCAGCAAACACCA	+1033 to +1052 of ORF slr1213
10JPM	62	CAAATTGAGCGCCACCGACA	+2515 to +2496 of ORF slr1212
11JPM	60	ACGACGGAAGGGGAAATTTC	+2317 to +2336 of ORF slr1212
12JPM	60	GTTAGTCTCCCGGTAGCTTT	+80 to +61 of ORF slr1214
13JPM	58	CCAGTCATCTTCCTTGAATC	+1050 to +1069 of ORF slr1213
14JPM	62	TGGCCACCAAACCTTGGCTA	+993 to +1012 of ORF slr1213

* Position of the target site is given with respect to the relevant ORF in CyanoBase.

Table 2-7:- Sequences and melting temperatures of the primers used for DNA sequencing in this study.

2.14.2. Analysis of sequence data

Sequence data was analysed using Chromas (version 1.45) and Seq Man (version 4.05; part of DNA star).

2.15. RNA methods

2.15.1. Preparation of materials for work with RNA

When working with RNA, the presence of RNases at any step can lead to failure of the experiment. It is therefore important that certain precautions are taken. At all times, a pair of disposable gloves were worn and changed at regular intervals. All disposable plastics such as Eppendorfs were used directly from their original packaging. ART filter tips (Merck-MBP, UK) were used for pipetting. Any glassware used was first baked at 180°C for a minimum of three hours. All solutions were made from chemicals kept separately from the main laboratory stocks, and the pH of solutions was measured using pH paper (BDH, UK). All solutions were made using water treated with dimethyl pyrocarbonate (DMPC, Sigma, UK) to inactivate RNases (see section 2.15.2).

2.15.2. DMPC treatment of dH$_2$O

10 ml DMPC (Sigma, UK) was added to 10 litres of water, mixed, and incubated at room temperature in a fume hood for 30 minutes. The water was then autoclaved at 121°C for 45 minutes to inactivate the DMPC.

2.15.3. Total RNA isolation from *Synechocystis* sp. PCC 6803

Total RNA was isolated from *Synechocystis* sp. PCC 6803 according to the method devised by Alley (1987), as described by Scanlan *et al.* (1993), as follows:

Synechocystis sp. PCC 6803 cells were harvested during the exponential growth phase by centrifugation at 8,000 rpm in a MSE Hi-Spin 21 centrifuge fitted with the 8x50 ml fixed angle rotor and washed in 20 ml wash buffer (0.05 M EDTA, 0.12 M NaCl) in sterile universals. The pellet was resuspended in 1.6 ml RNA extraction buffer (100 mM LiCl, 50 mM Tris.HCl, 30 mM EGTA, 1% (w/v) SDS pH7.5) and boiled in a Proline MicroChef ST44 microwave on power setting 2 for 20 seconds. The cells were immediately split into two Eppendorf tubes containing 0.8 ml saturated phenol pH 4.5 (BDH, UK), mixed to an emulsion and placed at 65°C for 5 minutes. The samples were centrifuged at full speed in a MSE micro centrifuge for 5 minutes, and the aqueous phase transferred to a fresh tube. Samples were again extracted with acidic phenol at room temperature, and then once with chloroform/isoamyl alcohol (24:1 v/v). The RNA was then ethanol precipitated (see section 2.9.6). The pellet was dissolved in 400 µl DNase buffer (100 mM sodium acetate, 10 mM MgCl$_2$ pH 5.6), to which 5 µl (50 units) DNaseI-RNase free (Roche Molecular Biochemicals, Germany) was added. The reaction was incubated for 1 hour at 37°C, before a second enzyme addition and incubation. The RNA was then extracted once with acidic phenol and once with chloroform/isoamyl alcohol. The RNA was ethanol precipitated, resuspended in 50 µl DMPC-treated distilled water containing 1 unit RNAguard® (Amersham Pharmacia Biotech, UK), and stored at –20°C.

The RNA yield was analysed spectrophotometrically and by denaturing formaldehyde agarose gel electrophoresis.

2.15.4. Separation of RNA using denaturing formaldehyde-MOPS agarose gels

Separation of RNA was carried out using two different gel systems described here and in section 2.15.5. The first system was carried out using denaturing formaldehyde-MOPS agarose gels as described in Sambrook *et al.* (1989). A gel (final volume 200 ml) containing 1.5% (w/v) agarose, 40 ml 5x FGR buffer (0.1 M MOPS pH7, 40 mM sodium acetate, 5 mM EDTA pH8) and 124 ml DMPC-treated water was melted in a microwave, and cooled to 65°C before the addition of 36 ml formaldehyde (2.2 M final) and 0.5 µg ml^{-1} ethidium bromide. The gel was mixed by swirling and poured. Samples (4 µl) were prepared in 0.5x FGR buffer, 50% (v/v) formamide, 17.5% (v/v) formaldehyde (20 µl total), heated to 65°C for 10 minutes, and cooled on ice before loading. 0.24-0.95 kb RNA ladder (GibcoBRL, USA) was used as a molecular weight marker. Gels were run at 5 V/cm for 4 hours in 1x FGR buffer with circulation. After running, gels were washed in DMPC treated water for 30 minutes to remove formaldehyde.

2.15.5. Separation of RNA using denaturing formaldehyde-HEPES agarose gels

Separation of RNA was also carried out using denaturing formaldehyde-HEPES agarose gels. A gel (final volume 250 ml) containing 1.5% (w/v) agarose, 25 ml 10x Hepes/EDTA buffer (0.5 M Hepes pH7.8, 10 mM EDTA pH8) and 190 ml DMPC treated water was melted in a microwave and allowed to cool to 65°C before the addition of 40 ml formaldehyde. The gel was mixed by swirling and poured. Samples were prepared with 1x loading buffer (5x = 50% (v/v) deionised formamide, 16% (v/v) formaldehyde, 1x Hepes/EDTA buffer, 10% (v/v) glycerol, 0.025% (w/v) xylene cyanol, 0.025% (w/v) bromophenol blue, 0.01% (w/v) ethidium bromide) heated to 65°C for 10 minutes and cooled on ice before loading. 0.24-0.95 kb RNA ladder (GibcoBRL, USA) was used as a molecular weight marker. Gels were run at 40 mA overnight, with running buffer (1x Hepes/EDTA buffer, 16% (v/v) formaldehyde) recirculation.

2.15.6. Preparation of RNA for use in RT-PCR

The method for total RNA isolation (see section 2.15.3) was not sufficient to rid the sample of all contaminating DNA. Therefore, in order to use the RNA for RT-PCR, the QIAGEN RNeasy miniprep kit was used to clean up the RNA according to the manufacturer's instructions. This kit was used in conjunction with the RNase-free DNase enzyme (Qiagen, UK) for the digestion of DNA whilst bound to the spin-column.

2.15.7. RT-PCR

RT-PCR was carried out using Expand reverse transcriptase (Roche Molecular Biochemicals, Germany), a genetically engineered version of M-MuLV reverse transcriptase. This enzyme was used according to the manufacturer's instructions, and the forward PCR step was carried out as in section 2.12 on page 73, with an annealing temperature of 55°C, extension time of one minute, and 35 cycles.

The primers used in the RT-PCR studies are shown in Table 2-8.

Primer	T_m (°C)	Sequence (5′ to 3′)	Target Site*
RT CpcB F	60	ATCGTTAACGATGTCCAGGG	+432 to +413 of ORF sll1577
RT CpcB R	60	TTCTGTTAACCGCATCACCG	+117 to +136 of ORF sll1577
RT Etr1 F	60	TCGCCTACTACTCTATTCCC	+128 to +147 of ORF slr1212
RT Etr1 R	60	ACAATAGCAGCCAATTGCGC	+614 to +595 of ORF slr1212
RT Etr-LumQ F	60	TTACAGGTGTGGGATAACGG	+2371 to +2390 of ORF slr1212
RT Etr-LumQ R	60	TATCCCGTCGATCTCTCCTT	+192 to +173 of ORF slr1213
RT CpcBA F	60	TCAGAAAGCGACCTTGAGAG	+58 to +39 of ORF sll1578
RT CpcBA R	60	AATGAAAGAAGCTGCCCTGG	+399 to +418 of ORF sll1577
RT Cob-Etr F	60	GCAGAGCAAAGACAACTCTG	+3151 to +3170 of ORF slr1211
RT Cob-Etr R	60	TTGCCAAAGGTAGCAATGGC	+81 to +62 of ORF slr1212

* Position of the target site is given with respect to the relevant ORF in CyanoBase

Table 2-8:- List of primers used in RT-PCR analyses.

2.16. Transfer of nucleic acids to a solid membrane

2.16.1. Southern blotting

The method used is essentially that described by Sambrook *et al.* (1989). Following agarose gel electrophoresis the DNA was partially hydrolysed by acid depurination

by soaking the gel in 0.25 M HCl until the bromophenol blue dye band turned yellow. Further denaturation of the DNA was achieved by soaking the gel in several volumes of 1.5 M NaCl, 0.5 M NaOH for two 30 minute washes. The gel was neutralised by soaking in several volumes 1 M Tris.HCl pH7.4, 3 M NaCl for two 30 minute washes. The DNA was transferred to Hybond N membrane (Amersham International, UK) according to Southern (1975, 1979) in 20x SSC (3 M NaCl, 0.3 M sodium citrate). To ensure complete transfer had occurred the gel was visualised under short-wave UV illumination. Before hybridisation, the DNA was crosslinked to the filter using a UV Stratalinker™ 2400 (Stratagene, USA).

2.16.2. Northern blotting

Following gel electrophoresis gels were washed for 4 x 15 minutes in DMPC-treated distilled water. The RNA was then transferred to Hybond N membrane (Amersham International, UK) according to (Southern, 1975; Southern, 1979). To ensure complete transfer had occurred the gel was visualised under short-wave UV illumination. Before hybridisation, the RNA was crosslinked to the filter using a UV Stratalinker™ 2400 (Stratagene, USA).

2.16.3. Dot blotting

Dot blotting was carried out using a blotting minifold (Schleider and Scheull Inc., Germany). Before blotting, samples were denatured by the addition of 0.2 M NaOH. A piece of Whatman filter paper was pre-wet in 2x SSPE (5x = (3 M NaCl, 0.2 M NaH$_2$PO$_4$·2H$_2$O, 20 mM EDTA pH 7.4) and placed on the manifold, with a piece of pre-wet Hybond N (Amersham International, UK) on top. Following blotting, the sample was chased with 200 µl 2x SSPE to neutralise the NaOH before hybridisation.

2.17. Nucleic acid labelling

2.17.1. Labelling DNA with Digoxigenin-11-dUTP

DNA was labelled with DIG-11-dUTP using the random primer method (Feinberg and Vogelstein, 1983). The standard random primed DNA labelling reaction was carried out according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany), except that reactions were allowed to proceed overnight at 37°C. The template was usually a PCR product or plasmid which were purified by passage through an agarose gel and subsequent extraction using the GeneClean kit (see section 2.9.4). The reaction was stopped by the addition of 2 μl 0.2 M EDTA (pH 8).

Labelling was also carried out using the DIG-High Prime kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany).

2.17.2. Labelling DNA with [α-^{32}P]-dCTP

Labelling DNA with [α-^{32}P]-dCTP was carried out using the Prime-a-Gene® labelling system (Promega, USA), with some modifications from the manufacturer's protocol. The kit is based upon the method of Feinberg and Vogelstein (1983) in which a mixture of random hexanucleotides is used to prime DNA synthesis *in vitro* from a linear dsDNA template.

The template was usually a PCR product or plasmid which had been purified by passage through an agarose gel and subsequent extraction with the GeneClean kit (see section 2.9.4). Each labelling reaction consisted of 1x labelling buffer, 20 μM unlabelled dNTPs (dATP, dGTP, dTTP), 25 ng denatured DNA template, 0.4 mg BSA, 0.925 MBq [α-^{32}P]-dCTP, 1 unit Klenow fragment DNA polymerase (Roche Molecular Biochemicals, Germany) and sterile distilled water to a final volume of 50 μl. The reaction was incubated at room temperature for 1 hour. Before labelled probes were used for hybridisation, the unincorporated nucleotides were first removed by passage of the reaction down a G25 sephadex column.

2.17.3. G25 sephadex columns

Sephadex columns were used for the removal of unincorporated nucleotides from labelling reactions. These columns have the advantage of yielding probes virtually free of unincorporated dNTPs and reducing the content of very short oligomers. This is useful when generating hybridisation probes, since optimal signal to noise ratios are achieved with probes 500-1500 bases in length (Berger and Kimmel, 1987). G25 sephadex (Amersham Pharmacia Biotech, UK) was prepared in TE pH 8 (10 mM Tris.HCl, 1mM EDTA pH 8) and sterilised at 121°C for 15 minutes. The column was prepared in a 1 ml syringe. The plunger was removed and the syringe plugged with a small amount of sterilised glass wool. Sephadex was then added to the syringe to the 0.9 ml mark, and centrifuged in a test tube at 1000 rpm in a Gallenkamp Labspin centrifuge with a 8x15 ml swing-out rotor to remove excess TE. Further sephadex was added until the syringe was filled to the 0.9 ml mark. The column was then equilibrated by repeated additions of a known amount of TE until the same amount was recovered by centrifugation. The radiolabelled probe was then passed through the column by centrifugation and collected in a 2 ml screw-top vial. 100 μl TE was then passed through the column to wash through any remaining probe, before disposal of the column.

2.17.4. RNA labelling with [α-^{32}P] UTP by *in vitro* transcription

Labelled RNA probes were created from PCR products which had a core T7 phage RNA polymerase promoter incorporated. The 23 bp core promoter sequence was engineered at the 5' end of one of the PCR primers (Mullis and Faloona, 1987; Stoflet *et al.*, 1988). Amplification of the target DNA yields PCR product which has the T7 phage RNA polymerase promoter upstream of the sequence of interest. The PCR product was then used in an *in vitro* transcription reaction using a Strip-EZ kit (Ambion, UK) according to the manufacturer's instructions to yield a radiolabelled (with [α-^{32}P]-UTP, Amersham International, UK) antisense RNA probe (see Figure 2-1). Before labelled probes were used for hybridisation, the unincorporated nucleotides were first removed by passage of the reaction down a G25 sephadex column (see section 2.17.3). The primers used in the PCR reactions to form the templates for *in vitro* transcription reactions are shown in Table 2-9.

T7 Etr1 F

Direction of transcription

5'-CTCTATTCCCTTAACTCTGC-3'

Target DNA

5'-CTCTATTCCCTTAACTCTGCTCTACTTTTTGCG........TTAGCTACCCTCAATCTGGCTCTAAA-3'
3'-GAGATAAGGGAATTGAGACGAGATGAAAAACGA........AATCGATGGGAGTTAGACCGAGATTT-5'

T7 Etr1 R

3'-GGAGTTAGACCGAGATTTAGGGATATCACTCAGCATAAT-5'

|
+1 Core T7 phage RNA
polymerase promoter
sequence

PCR

5'-CTCTATTCCCTTAACTCTGCTCTACTTTTTGCG........TTAGCTACCCTCAATCTGGCTCTAAATCCCTATAGTGAGTCGTATTA-3'
3'-GAGATAAGGGAATTGAGACGAGATGAAAAACGA........AATCGATGGGAGTTAGACCGAGATTTAGGGATATCACTCAGCATAAT-5'

|
+1 303bp PCR product

in-vitro transcription reaction

5'-pppGGGAUUUAGAGCCAGAUUGAGGGUAGCUAA........AGCAAAAAGUAGAGCAGAGUUAAGGGAAUAGAG-3'

Antisense RNA probe for the detection of ORF slr1212 transcripts by Northern hybridisation

Figure 2-1 :- Strategy for adding a core T7 phage RNA polymerase promoter by PCR for use in *in-vitro* transcription reactions.

Primer	T_m (°C)	Sequence (5′ to 3′)	Target Site*
T7 Etr1 F	58	CTCTATTCCCTTAACTCTGC	+138 to +157 of ORF slr1212
T7 Etr1 R	56	TAATACGACTCACTATAGGGAGGGATTTAGAG CCAGATTGAGG	+418 to +399 of ORF slr1212
T7 16S F	60	TGGGGAATTTTCCGCAATGG	+327 to +346 of rrn16S(1)
T7 16S R	58	TAATACGACTCACTATAGGGAGGGTACCGTCA GAACTTCTTCC	+434 to +415 of rrn16S(1)

* Position of the target site is given with respect to the relevant ORF in CyanoBase

Table 2-9 :- Primers used in the incorporation of T7 polymerase promoters onto PCR products for use in in vitro transcription reactions to yield anti-sense RNA probes.

2.18. Nucleic Acid Hybridisation

2.18.1. DNA:DNA hybridisation with DIG-labelled probes

Hybridisation of blots using DIG-labelled probes was carried out according to the manufacturer's recommendations (Roche Molecular Biochemicals, Germany). Chemiluminescent detection with an anti-digoxigenin alkaline phosphatase conjugated antibody was carried out in conjunction with either CSPD® or CPD-Star™ (Roche Molecular Biochemicals, Germany). Filters were exposed to X-ray film at room temperature, and developed using an Agfa Curix 60 automated developer.

2.18.2. DNA:DNA hybridisation with [α-^{32}P]-dCTP labelled probes

Prehybridisation of the blot was carried out in 5x SSPE, 5x Denhardt's (0.1% (w/v) Ficoll 400, 0.2% (w/v) polyvinylpyrrolidone-10, 0.1% (w/v) bovine serum albumin), 0.1% (w/v) SDS for 2-4 hours at 55-65°C. Herring sperm DNA was added to the probe at a concentration of 100 μg ml^{-1} hybridisation fluid, and both were denatured before use by boiling for 10 minutes. Hybridisation was carried out overnight for 12-16 hours at 55-65°C. For homologous probes, filters were washed in 2x SSPE, 0.1% (w/v) SDS for 2 x 15 minutes at room temperature, followed by 1-2 washes at the hybridisation temperature in 0.1x SSPE, 0.1% (w/v) SDS for 15 minutes each. Filters were exposed to X-ray film and stored at –70°C with an intensifier screen. The film was developed using a Agfa Curix 60 automated developer.

2.18.3. DNA:RNA and RNA:RNA hybridisation using $[\alpha\text{-}^{32}P]$-dCTP and $[\alpha\text{-}^{32}P]$-UTP labelled probes

Prehybridisation and hybridisation were carried out in 40 ml RNA hybridisation buffer (5x SSC (0.75 M NaCl, 0.075 M sodium citrate), 1x Denhardt's, 0.1% (w/v) SDS, 50% (v/v) deionised formamide, 50 mM phosphate buffer (25 mM disodium hydrogen phosphate, 25 mM sodium dihydrogen phosphate), 10 µg ml^{-1} Herring sperm DNA) at 50-65°C. Washes were carried out in 0.5-2x SSC, 0.1% (w/v) SDS depending on the stringency required.

Filters were exposed to X-ray film and stored at −70°C with an intensifier screen. The film was developed using a Agfa Curix 60 automated developer.

2.19. Uptake of ^{35}S-methionine by *Synechocystis* sp. PCC 6803

Uptake of ^{35}S-methionine was measured by incubating the cells with different concentrations of the radiolabelled compound (Amersham International, UK) (0.0185 and 0.037 MBq ml^{-1}, and taking samples over a time course (0.5, 1, 2, 4, 6, 8, 10, 12 hours)). Exponentially growing cultures of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells were harvested by centrifugation and resuspended in fresh BG-11 medium to a final OD$_{750}$ of 0.5. Following incubation with ^{35}S-methionine, 1 ml samples were vacuum filtered onto 0.2 µM polycarbonate filters (Whatman, UK) with a 0.45 µM cellulose acetate backing filter (Whatman, UK). The cells were rinsed with 1 ml 10 mM L-methionine, and the polycarbonate filters folded and placed into 1.5 ml Eppendorfs. Scintillation fluid (0.5 ml, Optiphase safe) was added to each, and scintillation counting was carried out with a Wallac TriLux 1250 MicroBeta liquid scintillation and luminescence counter.

Analysis of the uptake data allowed determination of the optimum parameters for use of ^{35}S-methionine in protein synthesis experiments (see section 2.19.1).

2.19.1. Examination of protein expression in *Synechocystis* sp. PCC 6803

Short-term ^{35}S-methionine incorporation experiments were carried out to examine changes in protein synthesis in *Synechocystis* sp. PCC 6803 in response to changes in external stimuli. Typically, cells were cultured to mid-exponential phase, and diluted as necessary to make-up 100 ml culture at an OD_{750} of 0.5.

A sample from this culture was taken and labelled for 2 hours with 0.037 MBq ml^{-1} ^{35}S-methionine, before the addition of L-methionine to a concentration of 100 μM, a saturating concentration for the methionine transporter in *Synechocystis* sp. PCC 6803 (Labarre *et al.*, 1987). Cells were incubated for a further 10 minutes before harvesting by centrifugation. Cell pellets were resuspended in 200 μl stacking gel buffer (0.5 M Tris.HCl pH 6.8) and stored at –20°C. Numerous samples were labelled during the course of an experiment, at specific points following changes in environmental stimulus.

60 μl samples (equivalent to 3 ml of culture OD_{750} 0.5) were boiled for 10 minutes with 1x denaturing mix (see section 2.22), before analysis on 6-24% (w/v) SDS polyacrylamide gels. Gels were coomassie stained (coomassie brilliant blue R250), dried and analysed by autoradiography.

The methodology for each experiment contained slight variations upon this core method, and these are detailed in the following sections.

2.19.1.1. Effect of a high light shift on protein synthesis

In this experiment the first 10 ml sample was incubated with ^{35}S-methionine (along with the remaining 90 ml 'parent' cultures) at 30°C with shaking in 35 μmol m^{-2} s^{-1} white light irradiance (sample name: 0-2 hours standard light) (provided by fluorescent light tubes). Two further 10 ml samples were taken from the parent culture for labelling. One was incubated under the same conditions (sample name: 2-4 hours standard conditions), whilst the other was incubated with 850 μmol m^{-2} s^{-1} white light irradiance (sample name: 2-4 hours high light) (provided with a 500 W halogen light). Cultures in the high light conditions were prevented from warming

up by placing bottles of cold water between the lamp and the flasks to filter the infra-red light. It was necessary to change these bottles every 10 minutes. The parent cultures were also incubated in high light conditions at this stage. A final 10 ml sample was taken and incubated for a further 2 hours in high light irradiance (sample name: 4-6 hours high light). The use of different light sources for the low and high fluence rates may have produced alterations in the spectral quality of the light.

2.19.1.2. Effect of ACC on protein synthesis

Samples (10 ml) were transferred to fresh 250 ml flasks and incubated with ^{35}S-methionine at 30°C with shaking in 35 μmol m^{-2}s^{-1} white light irradiance for 2 hours (sample name: 0-2 hours – ACC). ACC (5 mM; Sigma, UK) was then added to one of the parent cultures, and 10 ml of each was again transferred to a fresh flask and incubated with ^{35}S-methionine for 2 hours (sample name: 2-4 hours ± ACC). Final 10 ml samples of the parent cultures were again transferred to fresh flasks and incubated with ^{35}S-methionine for 2 hours with 35 μmol m^{-2}s^{-1} white light irradiance (sample name: 4-6 hours ± ACC).

2.19.1.3. Effect of gaseous ethylene on protein synthesis

Samples (10 ml) were transferred to 250 ml flasks and incubated with 0.037 MBq ml^{-1} ^{35}S-methionine at 30°C with shaking in 35 μmol m^{-2}s^{-1} white light irradiance for 2 hours (sample name: 0-2 hours no treatment). Two further 10 ml cultures of each cell type were set-up and incubated with 0.037 MBq ml^{-1} ^{35}S-methionine. These cultures were bubbled with gaseous ethylene (99.5% v/v purity) (Aldrich, UK) for 20 minutes of the 2 hour labelling period (sample name: 2-4 hours ethylene bubbled).

2.20. Large-scale preparation of thylakoid membranes from cyanobacteria

Cells were fractionated into a soluble component and a total membrane fraction using the following technique. A late-exponential phase culture (800 ml) was harvested by centrifugation at 8000 rpm for 10 minutes at 4°C in a Beckman J2-21

centrifuge with a JA10 fixed-angle rotor. The supernatant was discarded and the cell pellets washed twice in 20 mM TES pH 7.5. Following the second wash, the cell pellet was resuspended in 50 ml 10 mM TES pH 7.5, 2 mM EDTA containing freshly added 0.2% (w/v) lysozyme and 600 mM sucrose. The cells were mixed using a magnetic stirrer at 30°C for 2 hours, and harvested by centrifugation at 5000 rpm for 10 minutes at 4°C in an IEC Centra® MP4R centrifuge with an 816 fixed-angle rotor. The cell pellet was resuspended in 3-4 ml 20 mM TES pH 7.5 and passed four times through a french press (Aminco french pressure cell press) at a pressure of 1000 Pa. The cell suspension was then centrifuged at 6000 rpm for 10 minutes at 4°C to remove unbroken cells. The supernatant was removed and centrifuged at 35,000 rpm for 30 minutes at 4°C in a Beckman TL-100 ultracentrifuge with a TLA 100.3 fixed-angle rotor. The supernatant contains soluble proteins, and the pellet the membrane fraction. The pellet was resuspended in a suitable volume of 10 mM TES pH 7.5, 2 mM EDTA and stored at –20°C.

2.21. Determination of protein concentration

A Bradford protein assay kit (Bio-Rad) was used according to the manufacturer's recommendations for determination of the protein content of soluble fractions (Bradford, 1976).

2.22. Removal of lipids from membrane fractions

Membrane fractions were prepared by a method similar to that devised by Wessel and Flugge, (1984). This method removes membrane lipids which is important in ensuring good resolution of proteins when using SDS-PAGE analysis.

Methanol (400 µl) was added to 100-400 µl of protein sample in a 1.5 ml Eppendorf tube and mixed well by vortexing. Chloroform (200 µl) was then added, and the sample again vortexed. Sufficient water was then added so that the total volume of the sample and water was equal to 400 µl. The samples were centrifuged at 13,000 rpm in a MSE micro centrifuge for 5 minutes and the top phase discarded, being careful not to disturb the protein interface. Methanol (300 µl) was added to the sample and mixed by vortexing. The sample was again centrifuged for 5 minutes and the supernatant discarded. The pellet was air-dried for 10 minutes and

resuspended in 50 µl 1x denaturing mix (1.06% (w/v) SDS, 5.3% (v/v) glycerol, 1.96 mM Tris.HCl pH 6.8, 0.26% (v/v) β-mercaptoethanol, 0.04% (w/v) bromophenol blue, 36% (w/v) urea) by vortexing. Samples were placed in a boiling water bath for 10 minutes before use.

2.23. SDS Polyacrylamide gel electrophoresis

SDS-PAGE analysis of polypeptides was routinely performed with 6-24% (w/v) gradient gels (see Table 2-10) using either a Hoeffer Scientific Instruments SE400 or vertical Studier-type slab gel apparatus essentially according to Laemmli (1970). For western blotting, fixed percentage gels (typically 12% (w/v)) were run on a Bio-Rad mini-protean gel system.

Briefly, acrylamide, water, separating buffer, SDS, APS (freshly prepared) and TEMED were mixed together in the order described below, and poured into pre-assembled gel plates. The desired acrylamide concentration determined the relative contribution of the components. Bubbles were removed with the addition of water saturated butanol. Once polymerisation had occurred, butanol was removed by washing and the stacking gel was poured. Stacking gel was prepared in exactly the same way as the separating gel, except that stacking gel buffer was used. Upon polymerisation, gels were run in 1x running buffer (25 mM Tris.HCl, 0.192 M glycine pH 8.3, 0.1% (w/v) SDS), at 100 V, until samples had concentrated at the stacking : separating gel boundary. Mini gels were run at 150 volts for 2 hours at 4°C. Large gradient gels were run at 100 volts overnight at 4°C. Coomassie blue stain and silver staining were used to visualise the protein.

Prior to loading, protein samples were denatured by suspension in 1x denaturing mix (see section 2.22) and heated for 5 minutes in a boiling water bath. Wide range colour markers (Sigma, USA), broad range precision protein standards (Bio-Rad, USA) and Kaleidoscope prestained standards (Bio-Rad, USA) were routinely used as molecular weight standards.

Component	6%	24%
40% (w/v) acrylamide	2.3 ml	9 ml
2 M Tris.HCl pH 8.85	5.6 ml	5.6 ml
Distilled water	6.9 ml	141.5 µl
10% (w/v) SDS	150 µl	150 µl
TEMED	8.4 µl	8.4 µl
10% (w/v) APS	75 µl	75 µl

Table 2-10 :- Components of 6 and 24% (w/v) polyacrylamide gel solutions.

The composition of the stacking gel is shown in Table 2-11.

Component	Stacking gel
40% (w/v) acrylamide	1.3 ml
0.5 M Tris.HCl pH6.7	750 µl
Distilled water	3.8 ml
10% (w/v) SDS	60 µl
TEMED	6 µl
10% (w/v) APS	30 µl

Table 2-11 :- Composition of the stacking gel used for SDS-PAGE.

2.23.1. Staining of SDS-PAGE gels

2.23.1.1. Coomassie blue staining

The staining solution contained 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.1% (w/v) coomassie brilliant blue R250 (Bio-Rad). Gels were stained for 2-3 hours and then destained in 41.6% (v/v) methanol and 16.6% (v/v) glacial acetic acid. The destaining process was accelerated by the addition of foam bungs to the destaining solution which bound the coomassie blue.

2.23.1.2. Silver staining

Silver staining of SDS-PAGE gels was carried out using a Silver Stain Plus kit (Bio-Rad) according to the manufacturer's instructions.

2.23.2. Drying SDS-PAGE gels

SDS-PAGE gels were dried onto Whatman filter paper using a vacuum driven Hoeffer Scientific Instruments slab gel drier SE1160 at 80°C for 3 hours.

2.23.3. Development of dry ^{35}S-methionine labelled gels

Dry SDS-PAGE gels containing ^{35}S-methionine labelled proteins were exposed to Hyperfilm-βmax film (Amersham International, UK) and manually developed with an extended fixing step of 10 minutes.

2.23.4. Western blotting

SDS-polyacrylamide gels were blotted onto Hybond C nitrocellulose membrane (Amersham International, UK) using a Bio-Rad mini electroblotting cell in transfer buffer containing 2 mM Tris.HCl pH 8.3, 190 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS. Blots were then blocked in TBS-Tween 20 (20 mM Tris.HCl pH 7.5, 500 mM NaCl, 0.1% (v/v) Tween 20) containing 5% (w/v) milk powder. Three washes in TBS-Tween 20 followed before incubation with antibody diluted to the appropriate concentration in antibody buffer (TBS-Tween 20, 2% (w/v) milk powder, 0.0025% (v/v) Triton X-100) for 2.5 hours. The blot was then rinsed three times in TBS-Tween 20 followed by incubation with secondary antibody, anti rabbit IgG horseraddish peroxidase linked whole antibody raised in Donkey (Amersham International, UK), at a concentration of 0.03% (v/v) for 1 hour. Three washes in TBS-Tween 20 and one wash in distilled water followed, prior to antibody detection using the ECL kit (Amersham International, UK) according to the manufacturer's instructions.

2.24. Cyanobacterial Photosynthesis and dark respiration whole cell measurements

Photosynthesis and dark respiration measurements were performed in a Clark-type oxygen electrode (Rank Brothers, UK) at a temperature of 30°C in the presence of 10 mM sodium bicarbonate. Cells of *Synechocystis* sp. PCC 6803 were harvested and resuspended in fresh BG-11 medium to a chlorophyll *a* concentration of 5 μg ml^{-1}. Oxygen evolution was measured with saturating illumination (which was determined experimentally) at 350 μmol m^{-2} s^{-1}, and oxygen uptake was measured in the dark.

Calculations were made based on the assumption that oxygen saturated water contains 7.6 mg O_2 / l H_2O at 30°C and 760 torr (1 atmosphere). This was calculated using the on-line calculator at General Chemistry *Online!* (http://antoine.fsu.umd.edu/chem/senese/101/solutions/faq/predicting-DO.shtml).

2.25. Phototaxis assays

Phototaxis assays were carried out essentially according to Choi *et al.* (1999). A 50 μl spot of *Synechocystis* sp. PCC 6803 cells containing 5×10^9 cells ml^{-1} was placed on a BG-11 plate containing 5 mM TES (pH 8), 10 μM glucose and 0.3% (w/v) agar. Petri dishes were incubated in a box with lateral illumination from a fluorescent lamp at 1-10 μmol m^{-2} s^{-1} at 30°C. Plates were observed every 48 hours for a phototactic response.

2.26. Flash photolysis

Synechocystis sp. PCC 6803 wild-type and mutant cells were cultured in copper replete (1 μM) and deplete BG-11 medium at 30°C with 30 μmol m^{-2} s^{-1} white light irradiance. At mid-exponential growth phase, cells were harvested and resuspended to a final chlorophyll *a* concentration of 25 μM. Cells were placed in the dark for 5 minutes before they were exposed to 250 μS flashes of broad-band white light. The absorbance of plastocyanin and P700 were measured at 597 nm and 700 nm respectively.

2.27. Whole-cell 77K fluorescence emission spectroscopy

77K fluorescence emission spectra were measured in a Perkin-Elmer LS50 luminescence spectrometer. Cells (5 µM chlorophyll a in BG-11 medium) were injected into 4 mm diameter silica tubes, dark adapted for 10 minutes and quickly frozen in liquid nitrogen. The excitation and emission slit widths were 5 nm. Measurements were taken with 435 nm excitation (Soret absorption band for Chl a).

2.28. Absorption spectroscopy

Absorption spectra of *Synechocystis* sp. PCC 6803 whole cells were measured in an Aminco DW2000 spectrophotometer.

2.29. Laser photoacoustics

The detection of ethylene by laser photoacoustics (LPA) is based on its strong IR absorption at specific laser lines of a CO_2 laser in the 9-11 µm region (Zuckermann *et al.*, 1997). Absorption of light produces a local heating effect that is measured through its accompanying acoustical consequences. Using this technique, a detection limit of 6 ppt (by volume) can be achieved.

The LPA set-up consists of a high power CO_2 laser (100 W with an acoustic cell placed inside the laser cavity). Ethylene possesses a distinct fingerprint-like absorption spectrum in the CO_2 laser wavelength region (Brewer *et al.*, 1982). The strongest ethylene absorption is at the 10P14 CO_2 laser line (wavelength, 10.53 µm; absorption strength, 30.4 atm^{-1} cm^{-1}), and a much weaker absorption is observed on the 10P12 line (10.51 µm, 4.8 atm^{-1} cm^{-1}). The gas from the outlet of the sample cuvette flows continuously through the acoustic cell at atmospheric pressure. Since the absorption of IR radiation occurs in a fixed volume acoustic cell, an increase in pressure occurs. The pressure changes are detected as acoustical noise by a sensitive microphone mounted in the centre of the acoustic cell, and amplified by a lock-in amplifier. During a single concentration measurement, the corresponding microphone signals on both laser lines are determined. The difference yields the concentration corrected for contributions from other gases and from window

absorption. All gases were obtained from Hoek-Loos (Dieren, The Netherlands). Gas mixtures were prepared using mass flow controllers (Brooks model 5850E).

Figure 2-2 shows a diagram of the typical apparatus used. The gases were mixed using flow controllers, and passed through a catalyst to remove contaminating hydrocarbons. The gas was then passed through the sample cuvette and through potassium hydroxide, calcium chloride and liquid nitrogen scrubbers to remove carbon dioxide and water. The sample gas was then passed into and through the photoacoustic cell. The outflow gas was analysed by a Clark-type oxygen electrode and a gas flow meter.

Two types of measurements were carried out in this work: incubation measurements and flow-through measurements (see sections 2.29.1 and 2.29.2).

2.29.1. Incubation measurements

These measurements involved incubating cyanobacterial cells under a given environmental condition to allow any ethylene to accumulate. Cells (8 ml) were incubated in 12 ml serum bottles with Teflon coated butyl rubber stoppers (Owens Scientific, UK). When the samples were sealed, the headspace gas was removed and replaced with a 20% (v/v) O_2, 80% (v/v) N_2 low CO_2 gas mixture which was cleaned by organic oxidation over a platinum catalyst to remove contaminating hydrocarbons including ethylene. This reduced the background levels of ethylene to essentially zero. The samples were then incubated, and measured by taking 1 ml gas samples and injecting them into a sample cuvette attached to a laser, with a carrier gas flow rate of 0.5 litres/hour. Concentrations of ethylene were determined through peak height comparisons with ethylene standards of known concentration. Chlorophyll concentrations were determined as described in section 2.7.

2.29.2. Flow-through measurements

Flow-through measurements were carried out with a temperature-controlled gas-tight incubation chamber, in which cells were in the solid phase (cyanobacterial cells were filtered at a given cell density onto polycarbonate filters) which allowed equilibrium to be reached very quickly. A reservoir of BG-11 medium was maintained
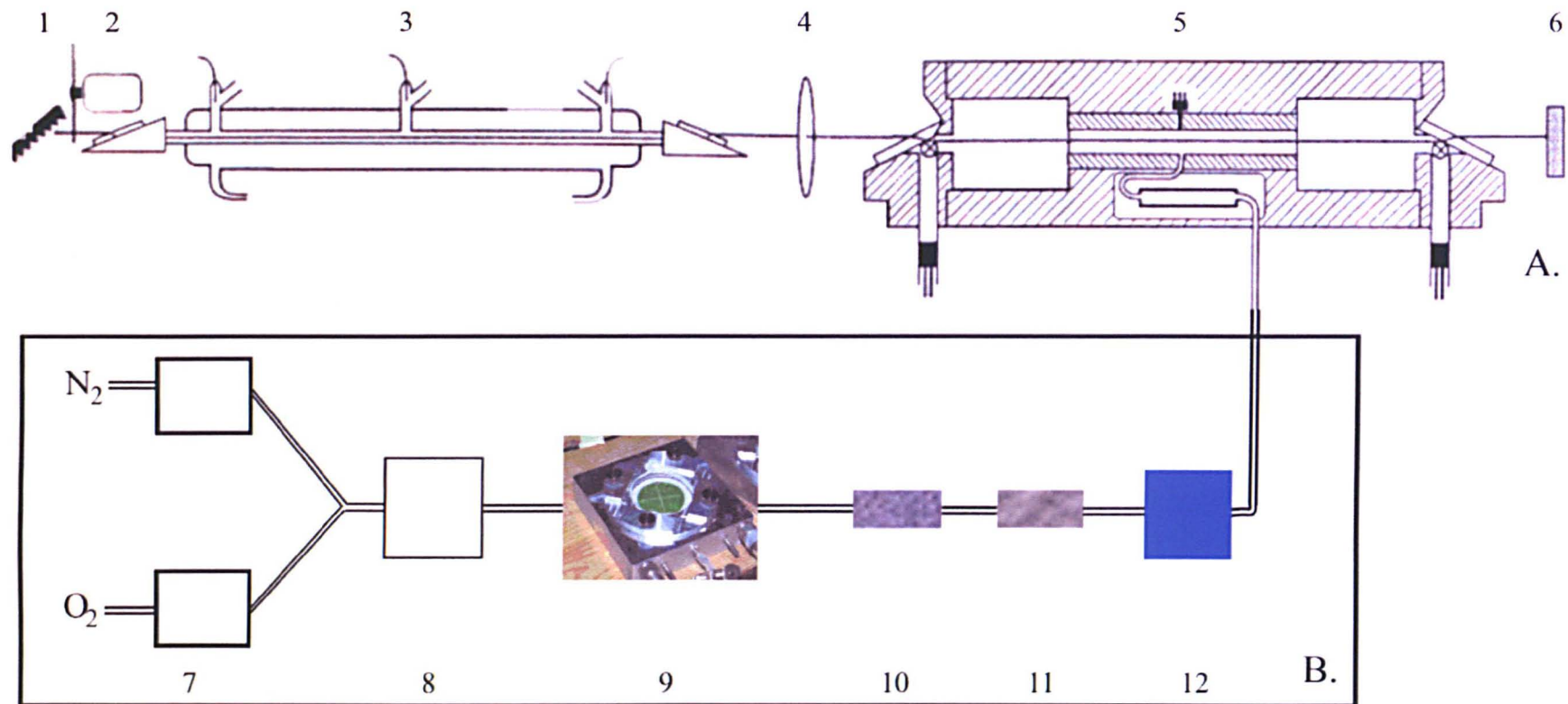
Figure 2-2 :- Diagrammatical representation of the apparatus used to measure ethylene comprising A. LPA and B. gas flow system. The system was made up of the following components: 1. grating to select wavelengths; 2. chopper to modulate light; 3. water-cooled CO_2 laser tube; 4. focusing lens; 5; photoacoustic cell; 6. front mirror; 7. mass flow controllers; 8. platinum catalyst; 9. temperature controlled sample cuvette; 10. KOH trap; 11. $CaCl_2$ trap; 12. liquid nitrogen trap. The outflow gas from the photoacoustic cell was analysed with a Clark-type oxygen electrode and a gas flow meter. Diagram adapted from Hekkert *et al.*, (1998).

underneath the filter, and this could be stirred to keep cells in equilibrium with any added substrates. In this way, cells could remain viable for many days. A slide projector (250 W halogen lamp) was used as a light source. Different light intensities were obtained by positioning slides with neutral-density filters in the light beam. The incubation chamber was connected to the photoacoustic cell by polystyrene tubing, and a carrier gas consisting of 20% (v/v) O_2 80% (v/v) N_2 low CO_2 was continuously passed through the cuvette to the acoustic cell at a flow rate of between 0.5 and 1.5 litres/hour. Contaminating hydrocarbons were removed by passing the gas mix over a platinum catalyst.

2.30. Statistical analysis of data

Data presented in this thesis is generally presented as a mean average plus or minus the standard deviation (SD) of the mean. Standard deviation was calculated as follows :-

$$SD = \sqrt{\frac{\sum (x - \bar{x})^2}{(n-1)}}$$

Where x = individual sample value

$\bar{x}$ = mean value of samples

n = sample size

Further statistical analysis of data was not performed as the sample numbers were generally too small to make any statistical conclusions drawn more meaningful than simple observation of non-overlapping means and SDs.

Chapter 3

Allelic replacement of ORFs slr1212 and slr1213

3. Allelic replacement of ORFs slr1212 and slr1213

3.1. Introduction

The cyanobacterium *Synechocystis* sp. PCC 6803 is readily amenable to genetic manipulations, and because of its ability to grow both photoautotrophically and photoheterotrophically has been widely used to study the mechanism of photosynthesis (Nakamura *et al.*, 1998). Foreign DNA is spontaneously taken up (Grigorieva and Shestakov, 1982), and homologous recombination occurs with a high frequency (Vermaas, 1996). Using appropriate constructs it is therefore possible to create gene mutations of various types in this organism. The use of such strategies provides a powerful technique to examine the role of individual proteins in cell physiology.

This chapter describes the strategy used to create specific gene knockout strains of *Synechocystis* sp. PCC 6803. The ORFs disrupted by allelic replacement encode Slr1212 and Slr1213, potential homologues of ethylene receptors and phytochromes of higher plants, and a response regulator of prokaryotic two-component signal-transduction systems (see Introduction). Broadly, the strategy used entailed gene cloning, construct design and assembly, cell transformation and recombinant selection, followed by the confirmation of segregation by PCR analysis and Southern blotting.

3.2. Methods

The sequence of events involved in construct development was as follows. The slr1212 and slr1213 ORFs were cloned following amplification using PCR with primers designed from the available genome sequence. The PCR products were cloned and analysed by restriction digestion, and sub cloned as necessary, before deletions and insertions in the ORFs were made using antibiotic cassettes. Transformants were selected and segregated by repeated exposure to a selective pressure, before subsequent confirmation using PCR analysis and Southern hybridisation.

At the initiation of these allelic replacement strategies, it was decided to delete a portion of each gene at the same time as the insertion was made to negate the risk of revertants arising. These initial studies would also provide information about

whether the two genes were essential for cell function. In such a case, a mutation would be lethal and full segregation would not occur.

3.2.1. Construct development

3.2.1.1. Cloning of ORF slr1212

Figure 3-1 outlines the strategy used to construct plasmid 1b (p1b), which was used for the allelic replacement of ORF slr1212. The primer set etr1 F/etr1 R (see section 2.12) was designed to amplify slr1212 based on the available genomic sequence. The PCR product of 2559 bp was analysed by agarose gel electrophoresis, and cloned using the Invitrogen® TOPO™ TA cloning® into the pCR®2.1-TOPO® vector, and designated plasmid 36 (p36).

As the TOPO vector lacks the required origin of replication to persist in *Synechocystis* sp. PCC 6803, it is suitable for use in allelic replacement strategies as a suicide vector. To create the desired knockout, p36 was digested by *Sma*I and *Hinc*II to remove a 650 bp fragment from the coding region of slr1212. An omega (Ω) resistance cassette which carries a selectable marker (*aadA*⁺; Spc^R/Sm^R) was removed from pHP45Ω (Prentki and Krisch, 1984) using *Sma*I and ligated into the *Sma*I-*Hinc*II junction of p36 (*Hinc*II and *Sma*I both cut DNA leaving compatible blunt ends, although once ligated together neither enzyme will cut that site). The 2 kb omega cassette is particularly suited for this application as it is flanked by short inverted repeats which carry transcription and translation terminators which should prevent any functional slr1212 protein being formed. The advantages of such an approach therefore, are a positive selection for the presence of the fragment, termination of RNA and protein synthesis beyond the site of insertion, and genetic stability of the resulting mutation. Following this cloning, 570 and 1317 bp of genomic DNA was left flanking the 5′ and 3′ ends of the omega cassette respectively so that homologous recombination could occur.

A.

slr1212 genomic region (8332 bp)

0   2000   4000   6000   8000

Etr1 F/Etr1 R PCR product

slr1211   slr1212   slr1213   slr1214

Recombination

Ω

Δslr1212

Ligate

*Sma*I *Hinc*II

Etr1 F/Etr1 R

*Sma*I   Ω   *Sma*I
*Bam*HI   *Bam*HI
*Hind*III   *Hind*III

*Sma*I cut Ω from pHP45Ω

B.

HindIII 234
BamHI 252
EcoRI 283

ColE1 ori

LacZ alpha

Etr1 F/Etr1 R

EcoRI 1207

BamHI 1641
HindIII 1643

Ampicillin[R]

**1b
7818 bp**

Kanamycin[R]

Omega

LacZ alpha

F1 ori

HindIII 3637
BamHI 3639
SmaI 3640

EcoRI 4211   Etr1 F/Etr1 R

Figure 3.1 :- Allelic replacement strategy for ORF slr1212. A. Scale diagram of the strategy used for the construction of p1b and its application in the allelic exchange of ORF slr1212. B. Physical map of p1b.

3.2.1.2.   Cloning of ORF slr1213

Figure 3-2 outlines the strategy to create plasmid pJIM7, used for the allelic replacement of ORF slr1213. In a procedure very similar to that used to disrupt ORF slr1212 (see section 3.2.1.1), the primer set LumQ F/LumQ R (see section 2.12) was used to amplify a 1105 bp genomic fragment containing the last 448 bp of ORF slr1212 and a portion of ORF slr1213. The PCR product was analysed by agarose gel electrophoresis and then cloned into the pCR®2.1-TOPO® vector to form pJIM5. For the construction of this mutant, a kanamycin cassette was used from pUIDK1 (Bardonnet and Blanco, 1992). The antibiotic cassette was different to that used for the mutation of ORF slr1212 so that double mutants could be selected without the possibility of recombination occurring between the two antibiotic resistance cassettes. The amplified genomic DNA was removed from pJIM5 by *Eco*RI and sub cloned into the unique *Eco*RI site of pBR322 to form pJIM6. This was done because the restriction sites intended for use in the insertion and deletion of the kanamycin cassette were present in the pCR®2.1-TOPO® vector, and because the TOPO vector already carries a kanamycin resistance gene. The kanamycin cassette was removed from pUIDK1 using *Nco*I and *Bgl*II and cloned into ORF slr1213 in pJIM6, with the deletion of a 146 bp coding fragment. This completed the construction of pJIM7, the construct used for the allelic replacement of ORF slr1213.

Restriction digest analysis was carried out at each stage of the cloning, and on the final construct before cell transformation.

3.2.2.   Cell transformation

Following transformation and selection for recombinant cells (see section 2.11.2), it was necessary to segregate the mutation onto every gene copy so that no wild-type copies remained as *Synechocystis* sp. PCC 6803 has multiple copies of its chromosome. This is achieved by repeatedly exposing cells to the selective pressure (antibiotic in this case) by patching cells on new plates. In these investigations, it was found that patching cells approx 3-6 times was normally sufficient to achieve complete segregation.

A.

slr1212 genomic region (8332 bp)

0   2000   4000   6000   8000

LumQ F/LumQ R PCR product

slr1211   slr1212   slr1213   slr1214

Recombination

Δslr1213

*Nco*I *Bgl*II

Ligate

*Bgl*II
Eco RV
Cla I
Eco RI
*Sma*I
*Hpa*I

*Bgl*II/*Nco*I cut kanamycin
cassette from pUIDK1

B.

BglII 6405
EcoRV 6403
ClaI 6401
EcoRI 6399
SmaI 6397
HpaI 6395

EcoRI 6871

EcoRV 185

LumQ F/LumQ R

Tetracycline

Kanamycin^R   pJIM7
6871 bp

ROP

NcoI 4855   LumQ F/LumQ R

Ampicillin^R   Ori

EcoRI 4359

Figure 3-2 :- Allelic replacement strategy for ORF slr1213. A. Scale diagram
of the strategy used for the construction of pJIM7 and its application in the
allelic exchange of ORF slr1213. B. Physical map of pJIM7.

3.3. Results

3.3.1. Disruption of ORF slr1212

Transformation of *Synechocystis* sp. PCC 6803 and *Synechocystis* sp. PCC 6803 FJ with p1b produced many spectinomycin-resistant colonies. Following the transformation 236 spectinomycin-resistant clones were selected, of which 25 were picked for further analysis.

A PCR based method was employed to screen colonies initially for the mutation and segregation. Primer set Etr1 F/Etr1 R was used to amplify DNA directly from cell patches (as described in section 2.12.2). In the construction of both mutant constructs, part of the coding DNA was removed and replaced with an antibiotic cassette, and as a result the size of PCR amplicon over each region was altered accordingly. The expected product sizes are shown in Table 3-1.

	Strain			
Primer set	Wild-Type	Δslr1212	Δslr1213	Δslr1212 Δslr1213
Etr1 F/Etr1 R	2535	3886	2535	3886
LumQ F/LumQ R	1105	1105	2556	2556

Table 3-1 :- Expected size of PCR amplicons (bp) in mutant screen.

As can be seen from this screen (see Figure 3-3 A), the expected PCR product sizes were observed in all cases, except in the Δslr1212 mutant in *Synechocystis* sp. PCC 6803 FJ, which was 0.6 kb larger than expected. It was also observed for PCRs from mutant strains that in all cases no wild-type-sized products could be seen, indicating that complete segregation had taken place and that the mutations were not lethal to the cell. Every colony of each mutant type tested appeared to be fully segregated. However, this technique may not be sensitive enough to detect whether segregation of a mutation is complete due to PCR bias, which could cause preferential amplification of the most abundant template i.e. wild-type or mutant.

To confirm that segregation was indeed complete, and to examine the FJ mutant further, Southern analysis was carried out. The restriction map of
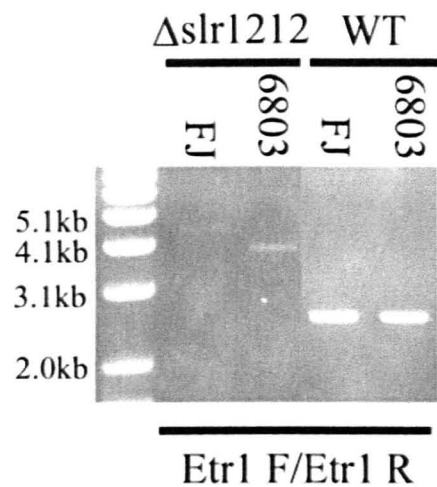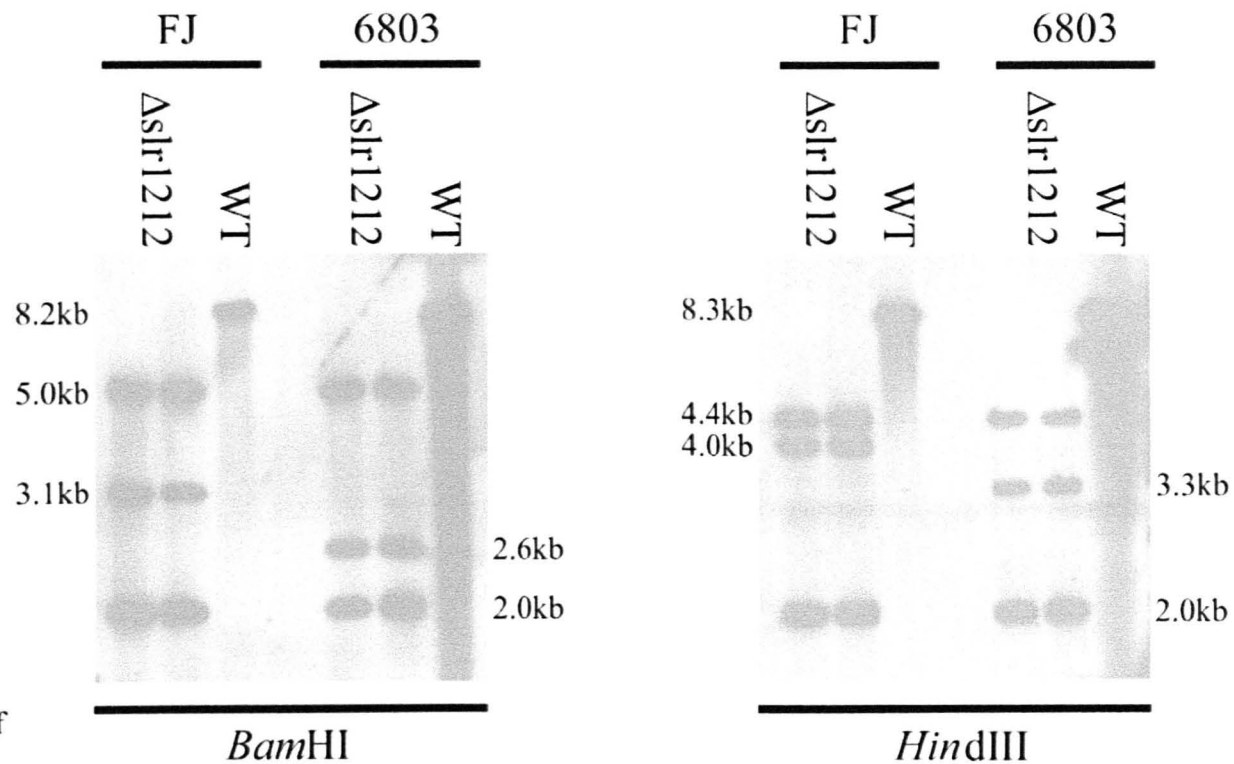
Figure 3-3 :- Analysis of Δslr1212 cells.
A. PCR analysis of cells to screen for segregation. B. Southern hybridisation of cells with a DIG-labelled p1b probe.

Synechocystis sp. PCC 6803 wild-type and mutant DNA used to confirm segregation is shown in Figure 3-4. Table 3-2 derived from this restriction map shows the fragment sizes expected from the relevant Southern hybridisations dependent upon the probe used.

	Probe		
	Etr1 F/Etr1 R	p1b	
	*Bam*HI	*Bam*HI	*Hind*III
Wild-type	8188	8188	8286
Δslr1212	4897, 2577	4897, 2577, 2065	4340, 3273, 2026
Δslr1213	9641		
Δslr1212 Δslr1213	6350, 2577		

Table 3-2 :- Expected fragment sizes from Southern hybridisation experiments to establish the segregation of the mutations in ORFs slr1212 and slr1213. Sizes shown are in bp.

Synechocystis sp. PCC 6803 wild-type and Δslr1212 DNA was probed with a DIG-labelled p1b (allelic exchange construct) probe (see Figure 3-3 B on page 105). Two digests were carried out, and in the case of Δslr1212 DNA, the complete absence of a wild-type-sized fragment showed that segregation was complete. The expected fragments were observed for the Δslr1212 mutant in *Synechocystis* sp. PCC 6803, but in *Synechocystis* sp. PCC 6803 FJ, one of the fragments was 0.5-0.7 kb larger than expected (for both digests). Interestingly, this fragment corresponded to the region of chromosomal DNA which should contain the gene deletion of 649 bp. Subsequent restriction analysis of the PCR products in Figure 3-2 A on page 103 showed that that 649 bp *Sma*I-*Hinc*II deletion had not occurred, and that the omega cassette had inserted at the *Hinc*II site. Clearly, one copy of the p1b construct contained the omega cassette inserted at the *Hinc*II site.

Synechocystis sp. PCC 6803 FJ was not retransformed as it was thought that the chance of reversion was very low.

slr1212 genomic region (12426 bp)

0   2000   4000   6000   8000   10000   12000

A.
slr1211   slr1212   slr1213   slr1214   slr1215   sll1112
*Hind*III *Hind*III *Bam*HI
95   677   1353
*Hind*III *Bam*HI
8963   9540

B.
slr1211   Spc^R/Sm^R slr1212   slr1213   slr1214   slr1215   sll1112
*Hind*III *Hind*III *Bam*HI
95   677   1353
*Bam*HI/*Hind*III   *Hind*III/*Bam*HI
3929   3949   5974   5994
*Hind*III *Bam*HI
10313   10890

C.
slr1211   slr1212   Kan^R slr1213   slr1214   slr1215   sll1112
*Hind*III *Hind*III *Bam*HI
95   677   1353
*Hind*III *Bam*HI
10417   10994

D.
slr1211   Spc^R/Sm^R slr1212   Kan^R slr1213   slr1214   slr1215   sll1112
*Hind*III *Hind*III *Bam*HI
95   677   1353
*Bam*HI/*Hind*III   *Hind*III/*Bam*HI
3929   3949   5974   5994
*Hind*III *Bam*HI
11767   12344

Scale :- 0.6 kb = 1 cm

Figure 3-4 :- A restriction map of *Synechocystis* sp. PCC 6803 wild-type and mutant DNA used in the confirmation of mutant segregation by Southern hybridisation. A. *Synechocystis* sp. PCC 6803 wild-type B. Δslr1212 C. Δslr1213 D. Δslr1212 Δslr1213 strains.

3.3.2. Disruption of ORF slr1213 in a *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 background

Synechocystis sp. PCC 6803 wild-type and *Synechocystis* sp. PCC 6803 Δslr1212 strains were then transformed with pJIM7 to create Δslr1213 and Δslr1212 Δslr1213 mutant strains respectively. From the kanamycin-resistant recombinants that were selected, 25 of each type were again chosen for further analysis. Following segregation, a PCR screen was again employed and the expected sizes of the PCR amplicons are shown in Table 3-1 on page 104.

The results of this screen (Figure 3-5 B) again show that segregation is apparently complete, and that the expected mutations are present in each of the strains. Subsequent Southern analysis was carried out using a $[\alpha\text{-}^{32}P]$-dCTP labelled probe. From the Southern analysis (see Figure 3-5 A) probed with a labelled Etr1 F/Etr1 R PCR product, with reference to the restriction map (see Figure 3-4 on page 107) and Table 3-2 on page 106, it was clear that in all cases cells contain the intended mutations and that following repeated selection, segregation is complete.

A.

WT — 6803, FJ

Δslr1212 — FJ, 6803

Δslr1213 — 6803, FJ

Δslr1212 Δslr1213 — FJ, 6803

8.2kb
5.0kb

9.6kb
6.4kb
3.2kb
2.6kb

*Bam*HI

B.

WT, Δslr1212, Δslr1213, Δslr1212 Δslr1213

2.5kb — 3.9kb

6803
Etr1 F/Etr1 R

WT, Δslr1212, Δslr1213, Δslr1212 Δslr1213

1.1kb — 2.6kb

6803
LumQ F/LumQ R

WT, Δslr1212, Δslr1213, Δslr1212 Δslr1213

2.5kb — 4.5kb

FJ
Etr1 F/Etr1 R

WT, Δslr1212, Δslr1213, Δslr1212 Δslr1213

1.1kb — 2.6kb

FJ
LumQ F/LumQ R

Figure 3-5 :- Analysis of Δslr1212, Δslr1213 and Δslr1212 Δslr1213 mutant strains. A. Southern hybridisation of cells transformed with the slr1212 and slr1213 knockout constructs. Hybridisation of *Bam*HI digested DNA with a ^{32}P-dCTP labelled Etr1 F/Etr1 R probe. B. PCR analysis of strains to show size differences in mutants due to partial gene deletion and insertion of an antibiotic cassette.

3.4. Discussion

This chapter describes the strategy that was used to disrupt two ORFs from the *Synechocystis* sp. PCC 6803 genome, slr1212 and slr1213.

The results gained from the analysis of these mutant strains have demonstrated that, except in the case of *Synechocystis* sp. PCC 6803 FJ Δslr1212, all the strains contain the expected mutations. Furthermore, it has been demonstrated that these mutations have fully segregated, and that none of the mutant strains have any wild-type copies of the genes. It can therefore be concluded that ORFs slr1212 and slr1213 are not essential for the survival of cells under standard conditions. Similar conclusions have been reached by other researchers for different genes such as *dnaA*, which is essential for initiating DNA replication in other bacteria (Richter *et al.*, 1998).

It is possible, however, that the ORF slr11212/slr1213 proposed two component sensor kinase system may have an essential function, which could be taken over by other proteins. Other members of the laboratory have demonstrated that *Synechocystis* sp. PCC 6803 is a very adaptable organism, and if a large enough selective pressure is applied to a cell it can adapt. An example of this occurs when ORF slr0228 is deleted, a putative homologue of the AAA protease FtsH. Disruption of this gene causes a 60% decrease in the cellular content of functional photosystem I (PSI), and western blotting revealed possible structural alterations in this complex. However, when cells were maintained in liquid culture in the presence of a selective pressure for long periods the phenotype was lost (Mann *et al.*, 2000).

Allelic replacement strategies have been used extensively in *Synechocystis* sp. PCC 6803 to study the function of many ORFs (examples include *cph1* (Garcia-Dominguez *et al.*, 2000), and *sigF*, an alternative sigma factor involved with pili formation (Bhaya *et al.*, 1999)), including proposed two component sensor kinase protein pairs. Two-component signal transduction systems clearly fulfil a very important role in *Synechocystis* sp. PCC 6803, given that eighty genes for two component signal transducers were identified from sequence analysis (Kotani and Tabata, 1998). These include twenty-six sensor kinases, thirty-eight response regulators and sixteen hybrid sensor kinases (proteins comprising both transmitter and receiver domains). Most of these genes are scattered throughout the genome making it difficult to pair specific sensor kinases with specific response regulators, in

contrast to *E. coli* where most two-component system genes which interact are in operons (Mizuno, 1997). ORFs slr1212 and slr1213 are therefore particularly interesting due to their close proximity, potentially regulated as part of an operon.

A recent example of the uses of allelic exchange strategies to study two-component sensor kinase systems in *Synechocystis* sp. PCC6603 involved the systematic disruption of putative genes for histidine kinases which identified two genes as components of the pathway for the perception and transduction of low-temperature signals. It was observed that the inactivation of either of these histidine kinases and a response regulator depressed the transcription of several low-temperature responsive genes (Suzuki *et al.*, 2000).

In conclusion, allelic exchange is a very powerful technique for determining the functioning of genes. The function of disrupted genes can be investigated using experiments designed from insights gained from protein sequence analysis (as in this study), or using experiments to further investigate a gene of interest identified from a mutant library using a suitable screen.

Chapter 4

Ethylene production by

Synechocystis sp. PCC 6803

4. Ethylene production by *Synechocystis* sp. PCC 6803

4.1. Introduction

Identification of an ORF from the *Synechocystis* sp. PCC 6803 genome database with homology to the ethylene-binding region of ethylene receptors of higher plants, the chromophore attachment domain of red/far-red light phytochrome photoreceptors, and histidine kinase proteins of two-component signal transduction systems was intriguing. It raised the possibility of a sensing system in *Synechocystis* sp. PCC 6803 involved with light and/or ethylene sensing/signalling.

The ethylene signalling mechanisms of higher plants depend not only upon the ability to sense and transduce an ethylene signal, but also upon the ability to produce ethylene. As discussed in the Introduction, all plants that produce ethylene do so via the Yang cycle, where AdoMet is converted to ACC and then to ethylene by ACC synthase and ACC oxidase respectively (reviewed by Yang and Hoffman (1984)).

As has already been stated (see section 1.4.4.2), many microorganisms also produce ethylene, via 2-oxoglutarate or L-methionine.

This chapter describes the work carried out using laser photoacoustic spectroscopy to study ethylene biosynthesis by *Synechocystis* sp. PCC 6803. Laser photoacoustic measurements are based upon the findings of Alexander Graham Bell that a pressure-wave (sound) can be detected from a substance that is irradiated by a rapidly interrupted beam of light (Bell, 1880). This photoacoustic effect is based on the generation of acoustic waves as a consequence of light absorption (Harren and Reuss, 1997). Absorption of an infrared photon excites a molecule into a higher energy state. Collisions between molecules transfer this energy to heat. Modulating the light intensity causes the sample temperature to rise and fall periodically. If the sample is a gas in a closed volume this temperature variation is accompanied by a pressure variation which creates a sound detectable by a sensitive microphone. At atmospheric gas pressures the collisional energy transfer is very efficient enabling sensitive detection of trace gases. The amplitude of the pressure changes is directly proportional to the number of absorbing molecules in a gas. Different molecules absorb infrared light maximally at different wavelengths. Ethylene absorbs

maximally on the 10P14 laser line. To distinguish ethylene from other molecules, absorption is also measured on the 10P12 laser line, where infrared absorption by ethylene is significantly reduced. The difference in absorption between the two laser lines allows the concentration to be determined (further technical details are available in section 2.29).

The application of LPA to biological systems is a relatively new phenomenon, but because it is very sensitive, versatile, and non-invasive, the technique is now being applied to many biological processes such as the respiration patterns of cockroaches (Bijnen *et al.*, 1996), nitrogen fixation by cyanobacteria (Zuckermann *et al.*, 1997), and ethylene biosynthesis by plants (Bessler *et al.*, 1998; Voesenek *et al.*, 1993).

4.2. Results

Initial experiments were conducted using gas chromatography analyses to examine ethylene production. However, this proved to be insensitive and problems were consistently encountered with background contamination. Therefore, LPA was used to search for evidence of an ethylene biosynthetic pathway in cyanobacteria.

The experiments carried out to investigate the production of ethylene by *Synechocystis* sp. PCC 6803 can essentially be split into two distinct groups. Firstly, flow-through measurements were carried out. In these experiments a carrier gas was continuously passed over the sample, which allowed real-time measurements to be taken, and this sensitivity (as low as 6 ppt for ethylene) is the biggest advantage of LPA. Secondly, accumulation experiments were carried where cells were incubated in serum bottles, usually overnight, to allow a build-up of produced ethylene before measurement.

4.2.1. Flow-through measurements of ethylene production

Flow-through measurements of ethylene were carried out using specially designed sample cuvettes through which the carrier gas could be passed. Attempts were first made to measure ethylene production by *Synechocystis* sp. PCC 6803 using a sample cuvette designed by Marc Staal for the incubation of liquid cultures of cyanobacteria to examine nitrogen fixation (Zuckermann *et al.*, 1997) (see Figure 4-1 A).

However, no evidence of ethylene release could be gained through the use of this cuvette. As a result, the cuvette shown in Figure 4-1 B was used, modified from an original idea of Marc Staal's.

The second cuvette has a number of advantages over the other used for measurement of ethylene release from liquid cultures. Cells are filtered onto a membrane filter and are held in the solid phase in contact with the carrier gas. An equilibrium is therefore established more quickly, mainly because released gases do not have to diffuse through liquid medium. Also, an increased biomass can be measured by filtering more cells, without any loss of light due to absorption by water. The filter was suspended on a fine metal mesh that was submerged in a reservoir of BG-11 medium. By keeping the filter wet, cells survived in excess of five days (data not shown). The reservoir was mixed by a small magnetic flea in the base of the cuvette. Additions to the reservoir could also be made (such as solutions of L-methionine) through an additional inlet. A problem encountered with this system was that the addition of substrates in this manner caused minor alterations to the headspace in the cuvette, and it was found that this caused a decrease in the measurements being taken. To circumvent this problem the volume of the reservoir was kept constant. To make substrate additions, a set volume of the reservoir was removed, and replaced with a substrate at the appropriate concentration to the original volume.

Using the apparatus described, cultures were screened for ethylene biosynthetic activity in the presence and absence of different putative ethylene precursors. In the absence of any substrate addition no evidence of ethylene biosynthesis could be found. Given that previous studies suggest that the majority of microorganisms in which ethylene biosynthesis has been characterised do so via L-methionine, the role of L-methionine in ethylene biosynthesis by *Synechocystis* sp. PCC 6803 was examined. However, no induction of ethylene biosynthesis was observed with either flow through or accumulation measurements (see section 4.2.2).

When the role of 2-oxoglutarate was examined, it was observed that ethylene biosynthesis was rapidly induced (see Figure 4-2). Interestingly, whilst a low level of ethylene evolution was observed when cells were incubated with 2-

A.
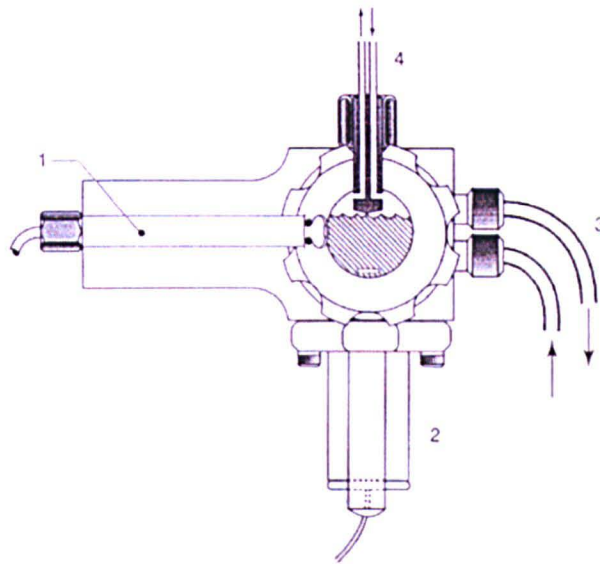
B.
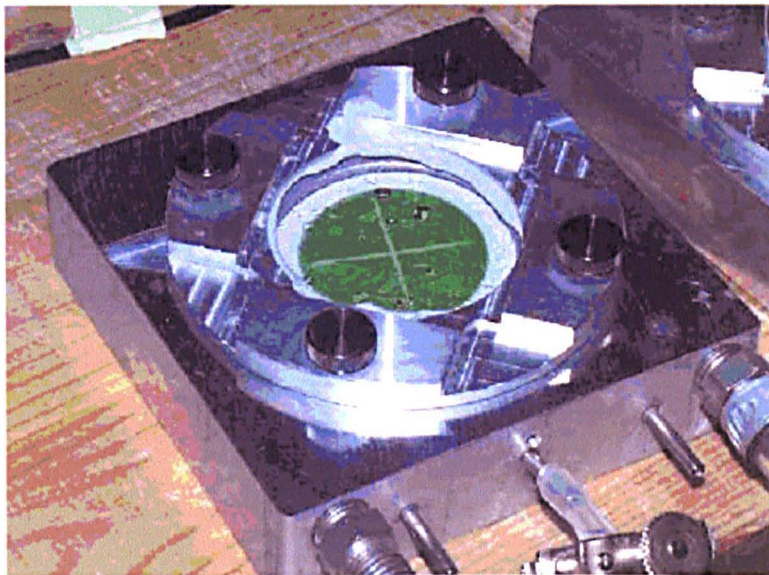
Figure 4-1 :- Sample cuvettes used for flow-through analysis of ethylene production using LPA. A. Schematic of the sample cuvette used for measuring ethylene release from liquid cultures, consisting of :- 1) oxygen electrode, 2) magnetic stirrer, 3) water inflow and exit, 4) gas inflow and exit. Culture volume was 8 ml (taken from Zuckermann *et al.*, (1997)). B. Photograph of sample cuvette for measurement of ethylene production by filtered cells. Both cuvettes were heated using a portable water heater.
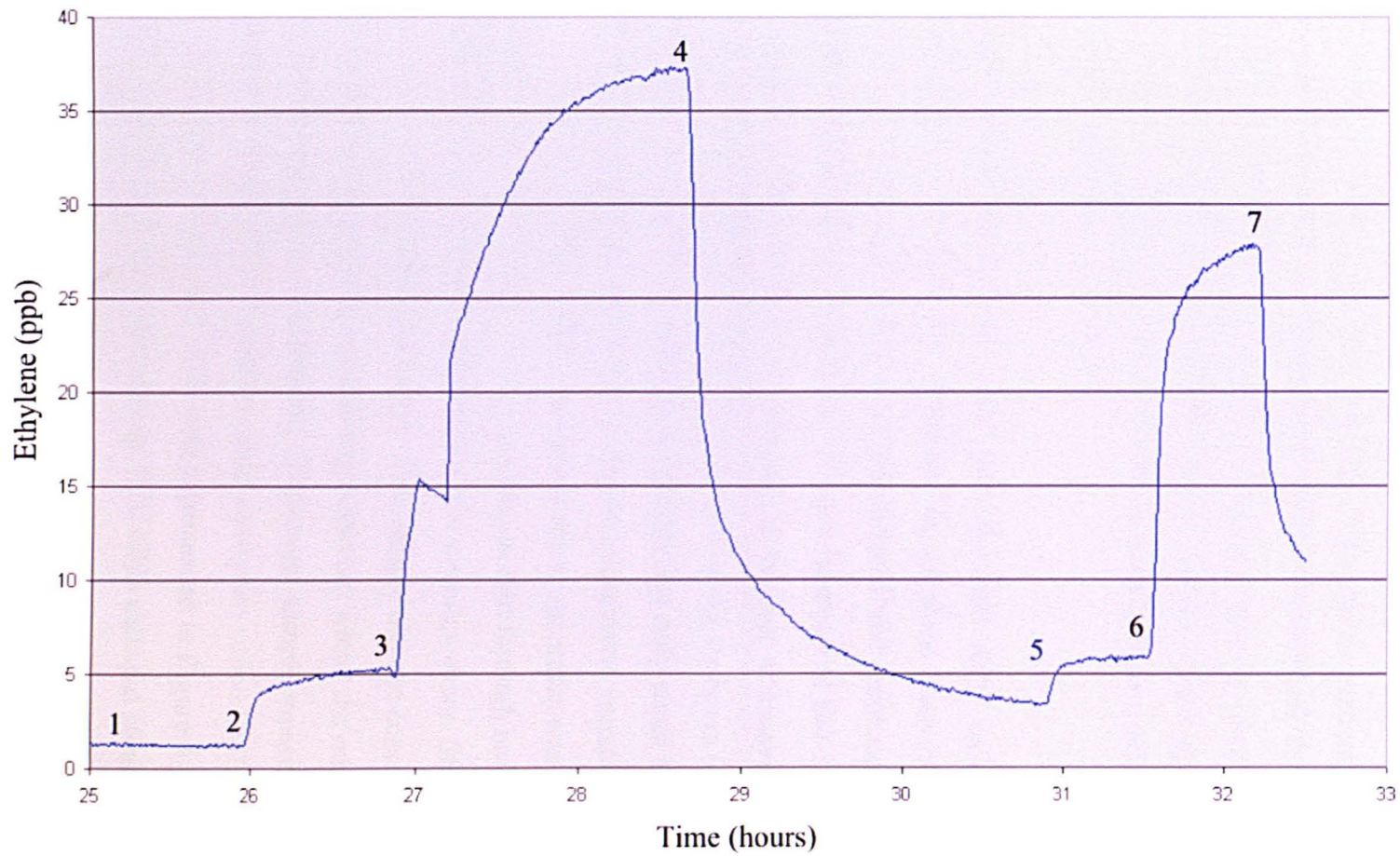
Figure 4-2 :- LPA measurement of ethylene production by *Synechocystis* sp. PCC 6803 on a membrane filter. 1) Cells plus 1 mM 2-oxoglutarate, 35 μmol m^{-2} s^{-1} white light, 2) 50 μmol m^{-2} s^{-1} white light, 3) 325 μmol m^{-2} s^{-1} white light, 4) dark, 5) 35 μmol m^{-2} s^{-1} white light, 6) 325 μmol m^{-2} s^{-1} white light irradiance, 7) dark incubation.

oxoglutarate at a 1 mM concentration, this biosynthesis appeared to be light dependent. In the presence of increasing light irradiances, the rate of biosynthesis rapidly increased. Whilst this effect was observed with duplicate cultures it remains difficult to rationalise, as cells incubated with 2-oxoglutarate for accumulation measurements did not produce any ethylene (see section 4.2.2). It would be surprising to identify an intact ethylene biosynthetic pathway in *Synechocystis* sp. PCC 6803 that utilised 2-oxoglutarate because the ethylene-forming enzyme required for this tends to be well conserved amongst species (Sato *et al.*, 1997), and a homologue has not been identified in the *Synechocystis* sp. PCC 6803 genome. Ethylene release was not observed with increasing light irradiance in the absence of 2-oxoglutarate (data not shown).

Despite apparently lacking ACC synthase and ACC oxidase, ACC was also investigated as a potential substrate for ethylene biosynthesis. As previously, *Synechocystis* sp. PCC 6803 cells were filtered and incubated at 30°C with white light at a fluence rate of 35 μmol m^{-2} s^{-1} on a reservoir of BG-11 medium. Following the addition of 1 mM ACC, an increase in the rate of biosynthesis was observed, which decreased to background after approximately six hours (see Figure 4-3 A). As the increase in the rate of ethylene evolution was only small however, an argument could be made that this result may be due to background variation.

In order to address this, the measurement apparatus was altered to incorporate a second identical cuvette. Carrier gas was passed through both cuvettes to a gating mechanism that switched between the two cuvettes every fifteen minutes, so that measurements were taken from each. One sample cuvette contained the sample cells and the appropriate substrate-containing reservoir, whilst the other contained only the sample reservoir (and the substrate). Following sample measurement, the data was corrected for background variation using a program called LMEANSO, developed at the University of Nijmegen. This data is presented in Figure 4-3 B. It was observed that ACC addition to *Synechocystis* sp. PCC 6803 cells did stimulate ethylene release via uncharacterised mechanisms.

A.
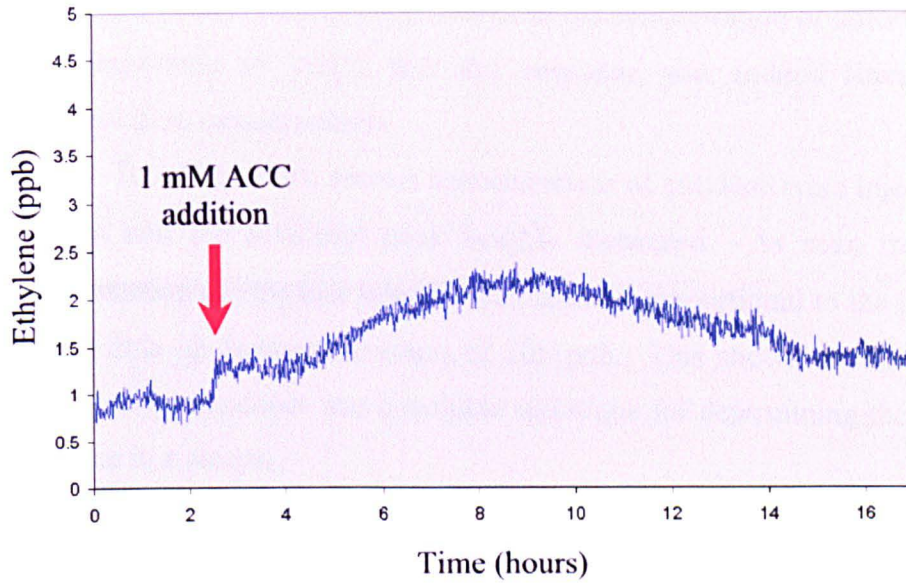
B.
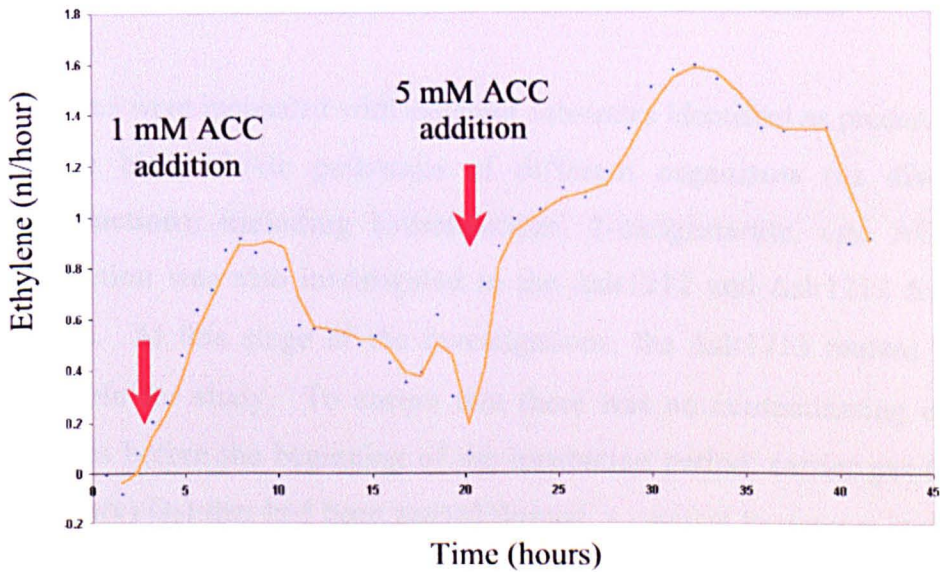
Figure 4-3 :- LPA flow-through measurements showing the effect of ACC upon ethylene biosynthesis by *Synechocystis* sp. PCC 6803 cells on a membrane filter. A. Effect of 1 mM ACC. B. Effect of 1 and 5 mM ACC. Blue dots represent measurements of ethylene release by cells corrected for background variation as determined by the use of an additional empty measurement cuvette. (see text for full details).

4.2.2. Accumulation measurements of ethylene production

Although the pressure changes caused by infra-red light absorption by ethylene molecules is theoretically proportional to the concentration of absorbing molecules, it was important to verify that the response was indeed linear before taking accumulation measurements.

To address this, known concentrations of ethylene were injected into a closed system and the resultant peak heights measured. As seen in Figure 4-4, the concentration of ethylene injected was directly proportional to the peak height in the output data up to concentrations of 100 ppb. This shows that the use of ethylene injections as standards was a suitable technique for determining the concentration of ethylene in a sample.

Preliminary measurements were carried out by incubating cyanobacterial cells on thin slivers of BG-11 solid medium in 5 ml serum bottles overnight. Samples were prepared as explained in section 2.29.1. However, these preliminary results indicated that agar releases ethylene over a period of time, and therefore this was an unsuitable method for examining ethylene production. To circumvent this problem, cells in liquid culture were incubated in serum bottles.

Cultures were incubated with different substrates identified as precursors of ethylene in the biosynthetic pathways of different organisms (as discussed in the Introduction), including L-methionine, 2-oxoglutarate, and ACC. Ethylene production was also investigated in the Δslr1212 and Δslr1212 Δslr1213 mutant strains. At this stage of the investigations, the Δslr1213 mutant strain was not available for study. To ensure that there was no contaminating ethylene in the samples before the beginning of the incubation period, carrier gas (80% (v/v) N_2, 20% (v/v) O_2) that had been passed through a catalyst to remove hydrocarbons was blown through the sealed serum bottles. All samples were incubated at 30°C overnight without shaking at a fluence rate of 50 μmol m^{-2} s^{-1} before analysis. Chlorophyll measurements were also taken, and ethylene production was expressed as nl/mg chl *a*/day.

It was immediately apparent from the data shown in Figure 4-5 that the addition of either L-methionine or 2-oxoglutarate had little or no effect on ethylene biosynthesis
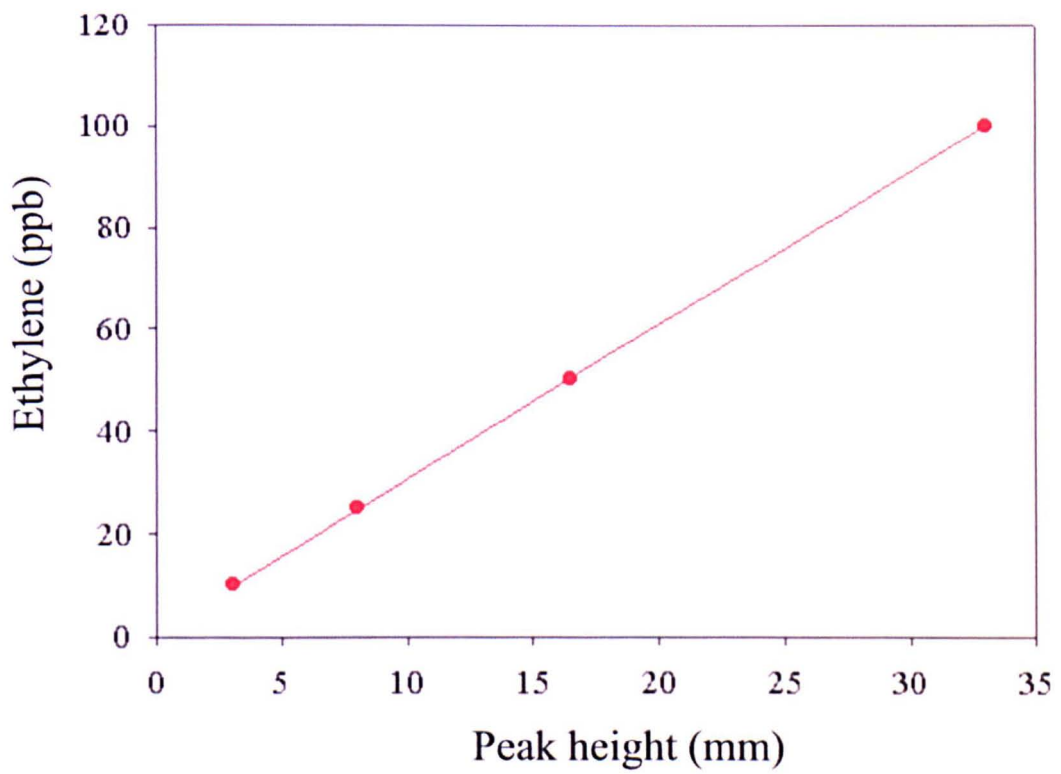
Figure 4-4 :- Calibration curve displaying the linear response of the LPA apparatus used to determine ethylene concentrations.
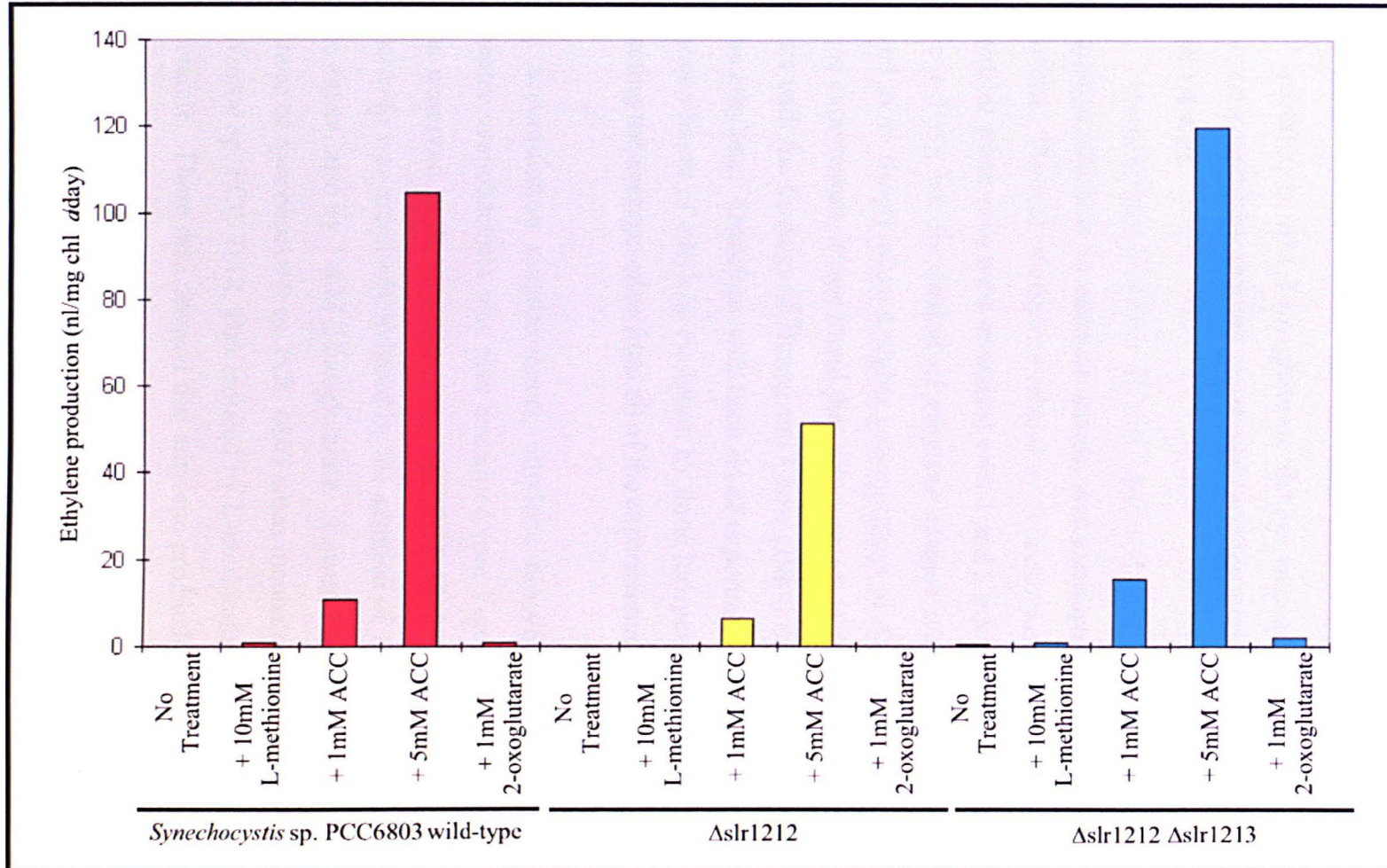
Figure 4-5 :- Laser photoacoustic measurement of ethylene production rates determined from *Synechocystis* sp. PCC 6803 wild-type, Δslr1212, and Δslr1212 Δslr1213 cells incubated with different substrates overnight at 30°C with illumination at a fluence rate of 50 μmol m^{-2} s^{-1}.

rates by *Synechocystis* sp. PCC 6803. Furthermore, it was observed that in the absence of any substrates, there was no detectable production of ethylene by cells incubated in the conditions described. This data concurred with the findings of Bleecker (1999) that *Synechocystis* sp. PCC 6803 does not produce detectable levels of ethylene. The absence of any ethylene production following incubation with both L-methionine and 2-oxoglutarate further indicated the absence of ethylene biosynthetic pathways that are conserved in other microorganisms (as discussed in section 1.4.4.2).

Interestingly however it was observed that incubation with ACC, the precursor of ethylene in vascular plants, was accompanied by a significant release of ethylene. The rate of ethylene release by *Synechocystis* sp. PCC 6803 appeared to be identical when cells were incubated with 1 and 5 mM concentrations of ACC (see Figure 4-3 B), but the period of ethylene release was sustained for longer and decayed more slowly when a higher concentration of ACC was used. In the course of these experiments it was found that solutions of ACC did release ethylene. This concurs with the findings of Huang and Chow (1984) that ACC slowly decomposes to form ethylene. Therefore, with each set of experiments this was accounted for by the measurement of ethylene evolution by three independent solutions of ACC, and subtracting the average value from all of the experimental data.

Using accumulation measurements, ethylene biosynthesis by other strains of freshwater cyanobacteria was also examined (see Figure 4-6). In contrast to the results observed with *Synechocystis* sp. PCC 6803, ethylene production by *Gloeotrichia* was massively induced by the addition of 10 mM L-methionine, and to a large extent, also by 1 mM 2-oxoglutarate. Lower biosynthetic rates (comparable with those of *Synechocystis* sp. PCC 6803 when incubated with ACC) were observed with *Nostoc* sp. PCC 7118, also induced by L-methionine, and to a lesser extent, 2-oxoglutarate. These data showed that ethylene production in these organisms could be induced separately by substrates from two different biosynthetic pathways. This phenomenon has also been previously demonstrated with the fungus *Penicillium digitatum* (Chalutz and Lieberman, 1977).
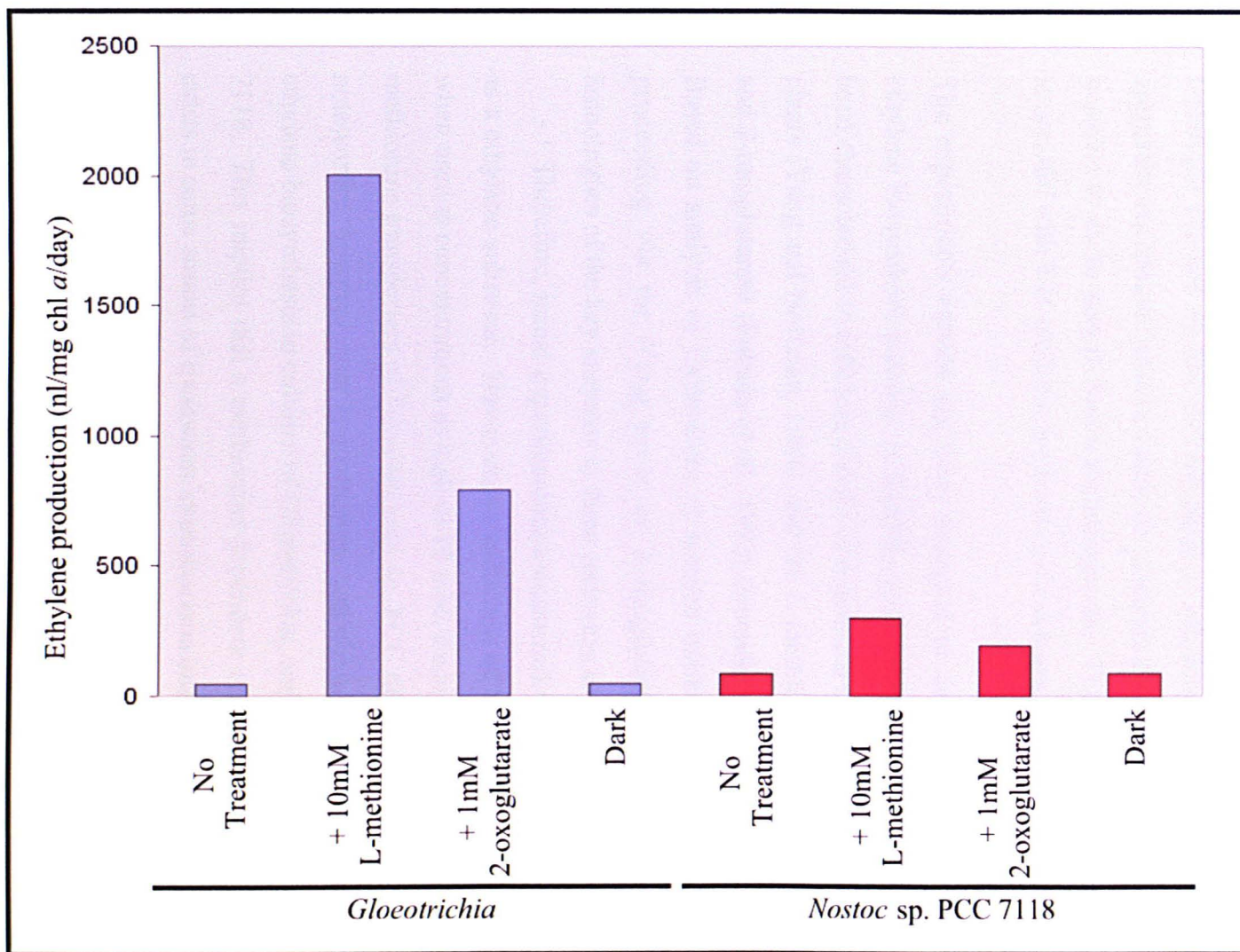
Figure 4-6 :- Laser photoacoustic measurement of ethylene production rates determined from cultures of *Gloeotrichia* and *Nostoc* sp. PCC 7118 incubated with different substrates overnight at 30°C with illumination at a fluence rate of 50 μmol m^{-2} s^{-1} (except dark-incubated cells).

4.3. Discussion

The data presented in this chapter shows the work carried out to ascertain the presence or absence of an ethylene biosynthetic pathway in *Synechocystis* sp. PCC 6803 and other freshwater cyanobacteria.

The experiments described were conducted using laser photoacoustic spectroscopy, a very sensitive technique for trace gas analysis. The sensitivity of this technique allowed on-line measurement of ethylene evolution. Also due to other specialist equipment present (such as catalysts for hydrocarbon removal), it was possible to make accumulation measurements. This approach did not prove to be successful with less sensitive apparatus such as a gas chromatograph.

The experiments carried out were designed to detect the presence of an intact ethylene biosynthetic pathway in *Synechocystis* sp. PCC 6803. Three pathways have been characterised in different classes of organisms to date: the Yang cycle of higher plants (Yang and Hoffman, 1984), and the L-methionine (Ince and Knowles, 1985) and 2-oxoglutarate (Fukuda *et al.*, 1992) dependent pathways in microorganisms. Based on analysis of CyanoBase, it seemed unlikely that biosynthetic pathways proceeding via the Yang cycle or 2-oxoglutarate would be discovered, as homologues of the key enzymes in these pathways could not be identified.

Therefore, initial experimentation examined whether L-methionine could act as a ethylene substrate. However, no induction of biosynthesis was observed even when used at concentrations as high as 10 mM, a concentration known to saturate the methionine transporters of *Synechocystis* sp. PCC 6803 (Labarre *et al.*, 1987). It is noteworthy, however, that L-methionine was found to induce a very high rate of ethylene biosynthesis in cultures of *Gloeotrichia*, and a lower rate in *Nostoc* sp. PCC 7118. This implies that a methionine-dependent ethylene biosynthesis mechanism exists in some strains of freshwater cyanobacteria and not others.

It is difficult to rationalise how 2-oxoglutarate was found to cause ethylene release when added to *Synechocystis* sp. PCC 6803 cells in the solid phase, but not when added to liquid cultures. It is possible that the release of ethylene caused by 2-oxoglutarate was the result of a stress response. All plants are known to release ethylene following environmental stresses such as flooding and pathogen invasion,

and the addition of 2-oxoglutarate could have resulted in the non-specific release of ethylene by virtue of its acidity. Cells cultured with 1 mM 2-oxoglutarate for three days were found to be non-viable (data not shown). It is also possible that 2-oxoglutarate addition caused membrane degradation, which in certain situations may also result in ethylene release (Dr. Frans Harren, pers. comm.), and hence would not be the result of a biosynthetic mechanism. However, this hypothesis does not address the light dependency of this phenomenon. A possibility is that increasing light irradiance contributed to membrane damage, in concert with 2-oxoglutarate.

When *Synechocystis* sp. PCC 6803 cells were incubated with ACC, it was observed that this caused a release of ethylene that could be observed with both flow-through and accumulation measurements. Given the absence of homologues of key enzymes required for this pathway in higher plants, and that L-methionine did not result in ethylene release, it seems likely that ACC is converted to ethylene via a novel mechanism or that as with 2-oxoglutarate, ACC addition results in a non-specific stress response (see section 5.6.3 for further experimentation regarding this). The Δslr1212 and Δslr1212 Δslr1213 mutant strains of *Synechocystis* sp. PCC 6803 generated in this study released ethylene in the same way as wild-type cells.

In summary, work described here demonstrated that *Synechocystis* sp. PCC 6803 could produce ethylene in response to 2-oxoglutarate and ACC. It is not clear, however, whether this is a result of direct biosynthesis in a physiological process, or indirectly induced by membrane degradation. Furthermore, it was found that two other strains of freshwater cyanobacteria, *Gloeotrichia* and *Nostoc* sp. PCC 7118, could produce ethylene via mechanisms not conserved in *Synechocystis* sp. PCC 6803.

Chapter 5

Phenotypic characterisation of the Δslr1212, Δslr1213, and Δslr1212 Δslr1213 mutants

5. Phenotypic characterisation of the Δslr1212, Δslr1213, and Δslr1212 Δslr1213 mutants

5.1. Introduction

This chapter addresses one of the main aims of the study, which is the phenotypic characterisation of the Δslr1212, Δslr1213 and Δslr1212 Δslr1213 mutants described in Chapter 3.

These experiments were designed based on insights into the possible functioning of this proposed two-component signal transduction system gained from analysis of the available protein sequences. As discussed in the Introduction, the most likely role for these proteins is an involvement with either light sensing and/or signal transduction, or ethylene sensing.

As results in Chapter 4 have already shown, *Synechocystis* sp. PCC 6803 and other freshwater cyanobacteria release ethylene in response to ACC, L-methionine and 2-oxoglutarate, and this suggests that ethylene may act as a signalling molecule for which there must be a sensor. Other investigations have revealed that Slr1212 has a functional ethylene-binding-domain (Rodriguez *et al.*, 1999). A logical next step therefore would be to characterise a role for ethylene production in *Synechocystis* sp. PCC 6803.

During the phenotypic characterisation of the mutants the following were examined: growth of the mutants under different light qualities and irradiances, characterisation of the photosynthetic machinery, light sensing as examined by phototactic responses, the role of slr1212 and slr1213 in copper homeostasis and the effect of ethylene and ACC addition on whole cell protein profiles.

5.2. Growth of mutants

Synechocystis sp. PCC 6803 is a phototroph, and as a consequence the ability to cope with a continuously changing light environment is essential. Photosynthetic organisms are equipped with an array of photoreceptors to sense and respond to altering intensity, duration, quality and direction of light (Chory, 1997). Acclimation to the light environment by higher plants involves many factors including "shade avoidance" responses such as germination, phototropism and induction of flowering. These responses are mainly regulated by phytochromes and cryptochromes (Ballare,

1999). Acclimation at the chloroplast level involves another set of responses governing the composition of the photosynthetic apparatus within each chloroplast. Interestingly, in a study of acclimation by *Arabidopsis thaliana* it was observed that photoreceptor mutants were capable of light-dependent changes in the composition of the chloroplasts, although evidence suggested that responses mediated by photoreceptors share regulatory components with acclimation, or regulate components which in turn regulate acclimation (Walters *et al.*, 1999). Until recently however, little has been known about the mechanisms of light sensing and signal transduction in cyanobacteria.

5.2.1. Analysis of growth characteristics

Growth was examined under low (13 μmol m^{-2} s^{-1}) and normal light irradiancies (30 μmol m^{-2} s^{-1}), red light (600-730 nm; 28 μmol m^{-2} s^{-1}) and blue light (350-580 nm; 10 μmol m^{-2} s^{-1}). Figure 5-1 shows the calculated doubling times.

Under all light regimes used, no significant differences were found in the growth rates between the wild-type and any of the mutant strains. Under white light, it was observed that doubling times were reduced by up to 55% when the irradiance was reduced from 30 to 13 μmol m^{-2} s^{-1}. This is an approximately proportional reduction in growth rate.

Growth rates were reduced under red light when compared with white light, despite a similar irradiance. This reduced growth rate could be due to a reduction in the amount of photosynthetically available light. *Synechocystis* sp. PCC 6803 has three main light-harvesting pigments: chlorophyll *a*. (λ_{max}=~686 nm), phycocyanin (λ_{max}=617 nm) and allophycocyanin (λ_{max}=650 nm) (Toole *et al.*, 1998). The transmission of 617 nm light through the red filters is only approximately 20% that of white light, and this may reduce the level of light-harvesting under these conditions, due to a lack of light harvestable by phycocyanin.

Under blue light, there was no observable alteration in the growth rates between wild-type and mutant strains. In comparison with cells cultured in low light conditions, the growth rates were slightly decreased (for wild-type, a doubling time of 33.95 hours ± 0.92 under blue light compared to 24.39 hours ± 1.43 in low white light) despite a similar irradiance. This is probably because of the reduction of light in the 580-650 nm region to a minimum, reducing light absorption by phycocyanin
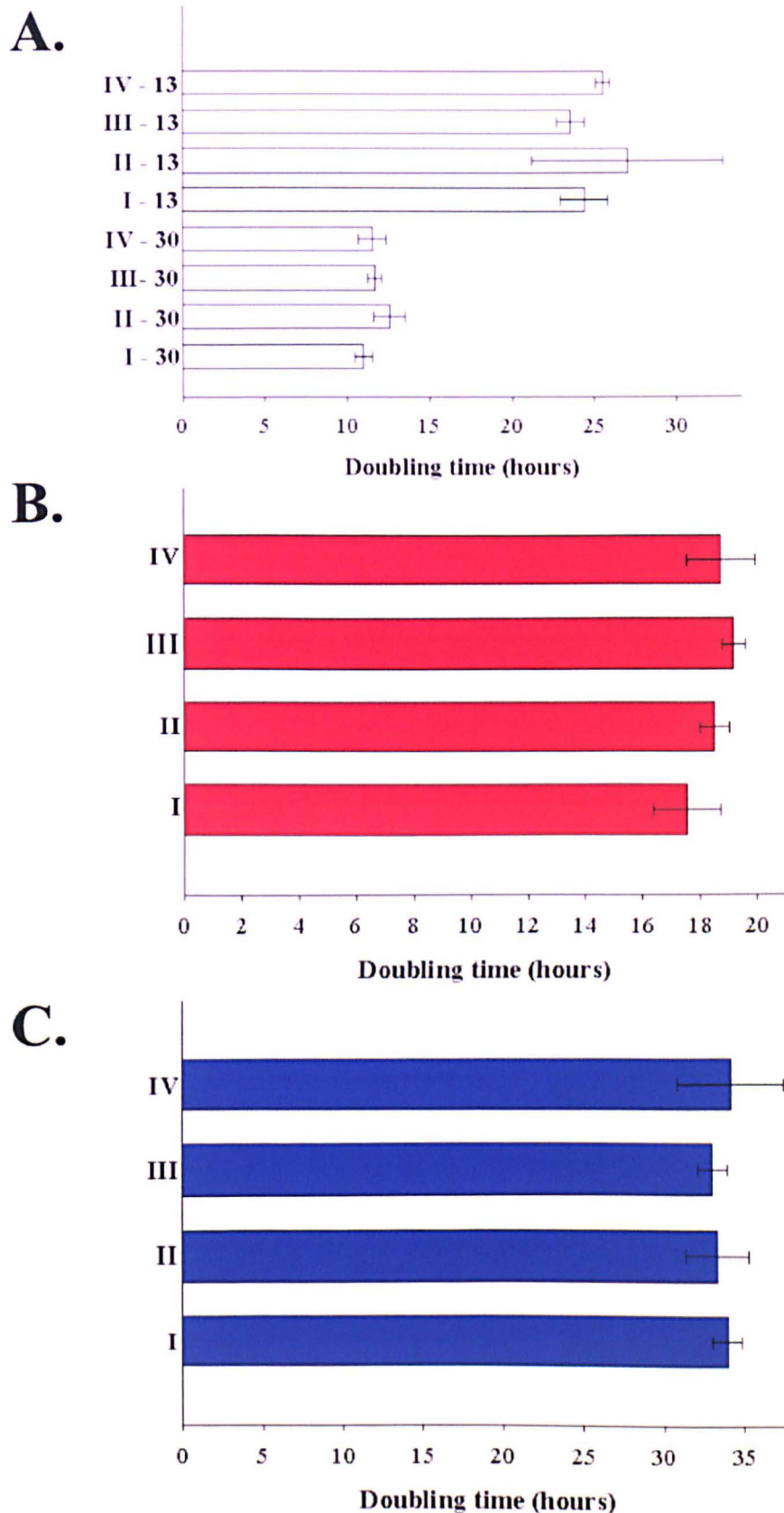
Figure 5-1 :- Doubling times of wild-type and mutant cells in different light qualities. A. White light at 30 and 13 μmol m^{-2} s^{-1} irradiance. B. Red light (600-730 nm) at 28 μmol m^{-2} s^{-1} irradiance. C. Blue light (350-580 nm) at 10 μmol m^{-2} s^{-1} irradiance. Data points are represented as means ± SD (n=3, from 1 experiment). I = Wild-type; II =Δslr1212; III =Δslr1213; IV =Δslr1212 Δslr1213.

and allophycocyanin.

Interestingly, it has also been reported that a Δslr1212 mutation created in a glucose-tolerant strain of *Synechocystis* sp. PCC 6803 has a lethal phenotype. The mutant strain was found to culture very poorly in any light quality, and could not be maintained. A phenotype for a Δslr1212 mutation in the PCC 6803 strain was not found (Wilde, 2000). The results from this study do not concur with these findings, when it proved possible to select for a Δslr1212 mutation in the glucose-tolerant strain *Synechocystis* sp. PCC 6803 FJ. This mutant did not show an impaired growth phenotype under any light regime (data not shown).

These results indicate that Slr1212 and Slr1213 may not play an obvious role in the adaptation of *Synechocystis* sp. PCC 6803 to a changing light environment. In such a situation, it might be expected to observe reduced growth rates of the mutants under certain light conditions when compared with wild-type cells. In the absence of a key sensor-kinase system involved in the sensing and adaptation mechanisms, the photosynthetic apparatus would not be optimised to maximise light-harvesting which would be reflected by a reduction in growth rate.

A role for these proteins in light sensing and adaptation responses should not be discounted however. The presence of a large number of phytochrome-like proteins in the genome indicates that a complex system might exist for sensing light quality, similar to the phytochrome A-E family of *Arabidopsis thaliana*. Mutants and overexpressor plants suggest that the individual phytochrome subtypes (A-E) have distinct, but overlapping roles (Smith, 2000; Whitelam and Devlin, 1997). There is also evidence of crosstalk between the pathways triggered by phytochrome A and B (Nagy and Schäfer, 1999). Therefore, in *Synechocystis* sp. PCC 6803 there may be interplay between the different sensing mechanisms, and redundancy may exist in the system. In such a complex system, removing one of the sensors and/or response regulators may cause a subtle phenotype which is difficult to identify.

Additionally, Slr1212 has a functional ethylene-binding domain (Rodriguez *et al.*, 1999). As discussed in the Introduction, ethylene controls a wide range of developmental and stress processes in higher plants including seed germination, fruit

ripening, stem elongation, drought and pathogen attack responses (Kieber and Ecker, 1993). The most applicable function to cyanobacteria however is the involvement of ethylene in promoting senescence in plants of both flowers and, in particular, leaves. Ethylene stimulates many activities associated with senescence including the decline of chlorophyll, proteins and starch, and an increase in the activity of many hydrolytic enzymes (Quirino *et al.*, 2000). The *etr1* mutant of *Arabidopsis thaliana* was shown to have a delayed onset of senescence (Grbic and Bleecker, 1995). It is generally accepted that all chloroplasts are derived from a single cyanobacterial ancestor (Cavalier-Smith, 2000; Wolfe *et al.*, 1994), and it is possible that they represent a prokaryotic ancestor of both phytochromes and ethylene receptors. It is quite possible therefore, that senescence, in particular chlorosis, may have an evolutionary relationship with cyanobacteria.

Although no alterations in the growth rates of the Δslr1212, Δslr1213 and Δslr1212 Δslr1213 mutants in *Synechocystis* sp. PCC 6803 were observed, the domain structure of Slr1212 and the predicted roles of these domains still suggests a possible role in light-sensing mechanisms and/or responses to light, and hence further characterisation of the photosynthetic machinery was carried out.

5.3. Characterisation of the photosynthetic machinery

To further characterise the Δslr1212 and Δslr1213 mutants a preliminary analysis of photosynthesis and the photosynthetic machinery was carried out, as rationalised in section 5.2.1.

77K fluorescence and scanning spectroscopy were employed to provide detailed information about the photosystems and their regulation. Oxygen electrode studies were used to measure photosynthetic electron transport rates.

5.3.1. Effect of light intensity on cell chlorophyll content

Preliminary analysis of *Synechocystis* sp. PCC 6803 strains with mutations in some of the phytochrome-like proteins identified in the genome has already been conducted by other research groups (Batschauer, 1998; Wilde *et al.*, 1997; Yeh *et al.*, 1997). It was observed that *cph1* mutants had a marked decrease in chlorophyll content per cell when cultured under high irradiance, leading to speculation that the

phytochrome-like proteins may have a role in regulating cellular metabolism in higher light irradiances (Fiedler *et al.*, 2000).

As the phytochrome-like proteins in *Synechocystis* sp. PCC 6803 have been implicated in regulating cell metabolism to light irradiance, this was examined in the mutant strains generated in this study. One litre cultures were exposed to a constant light environment for at least 40 hours to allow acclimation before chlorophyll measurements and cell counts were taken.

The results in Figure 5-2 show that there is no apparent alteration in the regulation of cellular chlorophyll content in the mutant strains. Furthermore, approximately equal levels of chlorophyll per cell were observed in cells cultured in both normal and high light irradiances. This observation concurs with studies in *Arabidopsis thaliana*, where alterations in chlorophyll content per unit leaf area when acclimated to high (400 μmol m^{-2} s^{-1}) and low (100 μmol m^{-2} s^{-1}) light irradiances were minor (Walters *et al.*, 1999).

5.3.2. SDS-PAGE and western analysis of the photosynthetic machinery

To further characterise the light-harvesting complexes of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 mutant cells, two approaches were taken. Firstly, SDS-PAGE analysis was carried out on soluble and membrane fractions to identify any major differences between wild-type and mutant strains. The proteins that comprise the light-harvesting apparatus of *Synechocystis* sp. PCC 6803 are very abundant. As a result, any major alterations to the photosystems may be identifiable by an analysis of total cell proteins. Secondly, a more directed approach was taken to analyse the cellular content of PSI by using an antibody specific to two of the constituent polypeptides of PSI reaction centres (PSI was chosen specifically for analysis due to results gained from 77K fluorescence spectroscopy studies in section 5.3.3 on page 137).

Based upon the previous analysis of ethylene receptors in tomato which indicated that expression was very low (Payton *et al.*, 1996), it was thought unlikely that the localisation of Slr1212 could be confirmed by SDS-PAGE analysis due to a low expression, although it was hoped that through comparison of cell fractions from wild-type and Δslr1212 cells that this might prove possible. The use of SDS-PAGE
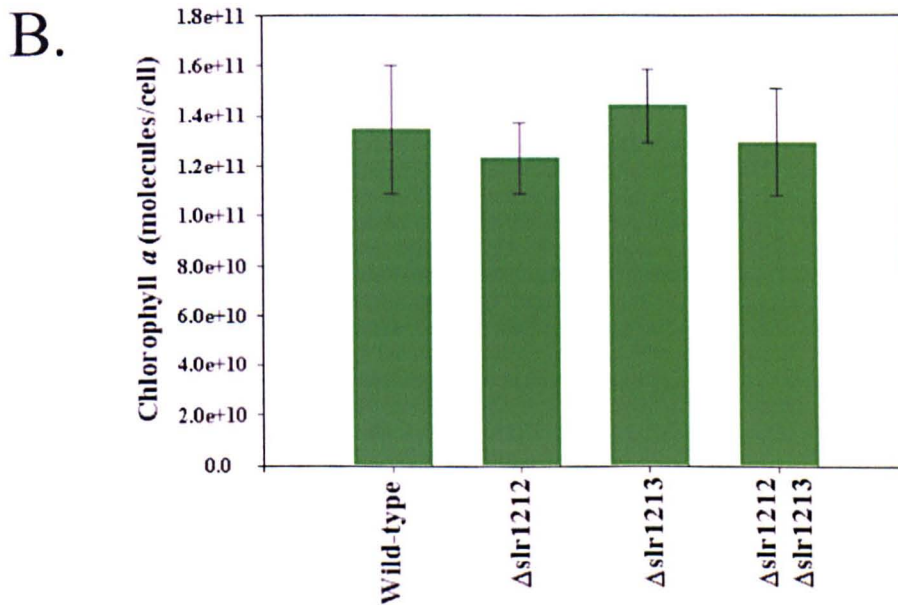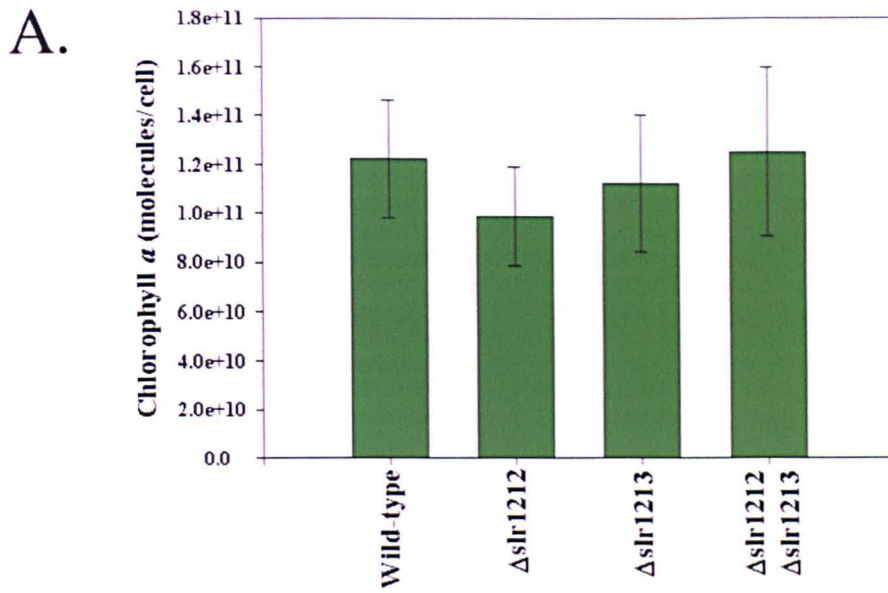
Figure 5-2 :- Chlorophyll content per cell was determined for *Synechocystis* sp. PCC 6803 wild-type and mutant strains cultured at 30°C at fluence rates of A. 35 μmol m^{-2} s^{-1} and B. 100 μmol m^{-2} s^{-1}. No alterations in the regulation of chlorophyll content per cell were observed for mutant strains. Higher irradiance had a negligible impact upon chlorophyll content. Data represented as means $\pm$ SD (n=3).

analysis could also reveal any other significant alterations in the protein profiles of wild-type and mutant cells. Soluble and thylakoid fractions were prepared from cells cultured in 30 μmol m^{-2}s^{-1} white light irradiance as described in section 2.20 on page 88, and analysed on a 6 to 24% (w/v) protein gradient gel (see Figure 5-3).

The predicted mass of Slr1212 based on sequence analysis is 97.3 kDa, and in this region there is no evidence of a protein present in the wild-type that is absent in the mutant (even with silver staining). This could suggest that ORF slr1212 was not expressed in those conditions, although subsequent analysis has proved that ORF slr1212 was transcribed in these conditions (see Chapter 6). It is obvious that more sensitive techniques such as western blotting (which would necessitate the over-expression of Slr1212, or peptide antibody production in order to produce antibodies) or immunotagging (attachment of an immunogenic epitope to the target) would be necessary to localise Slr1212 *in vivo*. Although specific antibodies exist against *etr1* of *Arabidopsis thaliana*, these were raised against the UNK domain which has only very limited similarity with the Slr1212 protein and are unlikely to cross-react (Bleecker, 1998; Schaller *et al.*, 1995). Under the growth conditions described above, no differences were observed in the protein profiles of the soluble fractions, but two major differences were seen in the membrane fractions, in proteins of approximately 32 and 10 kDa (see Figure 5-3 A-B). These proteins coincide with the sizes of known polypeptides from the reaction centres of PSI and PSII, specifically PsbO (33kDa) and PsaD (8kDa). Both of these proteins are present at reduced concentrations in the membranes of Δslr1212 cells, and although the evidence is circumstantial, this would be consistent with a reduction in the amount of PSI per cell in the mutant. However, it did not prove possible to duplicate this result from parallel cultures.

In an effort to characterise these alterations further, western blotting was carried out using a polyclonal antibody PSI-9 (Mann *et al.*, 2000) against PSI reaction centre polypeptides. Total membranes (thylakoid membranes and cell wall fraction) were prepared as described in section 2.20 from *Synechocystis* sp. PCC 6803 and Δslr1212 cells cultured under standard conditions (30 μmol m^{-2}s^{-1} white light irradiance). Membranes were analysed on both an equal protein and an equal chlorophyll basis (see Figure 5-3 C).

The western blot shows clearly that there is no significant reduction in the
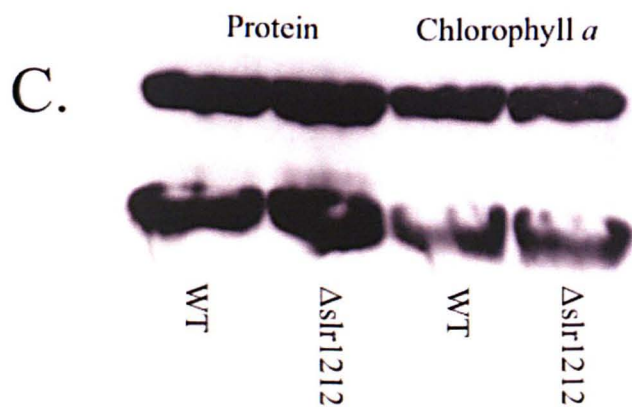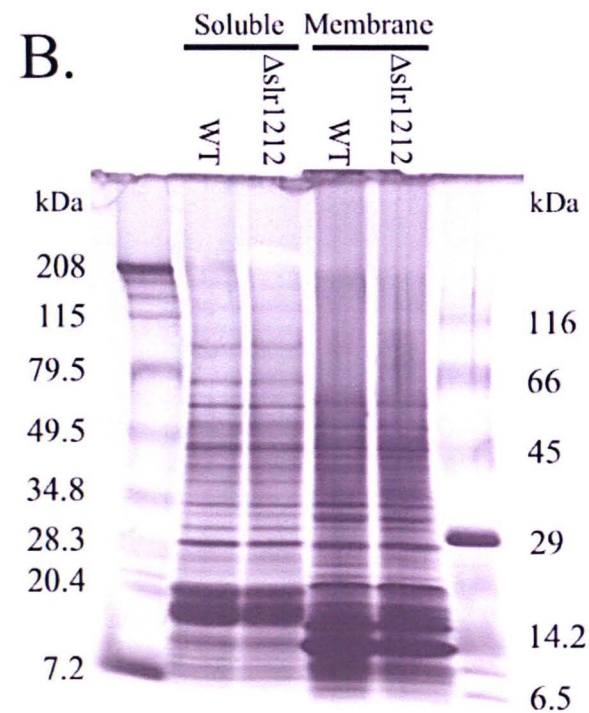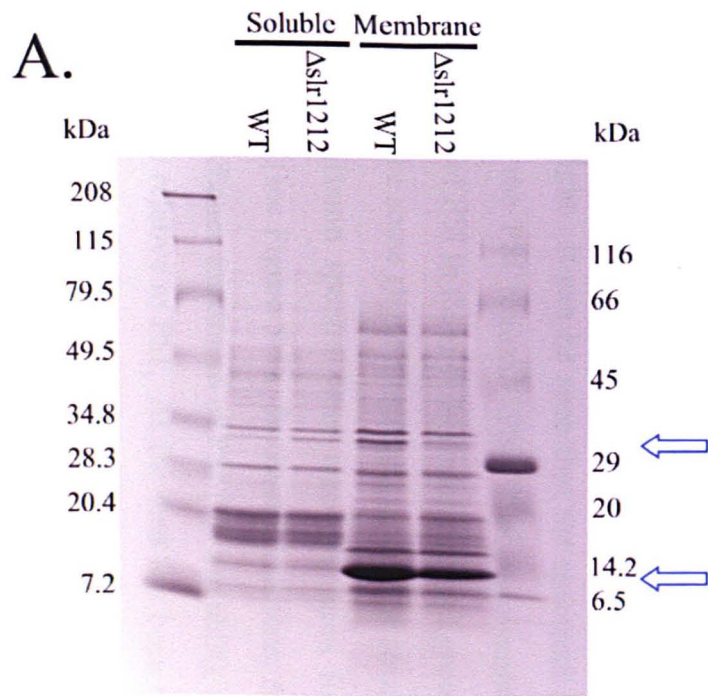
Figure 5-3 :- A-B. 6-24% (w/v) SDS-PAGE analysis of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cell fractions when cultured in 30 μmol m^{-2} s^{-1} white light irradiance. A. Coomassie and B. silver stained gels. The blue arrows highlight differences. 50 μg soluble protein was loaded per lane and 6 μg chlorophyll *a* was loaded per lane for membrane fractions. C. Western analysis of total membranes prepared from *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells cultured under standard conditions using PSI-9 antibody against two PSI polypeptides. Samples were loaded with 15 μg protein or 1 μg chlorophyll *a* per lane.

cellular content of PSI reaction centres in *Synechocystis* sp. PCC 6803 Δslr1212 cells in contrast to that implied from the SDS-PAGE analysis of cell membrane fractions when cultured in 30 μmol m^{-2} s^{-1} white light irradiance.

5.3.3. 77K fluorescence spectroscopy

Low temperature fluorescence and scanning spectroscopy were carried out to investigate further the potential role of Slr1212 in regulating light adaptation responses. Low temperature fluorescence spectroscopy can be used for a very sensitive analysis of photosystem structure and also photosystem stoichiometry. If Slr1212 acts as a sensor regulating cell adaptation to a changing light environment, it is likely that such responses would involve regulating the levels of photosystem proteins either through transcriptional or translational pathways, and/or possibly by acting upon protein degradation pathways, because *Synechocystis* sp. PCC 6803 cannot chromatically adapt to its light environment. Alterations in these pathways which affect the photosystems directly would be identified by these techniques.

Experiments were carried out on wild-type and Δslr1212 cells that had been cultured in normal light conditions (30 μmol m^{-2} s^{-1} white light) and high light conditions (90 μmol m^{-2} s^{-1} white light). Cells were acclimatised to their light environment for at least three days before measurements were taken. Figure 5-4 displays the emission spectra of wild-type and Δslr1212 cells cultured in 30 μmol m^{-2} s^{-1} white light. It can be seen that the spectrum for Δslr1212 cells is slightly red-shifted in the PSI region. This shift represents a 2 nm shift in the absorbance maximum of PSI in these cells under standard growth conditions. This shift suggests there may be some slight alterations in the PSI structure of Δslr1212 cells.

Emission spectra were also measured from cells cultured in high light conditions (see Figure 5-5). In these conditions, Δslr1212 cells maintain a red shift in the absorbance maximum of PSI (2.5 nm shift), and also show an increase in PSII absorbance relative to PSI. As the data has been normalised to the PSI absorbance maximum, this alteration could signify either an increase in the amount of PSII or a decrease in the amount of PSI per cell, relative to wild-type cells cultured in the same conditions. To determine which alternative is correct, scanning spectroscopy was carried out on both wild-type and Δslr1212 cells cultured in white light at both 30
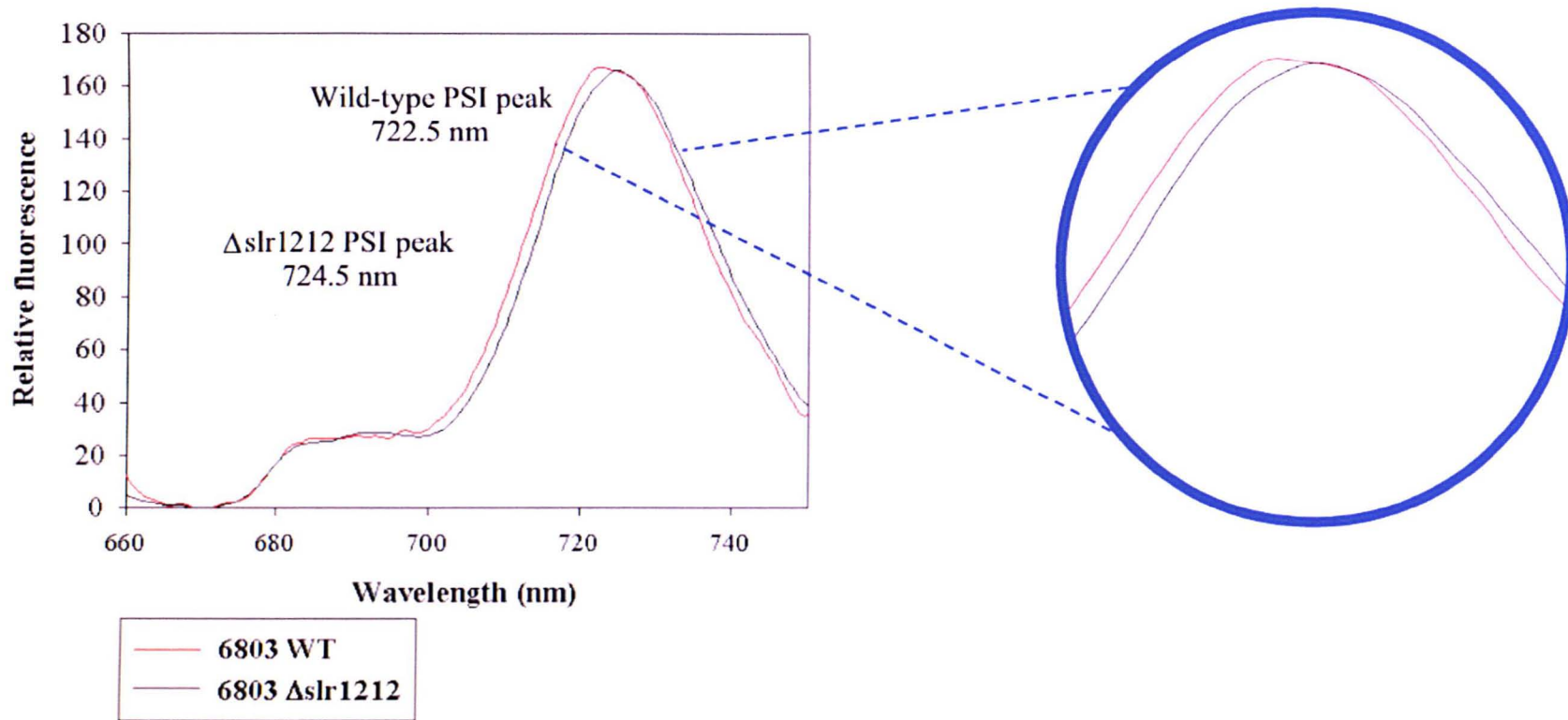
Figure 5-4 :- 77K emission spectra of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells cultured in 30 μmol m^{-2} s^{-1} white light irradiance. The dashed lines magnify the region between 710 and 730 nm to show the red shift. The excitation wavelength was 435 nm. Data normalised at 724.5 nm.
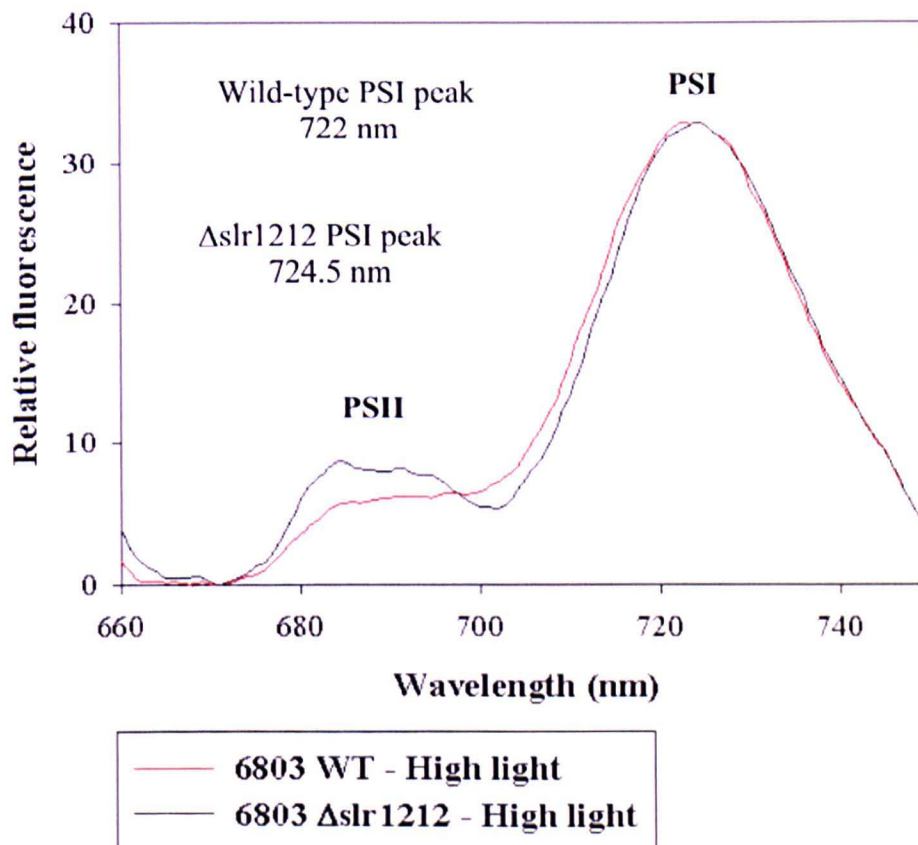
Figure 5-5 :- 77K emission spectra of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells cultured in 90 μmol m^{-2} s^{-1} white light irradiance. The excitation wavelength was 435 nm. Data normalised at 724.5 nm.

and 90 μmol m^{-2} s^{-1} irradiance.

Scanning spectroscopy measures absorbance by cells across a range of wavelengths, and from the maximum absorbance at 617 nm and 686 nm can be used to determine the ratio of phycocyanin to chlorophyll *a*.

	Standard light	High light
Wild-type	1 : 1.09	1 : 1.12
Δslr1212	1 : 1.12	1 : 0.77

Table 5-1:- Ratio of phycocyanin to chlorophyll a. of wild-type and Δslr1212 cells cultured under standard (30 μmol m^{-2} s^{-1}) and high (90 μmol m^{-2} s^{-1}) white light.

The data in Table 5-1 shows the calculated ratio of phycocyanin to chlorophyll *a* in wild-type and mutant cells. Although this ratio did not alter significantly in wild-type cells when cultured in normal and high light, when Δslr1212 cells were cultured in high light a substantial reduction in the amount of chlorophyll *a* relative to phycocyanin was observed. This data, along with the emission spectrum showing altered stoichiometry of the photosystems and a red shift in PSI absorbance is consistent with a reduction in the amount of PSI per cell, along with possible structural alterations in PSI.

When *Synechocystis* sp. PCC 6803 is shifted from a low to high light environment, the typical response of the cell is to switch off the production of PSI proteins, causing an increase in the ratio of PSII:PSI (Mullineaux, 2000). Therefore, the data suggests that Δslr1212 cells have an enhanced response to a high light shift. This is illustrated by the spectra in Figure 5-6, showing a small increase in the PSII:PSI ratio for wild-type cells when shifted from low to high light, and a much larger increase for Δslr1212 cells.

This result clearly implicates Slr1212 in light adaptation mechanisms in *Synechocystis* sp. PCC 6803, with an emphasis on adaptation to light intensity. In combination with the results from Chapter 4, demonstrating the ability of
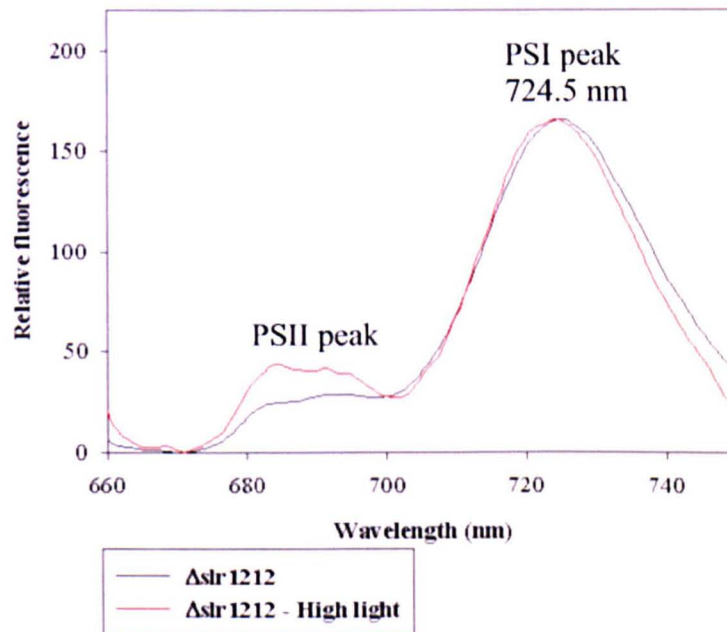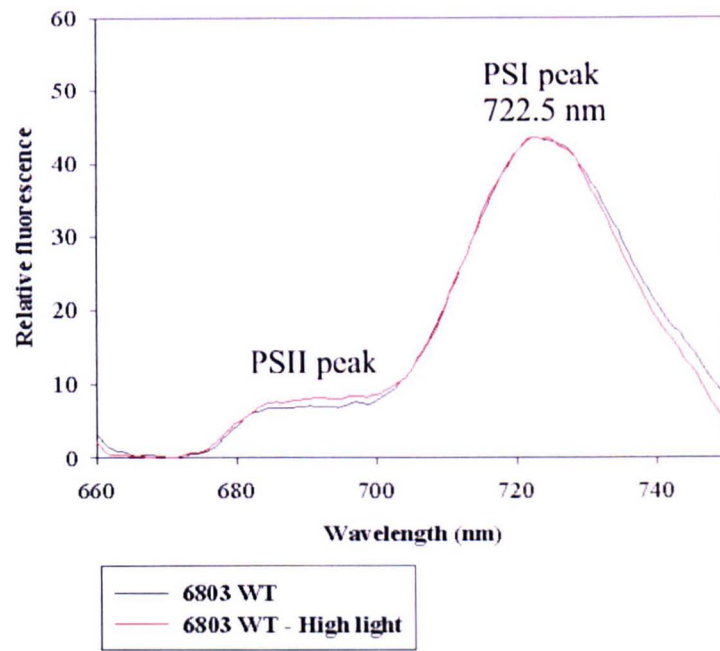
Figure 5-6 :- 77K fluorescence emission spectra of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells cultured in low (30 μmol m^{-2} s^{-1}) and high (90 μmol m^{-2} s^{-1}) white light irradiance highlighting the enhanced high light response by Δslr1212 cells.

Synechocystis sp. PCC 6803 to produce ethylene when incubated with specific substrates, this data increases the likelihood that Slr1212 may function as a receptor involved in both light sensing and ethylene sensing.

Although the western analysis carried out in section 5.3.2 did not reveal a reduction in the cellular content of PSI polypeptides under standard conditions, the 77K emission spectra and scanning spectra presented here suggests that the cellular content of PSI may be reduced under higher light intensities.

5.3.4. Oxygen evolution and respiration

The rates of CO_2-dependent oxygen evolution and oxygen uptake were also measured in *Synechocystis* sp. PCC 6803 and mutant strains. If there is an involvement for Slr1212 in adapting a cell to its light environment to maximise light-harvesting, it is possible that a mutant strain would show alterations in photosynthetic rates under saturating illumination. The fixation of carbon dioxide also regulates P_{max} (the light saturated rate of oxygen evolution) and this could also be light regulated. Photosynthetic oxygen evolution involves a large number of electron transport reactions involving the water-oxidizing complex, PSII, the cytochrome b_6f complex, PSI and low molecular weight electron carriers (Samuilov and Fedorenko, 1999). A regulatory role for Slr1212 and Slr1213 in any of these processes could affect electron transfer rates.

Dark incubated *Synechocystis* sp. PCC 6803 consumed molecular oxygen, and evolved oxygen in the presence of saturating illumination as shown in Figure 5-7 A. The rates of oxygen evolution under saturating light and oxygen uptake in the dark were measured for wild-type, Δslr1212, Δslr1213, and Δslr1212 Δslr1213 liquid cultures, as detailed in Figure 5-7. From these results, there is no indication that photosynthetic or respiratory electron transport are impaired in the mutant strains, showing that disruption of the ORFs slr1212 and slr1213 does not significantly affect the function of the photosynthetic apparatus in *Synechocystis* sp. PCC 6803 under the parameters of these experiments. It could perhaps be argued that dark respiration in the mutant cell lines is increased relative to wild-type cells, but there is overlap in the standard deviations and consequently significance cannot be attributed to this result.

A. 50 nmoles O$_2$

Light on   Light off   3 minutes

B.

C.
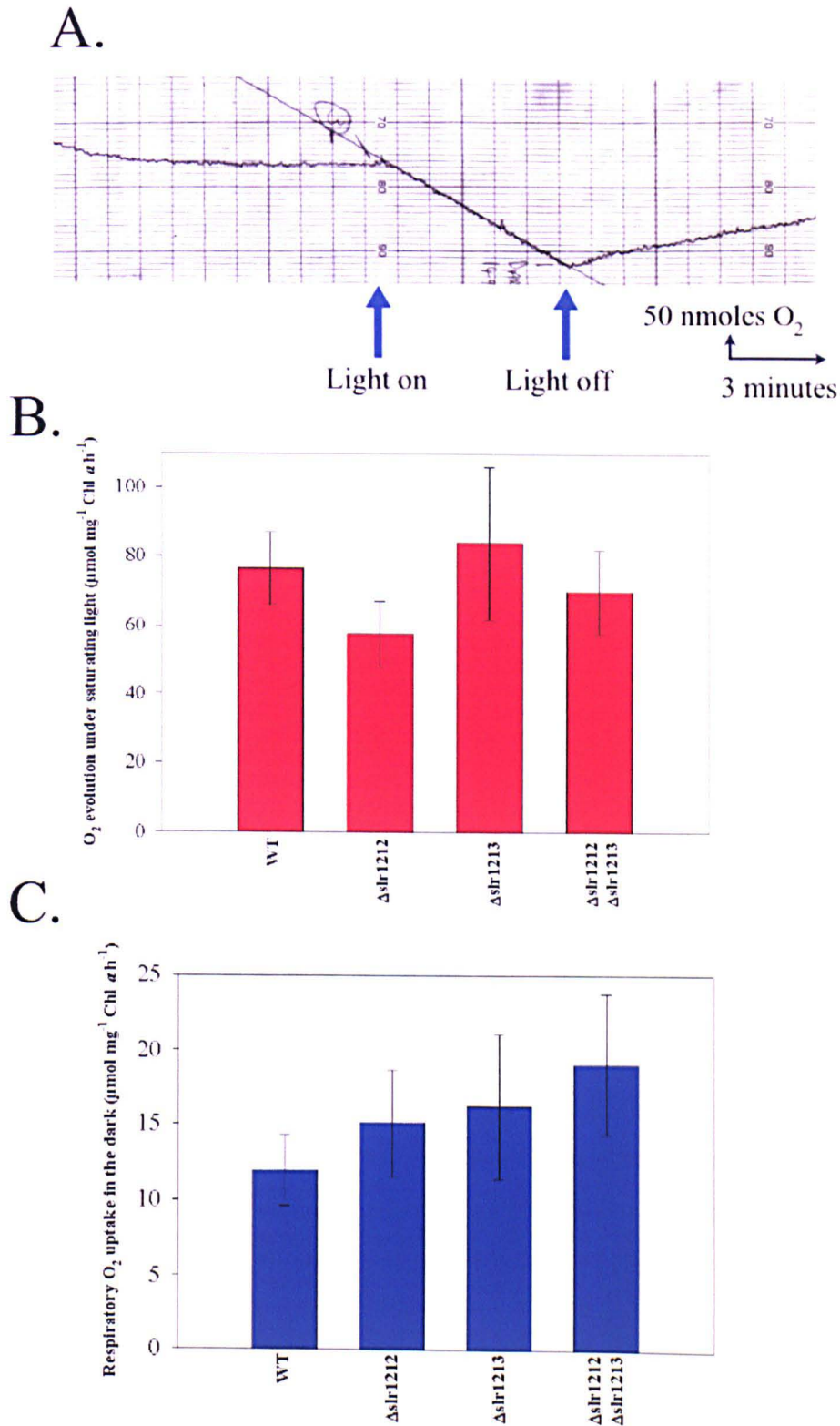
Figure 5-7 :- A. CO$_2$ dependent O$_2$ evolution by illuminated *Synechocystis* sp. PCC 6803 cells. B. Rates of O$_2$ evolution by cells under saturating illumination. C. Rates of respiratory O$_2$ uptake by cells in the dark. Data points represented as means ± SD (n=3-5, from 1-2 experiments.)

The figures gained for oxygen evolution and respiratory oxygen evolution are similar with those found in two independent studies. Emlyn-Jones *et al.* (1999) found that wild-type cells evolved oxygen in saturating light conditions at a rate of 211 ± 21 μmol mg^{-1} Chl *a* h^{-1}, and took up oxygen at a rate of 26 ± 2 μmol mg^{-1} Chl *a* h^{-1} in the dark. Meetam *et al.* (1999) reported whole chain electron transfer rates of 280 ± 40 μmol mg^{-1} Chl *a* h^{-1}. The figures reported in this study are slightly lower than these other studies, but this could be caused by slightly different growth conditions. Importantly, the samples in this study were all cultured and assayed in identical conditions. As reported in *Anabaena variabilis*, increasing light intensity did cause an increased rate of oxygen evolution up until a saturating level (data not shown) (Samuilov and Fedorenko, 1999).

5.4. Phototactic responses

It has been widely reported that the cyanobacteria display photomovement through gliding motility, in three major classes: photokinetic, phototactic and photophobic responses.

Gliding motility requires contact with a solid surface and occurs in a direction parallel to the long axis of the cell or filament, and provides a means for bacteria lacking flagella to travel in environments with a low water content such as in a microbial mat or soil (Spormann, 1999). In *Synechocystis* sp. PCC 6803 this movement has been found to require pili, normally abundant on the surface of wild-type cells. A mutation in an alternative sigma factor, SigF, has been shown to cause a loss of type IV pili and a non-motile phenotype (Bhaya *et al.*, 1999). Although the mechanistic action of gliding motility is not currently well understood, it is thought to require the constant secretion of slime to provide the thrust for locomotion (Hoiczyk and Baumeister, 1998).

Phototaxis is the movement of an organism with respect to the direction of light. Such a phenomenon has a clear involvement in assisting a phototrophic organism to position itself optimally for maximum growth. This differs from photokinesis which is the movement of an organism in the presence of light, but irrespective of direction, and photophobic responses which involve a reversal of movement, temporary stop, or a directional change upon a sudden change in fluence rate. In a study of different strains of *Synechocystis* sp. PCC 6803, the red/far-red

photoreceptor phytochrome was implicated in positive phototactic responses (Choi *et al.*, 1999). Previously, *Synechocystis* sp. PCC 6803 had been reported as having an unstable phototaxis phenotype (Castets *et al.*, 1986), it was later found that the ATCC 27184 strain (from the American Type Culture Collection) was not phototactic, whereas *Synechocystis* sp. PCC 6803 was. It has been reported that glucose-tolerant non-motile strains (such as ATCC 27184) have a 1 bp insertion in a 522 aa serine/threonine protein kinase (sll1575, designated *spkA*), splitting the protein into two non-functional ORFs and that this mutation causes the non-motile phenotype (Kamei *et al.*, 2000). In the study by Choi (1999), it was found that *Synechocystis* sp. PCC 6803 exhibited phototactic orientation by sensing light direction, not light intensity as determined by photomovement of cells towards the light source in both positive and negative light gradients. This is counter intuitive, as it would be expected that cells sense light intensity in order to position themselves to maximise light-harvesting. Movement in the direction of a light source irrelevant of light intensity does not seem to serve any physiological purpose. Interestingly, whilst the ATCC strain does not show phototactic responses on soft agar plates, random motility in liquid medium has been reported (Choi *et al.*, 1999), so it would seem that SpkA may have a role in the regulation of light sensing pathways, rather than in the regulation of motility. From the wavelength dependence of the phototactic response in *Synechocystis* sp. PCC 6803, a phytochrome-like photoreceptor was implicated.

In this study, biophysical measurements have suggested that Slr1212 may be involved with the light sensing mechanisms of *Synechocystis* sp. PCC 6803. As the predicted Slr1212 protein has strong similarity to phytochrome, it is therefore quite possible that Slr1212 and Slr1213 may be part of a sensory mechanism involved in the perception of light to position cells to maximise light-harvesting efficiency in the current environment. In the absence of one or more components of the system regulating these responses, abnormal movement such as slowed movement, or movement in a manner different to that of motile wild-type cells might be observed. A similar approach was used by Jiang (1998) to identify components of photoperception pathways, by using a phototactic assay to screen a library of Tn*5* mutants in *Rhodospirillum centenum*. It was demonstrated that such a screen could

be applied to identify components of photosensory pathways, and could be used for the genetic dissection of light sensing pathways in eubacteria.

Figure 5-8 shows two photographs of *Synechocystis* cells on soft agar plates (0.3% (w/v)) placed in a light gradient to analyse the phototactic response. In (A), it was observed that both the wild-type PCC strain, and the mutants generated in this study all exhibited a comparable phototactic response. However following a 10-day incubation, the rate of movement was less than expected and seemed to be due to either a too high cell density or volume plated. As a consequence, motility was inhibited by an increased surface tension. As observed with the wild-type cells with the streaking of a small portion of the original cell spot, once free of this surface tension the wild-type cells are highly motile and show a positive phototactic response. To further analyse this response, a 1000-fold reduction in cell number and 50-fold reduction in volume were used (2.5×10^7 cfu in 5 μl). In Figure 5-8 B, it was observed that both the Δslr1213 and Δslr1212 Δslr1213 strains exhibited a comparable phototactic response, and Δslr1212 and wild-type cells, whilst clearly exhibiting movement towards the light, had not moved to the same extent. This phenomenon was observed with different cell types in different experiments and it seemed that as previously reported, *Synechocystis* sp. PCC 6803 does exhibit an unstable phototaxis phenotype (Castets *et al.*, 1986; Choi *et al.*, 1999). However, all cell types did exhibit positive phototactic responses to a comparable extent overall. As reported, a glucose-tolerant wild-type strain was non-motile (see Figure 5-8 B) and did not exhibit a phototactic response.

These results show that Slr1212 does not have a direct involvement in light sensing with respect to phototaxis. However, as discussed in section 5.2.1 on page 129, it is possible that the role of just one of the several phytochrome-like proteins from *Synechocystis* sp. PCC 6803 may be subtle and could be masked by redundancy amongst the phytochrome family of proteins. A further more detailed analysis would be required in order to discern the specific individual role for each protein in light sensing mechanisms.
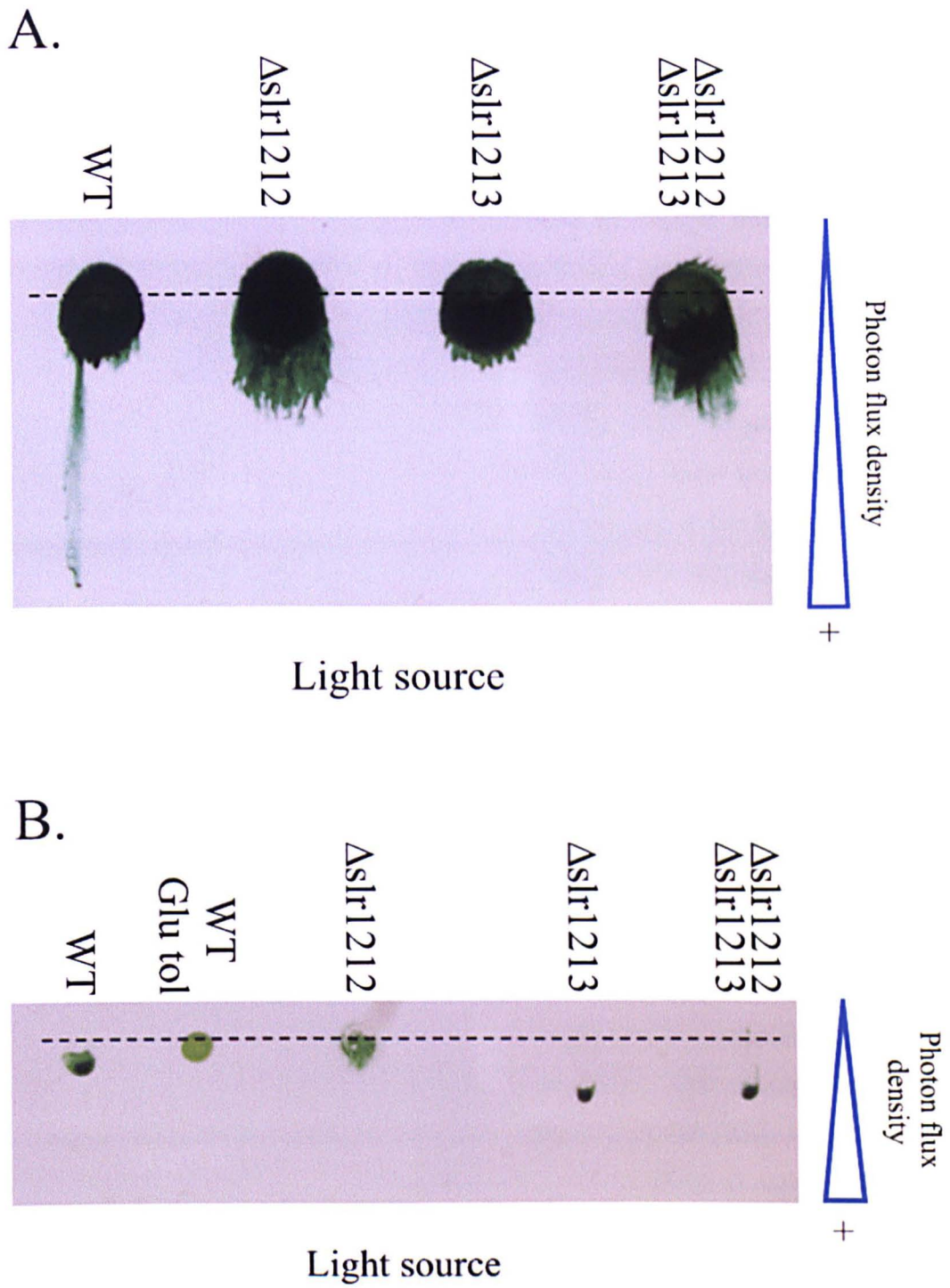
Figure 5-8 :- Phototactic response of *Synechocystis* sp. PCC 6803 cells in white light. A. 2.5×10^8 cfu / spot in a 1-5 µmol m^{-2} s^{-1} light gradient. B. 2.5×10^7 cfu / spot in a 1-10 µmol m^{-2} s^{-1} light gradient.

5.5. Examining a role for slr1212 in copper homeostasis

In plants, copper is an essential trace element because it participates in photosynthetic electron transport and is a cofactor for several oxidizing enzymes (Fathy and Falkner, 1997). Transition metal ions such as copper, iron, and molybdenum are important mainly by virtue of their ability to exist in multiple oxidation states *in vivo*. If the concentration of copper becomes too high for intracellular chaperones to cope with, it can have a toxic effect due to its inherent redox properties. Copper may indirectly cause damage to a biological system due its role in the production of free radicals, and therefore the level of free copper in the cell must be kept to a minimum, and its transport tightly regulated (Rogers *et al.*, 1991). As discussed in section 1.4.3, Δslr1212 cells have been found to have an increased resistance to the toxicity effects of copper, and it was hypothesised that this may be due to the N-terminal ethylene-binding domain originally functioning as a copper-binding domain (Esch *et al.*, 1998).

A hypothesis was formulated based upon this data that the Slr1212 protein may function as a negative regulator of *pacS* (sll1920). PacS has been identified as a CPx-type ATPase ion pump that exports copper ions from the cell cytoplasm into the external environment (Nigel Robinson, University of Newcastle). Δ*pacS* cells were found to be more sensitive to copper toxicity than wild-type cells, as a result of being unable to export copper ions as efficiently (Robinson, 1999). As Δslr1212 cells have been reported to exhibit a copper-resistant phenotype, it is possible that Slr1212 and Slr1213 may negatively regulate *pacS* expression. For example, conditions of increased copper concentrations would be sensed by Slr1212, leading to Slr1213 enhancing (or derepressing) the expression of *pacS* leading to copper export. In low copper concentrations, the expression of *pacS* would be repressed. In the absence of Slr1212 the expression of *pacS* would not be regulated, leading to increased copper export and cells more resistant to copper toxicity.

This section describes efforts to confirm this copper resistant phenotype with the Δslr1212 mutant created in this study. Also, the role of slr1212/slr1213 in copper scavenging/sensing was investigated using flash photolysis to examine the copper regulated genetic switch controlling plastocyanin/cytochrome c_{553} expression.

5.5.1. Growth of mutants at high copper concentrations

It has been reported that the minimum inhibitory concentration (MIC) of copper for *Synechocystis* sp. PCC 6803 at a cell density of 10^6 cfu ml^{-1} is 0.7 µM (Robinson, 1999). To test this, wild-type and mutant cells were inoculated in triplicate to 10^6 cfu ml^{-1} in test tubes containing BG-11 medium with concentrations of copper sulphate from normal levels in BG-11 medium (0.316 µM) through to 10 µM. Following incubation with shaking for seven days, tubes were scored for growth.

Figure 5-9 A displays the results, and it can be seen that there is no significant difference between wild-type and mutant cells in resistance to copper toxicity, and that all strains grow well at concentrations of copper sulphate up to 3 µM.

To investigate more closely the effects of copper toxicity on the strains, growth curves were carried out and doubling times calculated. These results are displayed in Table 5-2.

Strain	Copper concentration ($CuSO_4 \cdot 5H_2O$ µM)			
	BG-11 (0.316)	3	3.5	4
Wild-type	10.86 ± 0.48	23.26 ± 3.3	38.42 ± 14.95	-
Δslr1212	12.58 ± 0.96	13.49 ± 1	12.68 ± 0.63	19.62 ± 0.63
Δslr1213	10.68 ± 0.55	28.02 ± 2.43	46.76, 46.02	-
Δslr1212 Δslr1213	11.57 ± 0.85	12.57 ± 1.08	13.01 ± 0.75	12.27 ± 0.71

Table 5-2 - Doubling times of strains cultured in the presence of increasing concentrations of copper sulphate. A dash denotes that cells did not survive. Data points are calculated as means ± SD (n=3-8, from 1-3 experiments.)

From this data, it can be observed that the wild-type and Δslr1213 strains have a MIC of approximately 3.5 µM copper sulphate. It should be noted that the average for the wild-type at 3.5 µM is not truly representative, as it is calculated from five samples which were cultured under these conditions, whilst three further cultures did not survive. Frequently, wild-type and Δslr1213 strains had extended lag phases in 3.5

A.

WT   Δslr1213

0.32 1.1 2 3 4 5 10   0.32 1.1 2 3 4 5 10

Copper concentration (μM)

Δslr1212   Δslr1212
                                      Δslr1213

B.

3 μM

WT   Δslr1212 Δslr1212   Copper
      + spec   - spec   concentration

4 μM

WT   Δslr1212 Δslr1212
      - spec   + spec
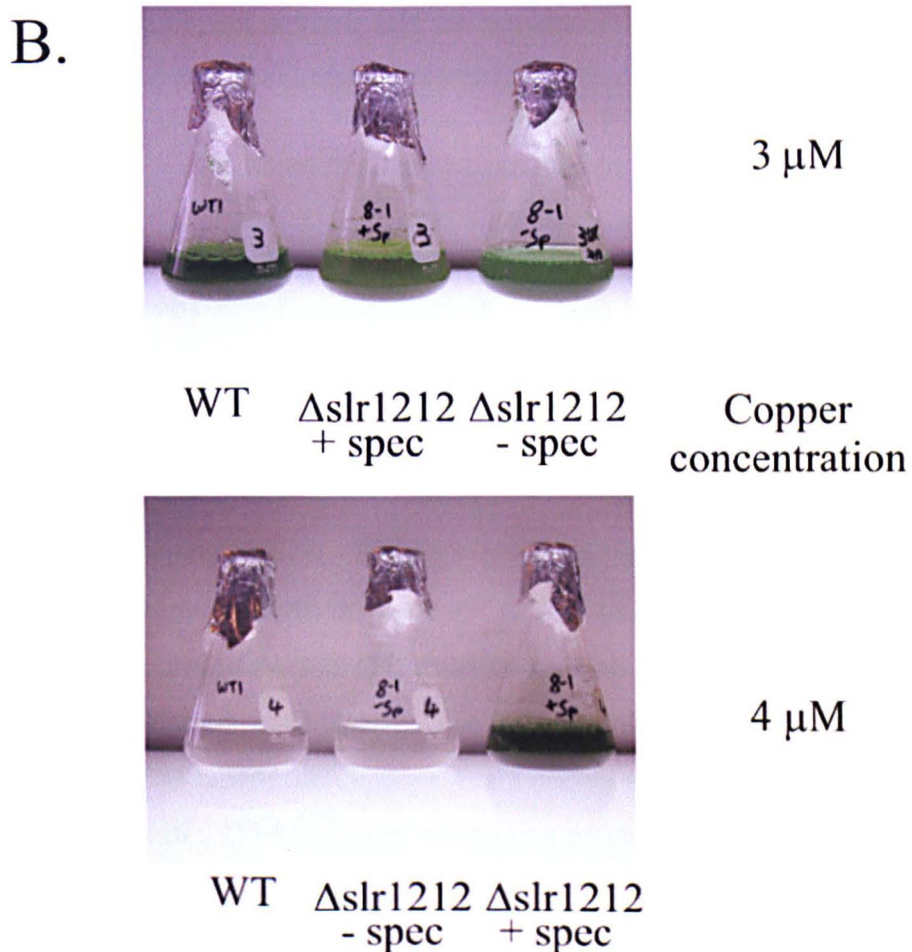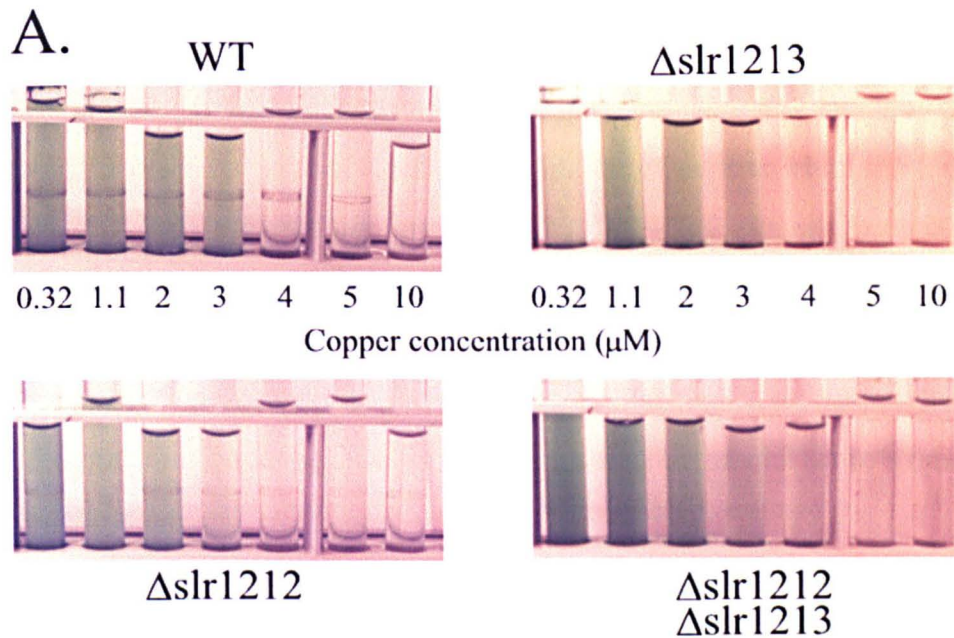
Figure 5-9 :- Examining the effects of copper toxicity on the growth of *Synechocystis* sp. PCC 6803 wild-type and mutant cells. A. The growth of cells in concentrations of copper upto 10 μM reveals that wild-type and mutant cells have an MIC of ~4 μM. B. Photographs of cells ± 25μg ml^{-1} spectinomycin showing the influence of antibiotic addition on the copper resistant phenotype.

µM copper sulphate of up to six days before growth was observed. In these situations, doubling times were calculated from the time period once growth had commenced. A 4 µM concentration of copper sulphate was lethal to wild-type and Δslr1213 cells, as demonstrated by the failure of cells to culture when transferred back to BG-11 medium containing normal levels of copper (data not shown). In concordance with the findings of Esch *et al.* (1998), an increased resistance to copper toxicity was observed for the Δslr1212 strain, as well as the Δslr1212 Δslr1213 strain. Growth of the mutant strains seemed unaffected at copper concentrations up to 4 µM, although the doubling times of the Δslr1212 strain were reduced at this concentration.

It is interesting to observe that although the Δslr1212 mutant exhibits a copper-resistant phenotype, the Δslr1213 strain does not. This suggests that slr1213 may play no role in copper sensing and the regulation of *pacS*, whilst slr1212 could negatively regulate *pacS* expression in conjunction with an unknown response regulator(s).

5.5.2. Involvement of Slr1212 in regulating plastocyanin and cytochrome c_{553} expression

Along with an increased sensitivity to copper toxicity, the Δ*pacS* strain in *Synechocystis* sp. PCC 6803 also loses control of the genetic switch regulating plastocyanin and cytochrome c_{553} (Robinson, 1999). Plastocyanin is a soluble, low molecular weight copper protein. The functional replacement is a soluble iron-heme protein, cytochrome c_{553}. Both proteins act as mobile electron carriers connecting the cytochrome b_6/f complex with PSI reaction centres. Cupric (Cu^{2+}) plastocyanin oxidizes the membrane-bound cytochrome b_6/f complex and then diffuses to the reaction centre of PSI where the cuprous (Cu^+) plastocyanin reduces $P700^+$ (Bovy *et al.*, 1992; Briggs *et al.*, 1990). Under conditions of copper deficiency, cytochrome c_{553} replaces plastocyanin as the reductant of $P700^+$; the availability of copper in the growth medium controls which protein is synthesised (Sandmann, 1986; Zhang *et al.*, 1992).

Δ*pacS* mutants have elevated levels of plastocyanin, even in conditions of low copper concentrations. If the hypothesis that Slr1212 regulates *pacS* expression in response to the copper concentration of the cellular environment is correct, it

would be expected to observe a similar malfunction in the coordination of expression of these electron carriers.

Using a technique called laser flash photolysis, it is possible to take a biophysical measurement to determine whether plastocyanin or cytochrome c_{553} is acting as the electron carrier between the cytochrome b_6/f complex and P700. This technique is based upon small absorbance changes caused by the oxidation and reduction of P700 and plastocyanin following pulses of light. Cells were cultured in copper deplete and replete (1 μM CuSO$_4$·5H$_2$O) conditions. Cells were dark incubated before the initiation of measurements, and then provided with very brief pulses of light. Under copper replete conditions such as those shown in Figure 5-10 A, P700 (the lower line) was reduced following dark incubation. Following a brief light pulse P700 rapidly became oxidised as electrons were transported downstream, and can then be observed to again become reduced. Plastocyanin (the upper line) became reduced following the light pulse as electrons were received from upstream complexes, and then became oxidised as it passed electrons onto P700 (which in turn became re-reduced). Following each pulse of light, P700 required longer periods of time to become reduced again, due to a shortage of electrons in the upstream complexes.

When measurements were taken with wild-type cells cultured in copper deplete conditions (see Figure 5-10 B), it was observed that as expected there was a complete absence of plastocyanin (signified by no alteration in the absorbance measurements). Despite this, P700 still becomes oxidised and reduced following each light pulse, indicating that a protein other than plastocyanin is acting as the electron carrier between the cytochrome b_6/f complex and P700. As discussed, the alternative electron carrier is known to be cytochrome c_{553}.

As shown, this technique allows a very sensitive, non-invasive, real-time examination of a key metabolic pathway in photosynthetic organisms.

When these measurements were carried out with *Synechocystis* sp. PCC 6803 Δslr1212 cells cultured under copper replete and deplete conditions (see Figure 5-10 C and D respectively), a similar pattern was observed. In cells cultured in copper replete conditions, the reduction and oxidation of plastocyanin was measured following each light pulse, indicating that this protein was acting as the electron carrier. With cells cultured in copper deplete conditions, no alterations in absorbance
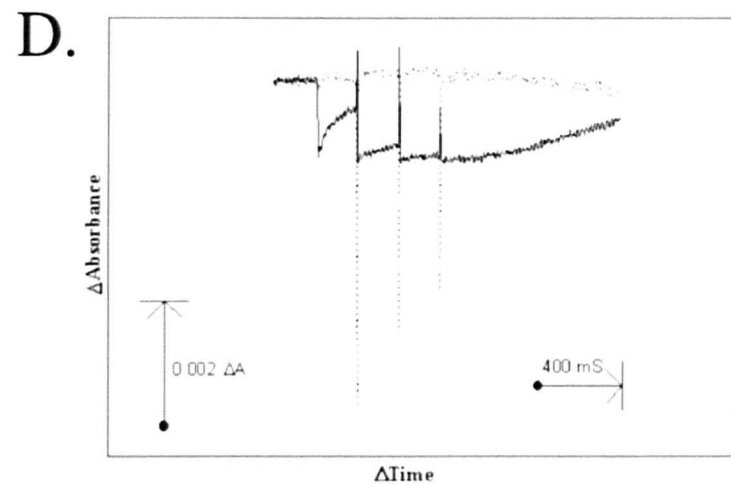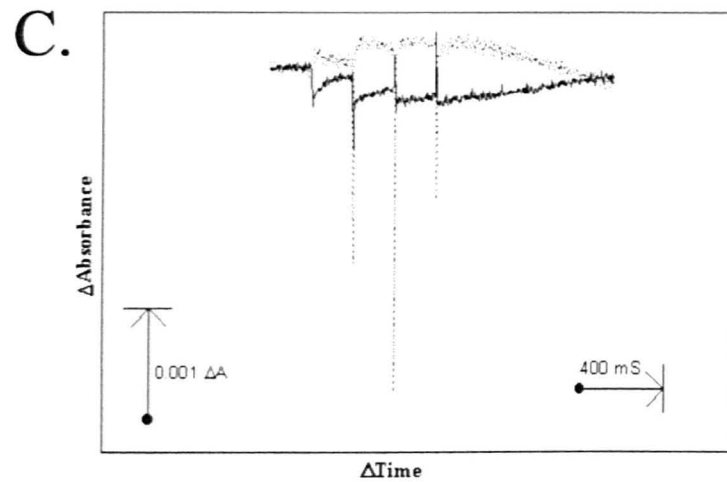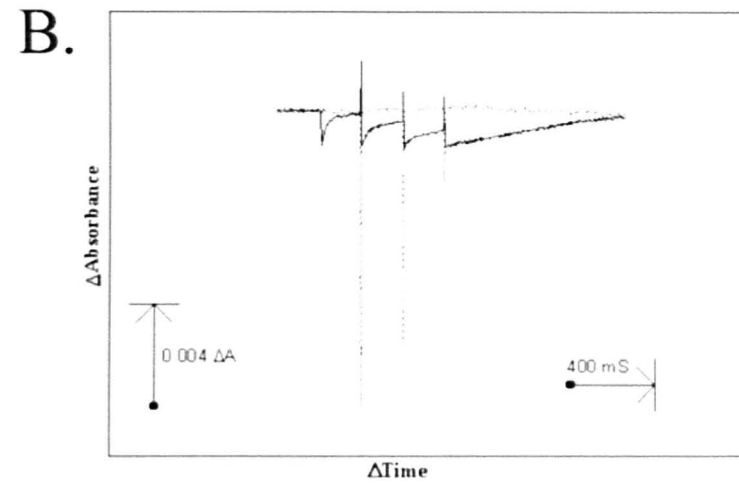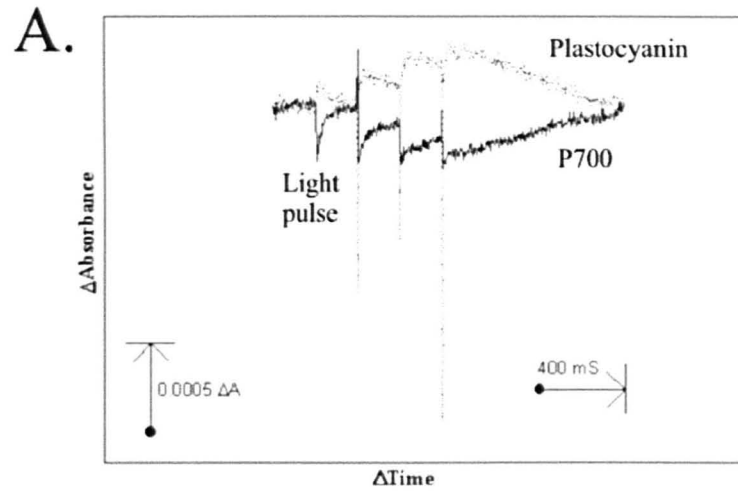
Figure 5-10 :- Laser flash photolysis of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells to determine the presence of plastocyanin. A. Wild-type cells cultured in copper replete (1 μM) and B. deplete conditions. C. Δslr1212 cells cultured in copper replete (1 μM) and D. deplete conditions.

at the plastocyanin wavelengths were observed, whilst P700 oxidised and reduced as normal following each light pulse. This result was consistent with the presence of an electron carrier other than plastocyanin

This result shows that the Δslr1212 strain regulates the expression of plastocyanin and cytochrome c_{553} in the same manner as wild-type cells when exposed to copper replete and deplete conditions. This implies that Slr1212 has no apparent role in the regulation of *pacS*. If a phenotype had been identified, a Δslr1212 $\Delta pacS$ strain could have been generated for epistasis studies.

5.5.3. Copper toxicity effects

Before conclusions could be made about the role of Slr1212 in copper homeostasis, it was necessary to test whether the copper resistant phenotype was affected by the presence of antibiotics in the medium (kanamycin and spectinomycin in this case). It has been reported that various antibiotics can complex with copper (II) ions such as ampicillin (Mukherjee and Ghosh, 1996), rifampicin (Bontchev *et al.*, 1997), tobramycin (Jezowska-Bojczuk *et al.*, 1998), geneticin (Jezowska-Bojczuk *et al.*, 1998), kanamycin B (Jezowska-Bojczuk *et al.*, 1998), and quinolones (Alvarez *et al.*, 1997; MendozaDiaz *et al.*, 1996).

To test this, cells were sub-cultured twice, subsequently, in the absence of antibiotics, before inoculation into copper containing (4 µM) BG-11 medium with and without antibiotic. It was found that whilst kanamycin had no effect on the growth of Δslr1213 cells, in the absence of spectinomycin Δslr1212 cells had the same susceptibility to copper toxicity as wild-type cells. This is illustrated in the photographs in Figure 5-9 B on page 151.

Two possibilities exist to explain the loss of the copper resistance phenotype in the absence of spectinomycin. Firstly, spectinomycin may bind a large proportion of, or all of the copper added to the medium, or secondly, the Δslr1212 mutation may not be stable in the absence of a selective pressure (unlikely, because even if the resistance cassette was lost, Δslr1212 has a deleted region which should prevent full length functional protein being produced). The former option seems more likely, as many aminoglycosides (including kanamycin) have been shown to complex with copper (II). Although technically an aminocyclitol, spectinomycin is very similar in

structure to many aminoglycosides (though lacking an aminosugar). To determine which possibility was correct, wild-type and Δslr1212 strains were cultured in the absence of antibiotics, and then 1000 cells were plated onto BG-11 agar plates with and without spectinomycin. Viable counts revealed that even following three sub-cultures in the absence of spectinomycin, resistance was retained (data not shown). This demonstrated that the presence of spectinomycin in the medium, and not the Δslr1212 mutation in the cell was responsible for the observed copper resistance phenotype. It also demonstrated that the mutations generated were stable in the absence of the selective pressures over a number of sub cultures.

It is interesting that spectinomycin binds copper preventing toxic effects, whilst kanamycin does not, as it has previously been reported that some forms of kanamycin can complex with copper (II) (Jezowska-Bojczuk *et al.*, 1998). As a result, future experiments with the mutants generated in this study were carried out in the absence of antibiotics (including the flash photolysis measurements in section 5.5.2).

In conclusion therefore, no evidence was gained to suggest that either Slr1212 or Slr1213 has any role in the regulation of *pacS*, and furthermore in contrast to the findings of Esch *et al.* (1998), it was found that the Δslr1212 mutation does not confer an increased resistance to copper toxicity upon the cell. The Δslr1212 construct used by Anthony Bleecker's group was made by inserting a kanamycin resistance cassette from Tn*903* into the *Sca*I site of slr1212 (214 bp beyond +1) (Rodriguez *et al.*, 1999). The possibility that a Δslr1212 mutation does not confer copper resistance, and that copper binding by antibiotics is responsible for this phenomenon remains.

5.6. Examination of protein synthesis in *Synechocystis* sp. PCC 6803

Data published by Anthony Bleecker's group has demonstrated that Slr1212 can bind ethylene (Rodriguez *et al.*, 1999), and work in this study has demonstrated the ability of *Synechocystis* sp. PCC 6803 to produce ethylene, particularly in response to ACC (see Chapter 4). Taken together, these results implicate ethylene as a signalling molecule in this bacterium. However, determining the precise nature of this signalling mechanism and its role in cell physiology is more difficult. In an effort to address the latter, ^{35}S-methionine was used to label proteins synthesised during a given time course in response to alterations in the cell environment, including the addition of gaseous ethylene to the extracellular environment.

Such an approach has already been taken with this organism to examine cell responses to salt shock (Fulda *et al.*, 1999).

5.6.1. Uptake of ^{35}S-methionine

Before such experiments could be undertaken, it was first necessary to determine the rate at which *Synechocystis* sp. PCC 6803 could actively transport ^{35}S-methionine, to determine a suitable concentration of isotope to use.

Using the method described in section 2.19 (see page 86), *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells were incubated with ^{35}S-methionine at 0.0185 and 0.037 MBq ml^{-1} with and without 10 mM unlabelled L-methionine. Following scintillation counting, rapid incorporation of ^{35}S-methionine by both wild-type and Δslr1212 cells was observed. In the presence of 10 mM L-methionine, no incorporation of labelled methionine was observed (see Figure 5-11 A). It can also be seen that after approximately 16 hours, the amount of incorporated ^{35}S-methionine peaked and then began to fall, whilst the cells continued to grow normally (see Figure 5-11 B). This indicates that within 16 hours the cells have taken up all of the original 0.037 MBq ml^{-1} ^{35}S-methionine. Interestingly, in all of the uptake experiments carried out (and as represented by the averages in Figure 5-11 A), Δslr1212 cells exhibited a slightly reduced incorporation of ^{35}S-methionine relative to wild-type cells. If the disruption of ORF slr1212 reduced the uptake rate of ^{35}S-methionine, it would be expected that the incorporation of all the isotope would take longer and hence peak later. However, the incorporation of ^{35}S-methionine by
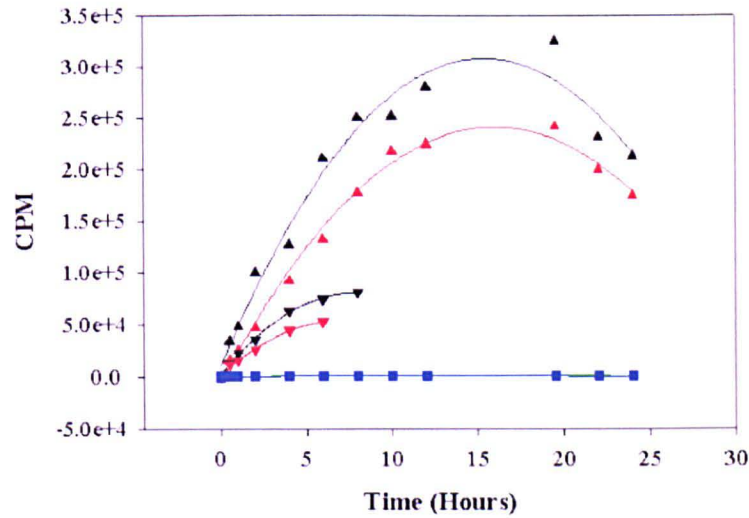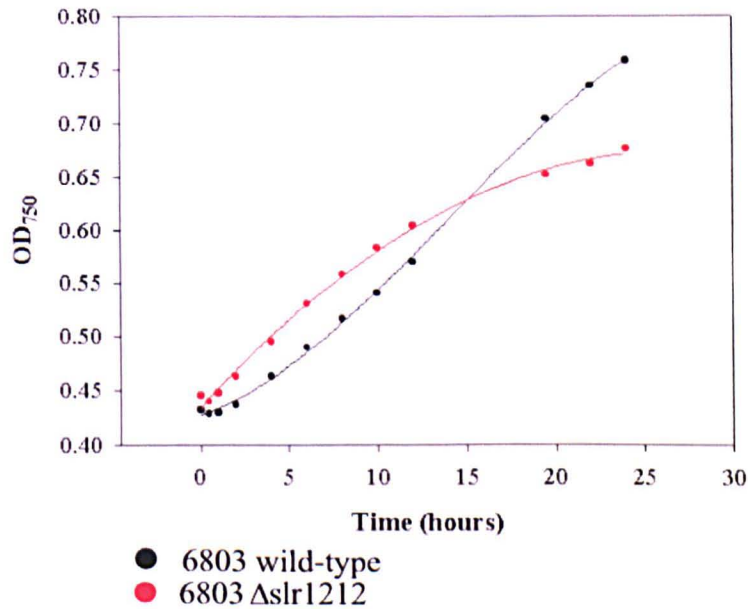
Figure 5-11 :- *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells can efficiently transport ^{35}S-methionine. A. Uptake of ^{35}S-methionine by *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells ± 10 mM L-methionine. B. Growth of wild-type and *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells in the presence of 0.037 MBq ml^{-1} ^{35}S-methionine. All data shown is an average of two data points.

Synechocystis sp. PCC 6803 wild-type and Δslr1212 cells peaked at approximately the same time, although Δslr1212 cells had incorporated less total ^{35}S-methionine.

This experiment clearly demonstrated that *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells can actively transport and incorporate ^{35}S-methionine. Furthermore, it has been demonstrated that increasing the concentration of isotope from 0.0185 to 0.037 MBq ml^{-1} approximately doubles the rate of incorporation by cells. The addition of 10 mM L-methionine to a culture incubated with ^{35}S-methionine prevents any significant incorporation of the radiolabelled amino acid. Finally, it was shown that the addition of ^{35}S-methionine in concentrations up to 0.037 MBq ml^{-1} does not inhibit the normal growth of cells. From the data, it was decided that sufficient incorporation of ^{35}S-methionine could be achieved for use in labelling experiments by incubating cells with 0.037 MBq ml^{-1} for 2 hours. As the methodology varied slightly with each experiment carried out, any alterations from that described in section 2.19.1 are included in a subsequent separate methods section.

5.6.2. Effect of a high light shift on protein synthesis

Evidence gained from 77K fluorescence spectroscopy (see section 5.3.3) suggested that Slr1212 may be involved in the acclimation of *Synechocystis* sp. PCC 6803 cells to a high light environment. Such a role could involve alterations in the transcription of different genes, ultimately affecting the translation of some proteins involved in high light acclimation. This experiment was designed to examine the response of *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells to a sudden change in their light environment from 35 μmol m^{-2} s^{-1} to 850 μmol m^{-2} s^{-1} white light irradiance. Methodology is located in section 2.19.1.1 on page 87.

5.6.2.1. Results

As seen in Figure 5-12, when *Synechocystis* sp. PCC 6803 cells are shifted from a low to a high light environment, there are several prominent alterations in the protein expression profiles, as highlighted by the blue arrows. These alterations involve the down regulation of several proteins (approx. molecular weight 57, 54, 25, and 10 kDa) and the up regulation of one protein (approx. molecular weight 33.5 kDa), and
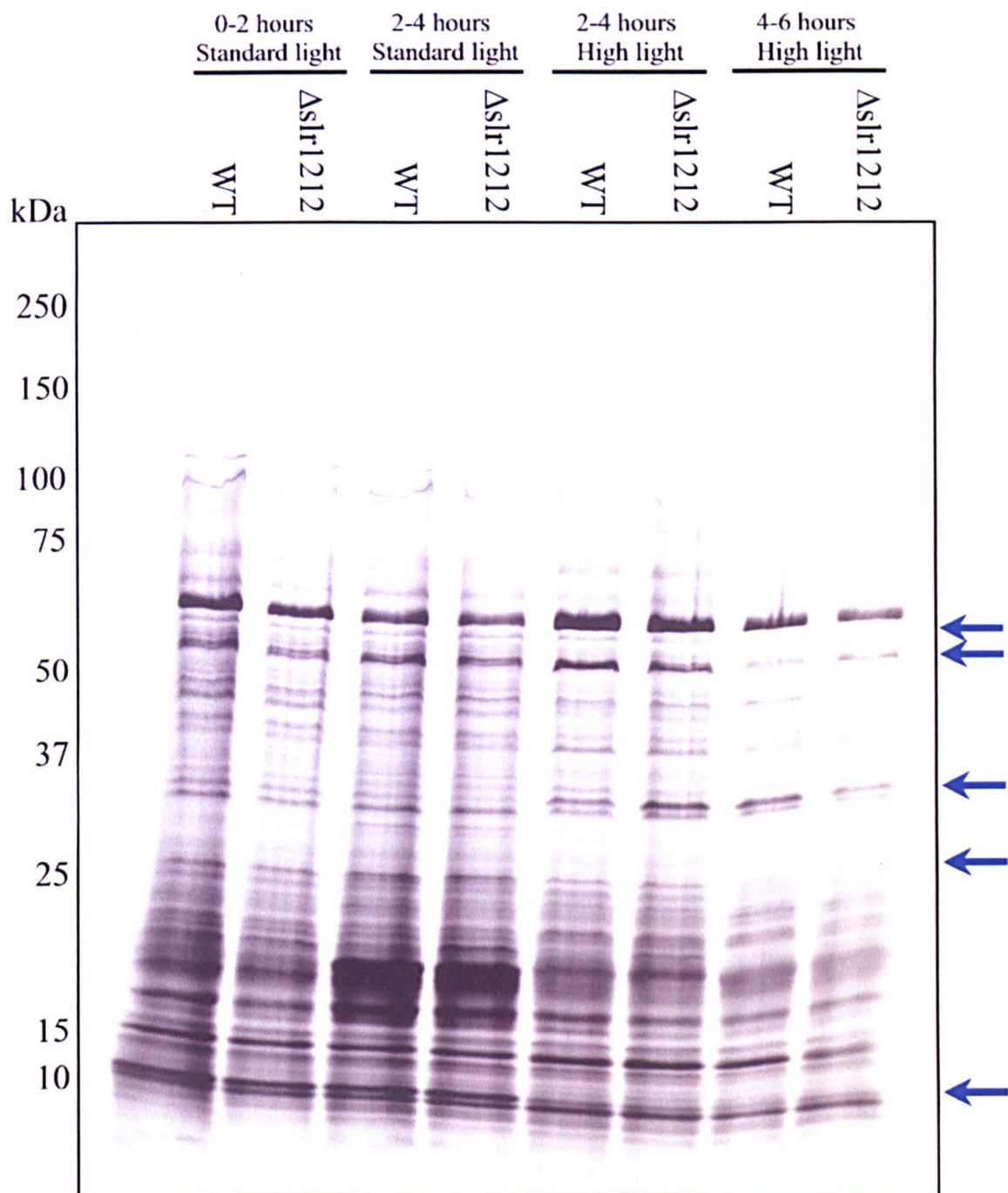
Figure 5-12 :- Autoradiography of a 6-24% (w/v) SDS polyacrylamide gel showing the effects of a light shift from 35 µmol m^{-2} s^{-1} to 850 µmol m^{-2} s^{-1} white light irradiance on whole cell protein profiles in *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells as analysed by a pulse-chase with 0.037 MBq / ml ^{35}S-methionine. An equivalent of 3 ml OD$_{750}$ 0.5 liquid culture was loaded per lane. Blue arrows highlight any significant alterations in protein expression.

these changes presumably aid the cell to cope with the extreme environment in which it has been placed. However, there are no obvious differences in the responses of wild-type and Δslr1212 cells to this environmental change. This result implies that Slr1212 has no role in the acclimation of cells to a high light environment. However, such a result contradicts the findings that Slr1212 does have a role in this process as determined by 77K fluorescence spectroscopy (see section 5.3.3 on page 137). One suggestion for this contradiction could be that the loss of Slr1212 takes longer than four hours to affect the cells physiology to adapt to a higher light environment. Cultures were adapted to a higher light environment over a period of days before 77K spectra were measured. Also, 77K fluorescence spectroscopy is a much better technique for examining subtle changes which occur in the PSI and PSII reaction centres.

5.6.3. Effect of ACC on protein synthesis

It was demonstrated using LPA that ACC, the precursor of ethylene in higher plants, also leads to a significant release of ethylene by *Synechocystis* sp. PCC 6803. Interestingly, analysis of CyanoBase did not reveal a homologue of ACC oxidase (the enzyme responsible for catalysing the conversion of ACC to ethylene in higher plants). Hence, the conversion of ACC to ethylene in *Synechocystis* sp. PCC 6803 may involve some unique components different from those in higher plants.

In an effort to identify some of the enzymes responsible for the conversion of ACC to ethylene, ^{35}S-methionine was used to study protein synthesis in cells following the addition of 5 mM ACC (this concentration was found to induce a significant release of ethylene). Methodology is located in section 2.19.1.2 on page 88.

5.6.3.1. Results

As seen in Figure 5-13, when *Synechocystis* sp. PCC 6803 and Δslr1212 liquid cultures are incubated with 5 mM ACC, protein synthesis halts immediately. Observation of cultures after 72 hours found cell death (cultures could not be revived by sub-culturing). This phenomenon is very interesting, as under these same
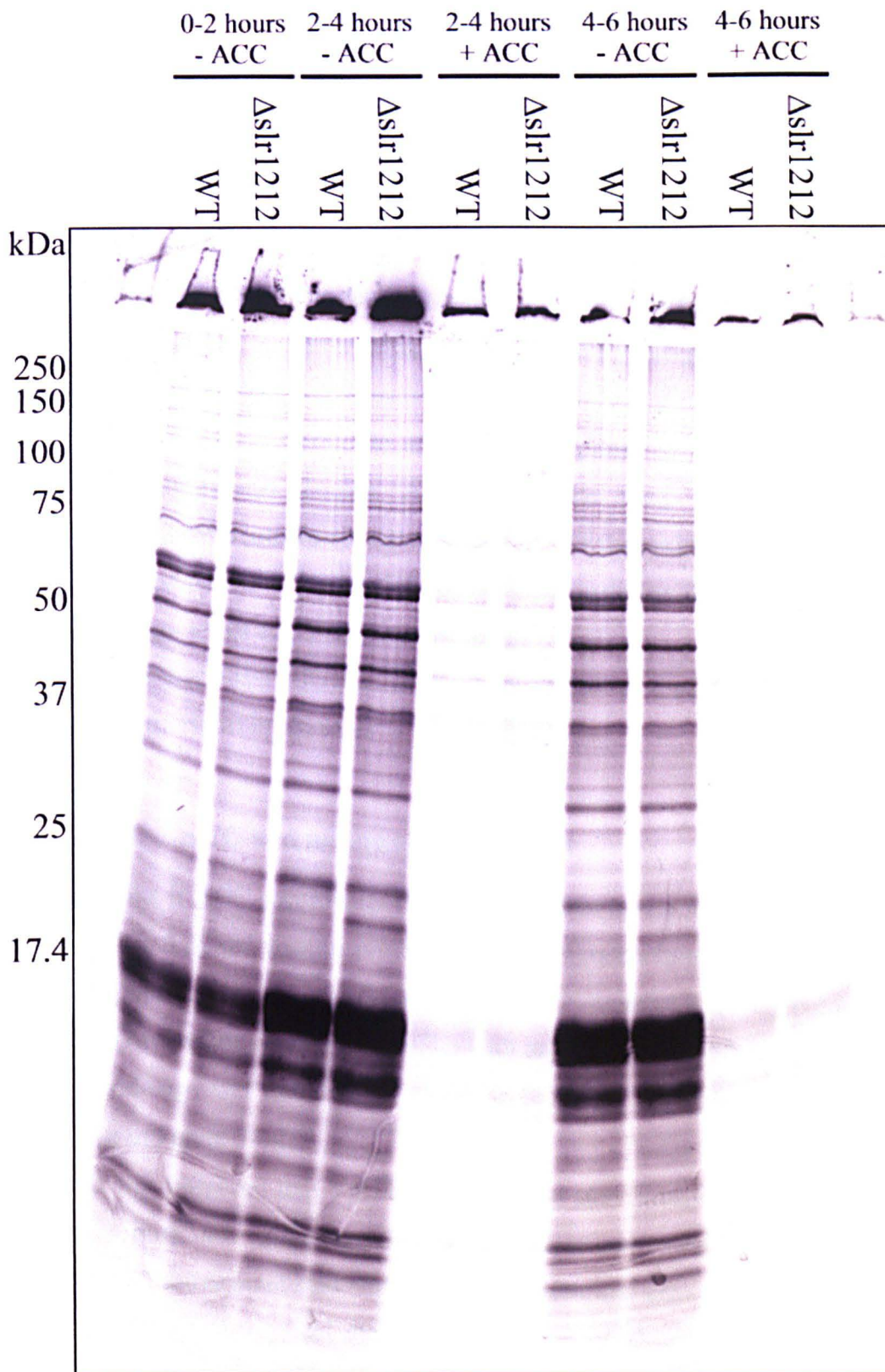
Figure 5-13 :- Autoradiography of a 6-24% (w/v) SDS polyacrylamide gel showing the effects of 5 mM ACC on whole cell protein profiles in *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells as analysed by pulse-chase with 0.037 MBq / ml ^{35}S-methionine. An equivalent of 3 ml OD$_{750}$ 0.5 liquid culture was loaded per lane.

conditions it has been shown that cells release a substantial concentration of ethylene. This raises three possibilities: firstly that ACC is not an intermediate in an ethylene biosynthetic pathway, and that the ethylene release observed is actually a result of membrane degradation. It has been reported previously that lipid peroxidation (membrane breakdown) in humans produces traces of ethylene, ethane and pentane in exhaled air (Haliwell and Gutteridge, 1985). It has since been found that the resulting ethylene can be measured on the surface of the skin following trauma such as UV radiation (http://www.sci.kun/nl/tracegasfac). Secondly, it is possible that the observed release of ethylene is not a direct result of the ACC addition. Higher plants release ethylene in response to many stresses including physical wounding and flooding. As ACC is acidic, it is possible that *Synechocystis* sp. PCC 6803 cells release ethylene in response to the sudden stress. Finally, ACC could be a component of an ethylene biosynthetic pathway in *Synechocystis* sp. PCC 6803, although lethal at a 5 mM concentration.

A further possibility for the apparent impact of 5 mM ACC on cells is that methionine transport is blocked.

5.6.4. ACC and amino acid transport

To determine if ACC specifically blocks methionine transport as opposed to causing severe cell stress and halting protein synthesis, *Synechocystis* sp. PCC 6803 wild-type cells were incubated with 0.037 MBq ml^{-1} ^{35}S-methionine, ^{14}C-alanine and ^{14}C-aspartate (Amersham international, UK) with and without 5 mM ACC (Sigma, UK). The methodology was exactly as described in section 2.19 on page 86, and the results are shown in Figure 5-14.

It was observed that 5 mM ACC does not specifically block the transport of ^{35}S-methionine, but prevents cell growth and as seen previously, halts protein synthesis. Cultures incubated with ACC show marginal growth for the first 6 hours, before a decrease in optical density is observed. Cultures incubated with ACC exhibit very low rates of incorporation of the amino acids tested. It was observed that *Synechocystis* sp. PCC 6803 wild-type cells cannot actively uptake and incorporate ^{14}C-aspartate. Addition of ^{14}C-aspartate did not affect the growth rate of the cells, and it is likely that *Synechocystis* sp. PCC 6803 lacks uptake mechanisms capable of transporting this amino acid. This result concurs with another study
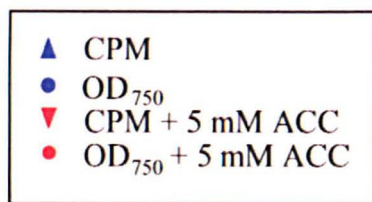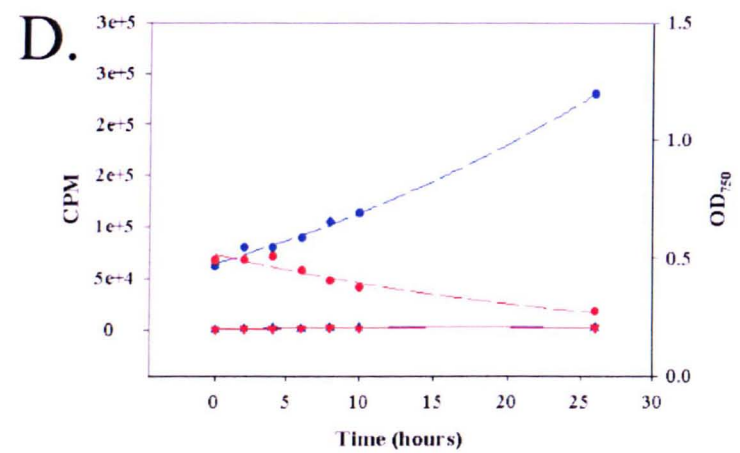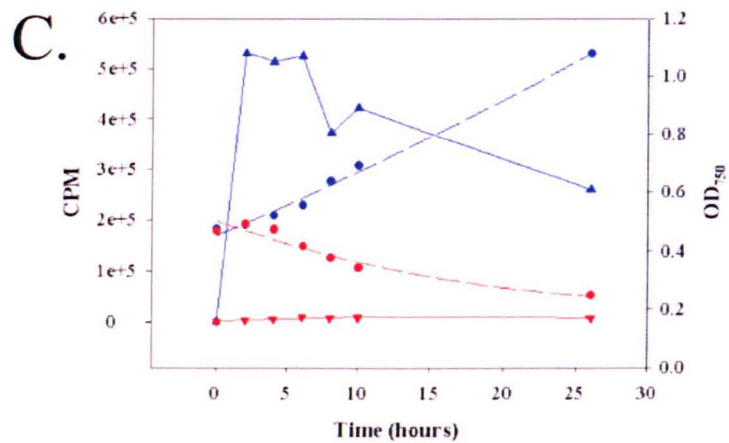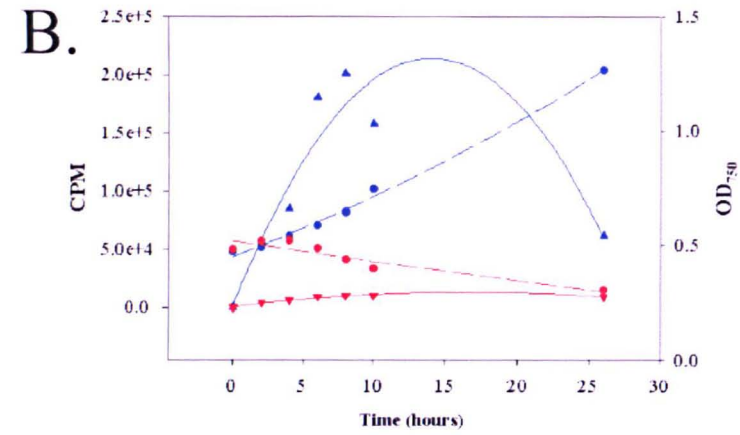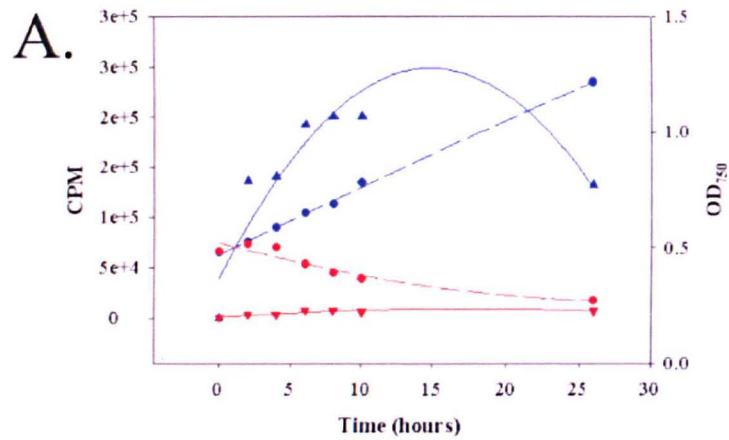
Figure 5-14 :- Effect of 5 mM ACC on the uptake of amino acids by *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells. A. Uptake of ^{35}S-methionine by wild-type cells. B. Uptake of ^{35}S-methionine by Δslr1212 cells. C. Uptake of ^{14}C-alanine by wild-type cells. D. Uptake of ^{14}C-aspartate by wild-type cells.

(Montesinos *et al.*, 1997).

This result shows that ACC is toxic to *Synechocystis* sp. PCC 6803 at a concentration of 5 mM, and suggests that the accompanying release of ethylene is either a non-specific stress response by the cell, or that ACC is part of the biosynthetic pathway (although lethal at these concentrations).

5.6.5. Effect of gaseous ethylene on protein synthesis

The ^{35}S-methionine pulse-chase method developed in this study was used to try and identify components of an ethylene signalling pathway in *Synechocystis* sp. PCC 6803. If ethylene acts as a signalling molecule in *Synechocystis*, then any regulatory mechanisms at the transcriptional and/or translational level might be identifiable with such an approach. Methodology can be found in section 2.19.1.3 on page 88.

5.6.5.1. Results

The autoradiograph in Figure 5-15 shows that when *Synechocystis* sp. PCC 6803 wild-type cells are bubbled with ethylene, a protein of approximately 20 kDa is up-regulated and Δslr1212 cells do not share this response. Whilst such a response would be indicative of either a component involved in ethylene signalling or a response to the ethylene signal, it did not prove possible to duplicate the result.

In an attempt to identify any short-term alterations in protein expression, 100 ml cultures were bubbled with ethylene, and 10 ml samples taken at time intervals between 0 and 30 minutes were labelled for 2 hours with 0.037 MBq ml^{-1} ^{35}S-methionine as previously described. As observed in Figure 5-16 on page 166, bubbling the cells with ethylene before a labelling period did not affect the pattern of protein synthesis during this period.
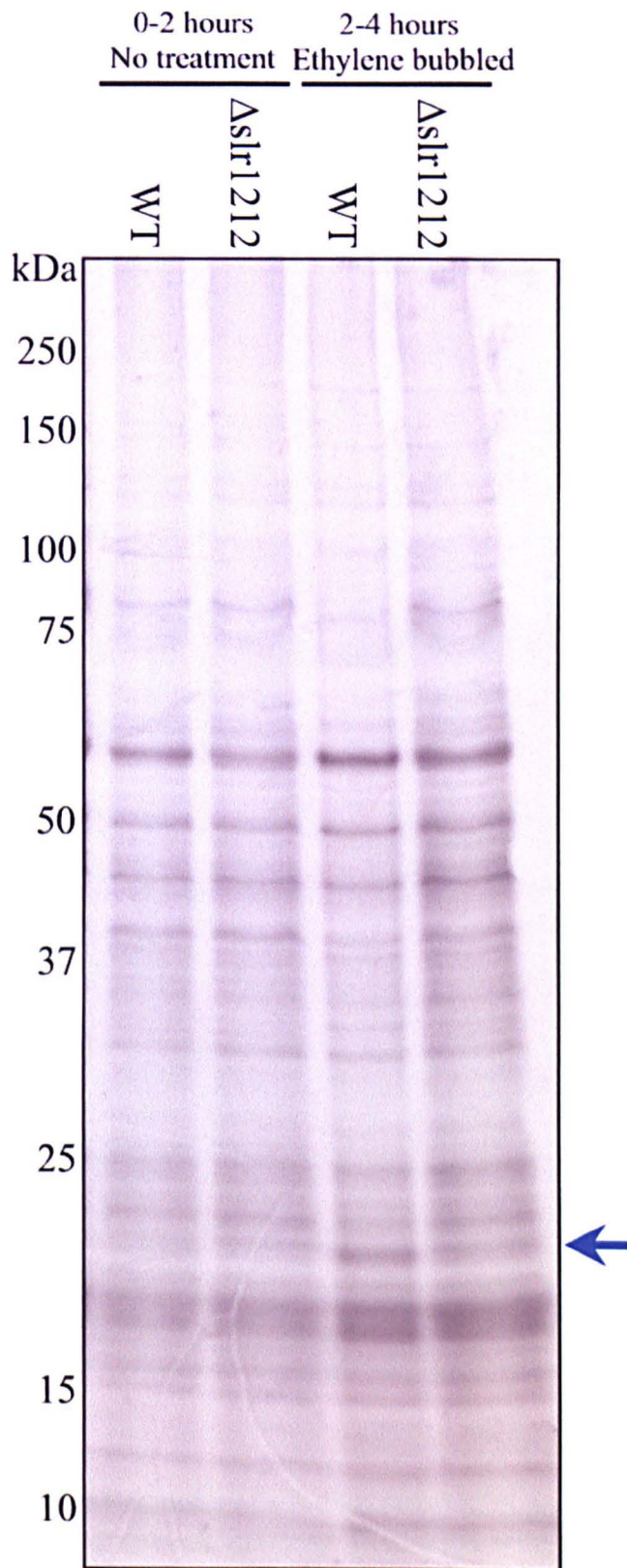
Figure 5-15 :- Autoradiography of a 6-24% (w/v) SDS polyacrlyamide gel showing the effects of bubbling cells with 99.5% (v/v) ethylene for 20 minutes during the 2 hour labelling period of a pulse-chase experiment with 0.037 MBq / ml ^{35}S-methionine. Samples were loaded on an equal counts basis. Differences are highlighted by blue arrows.
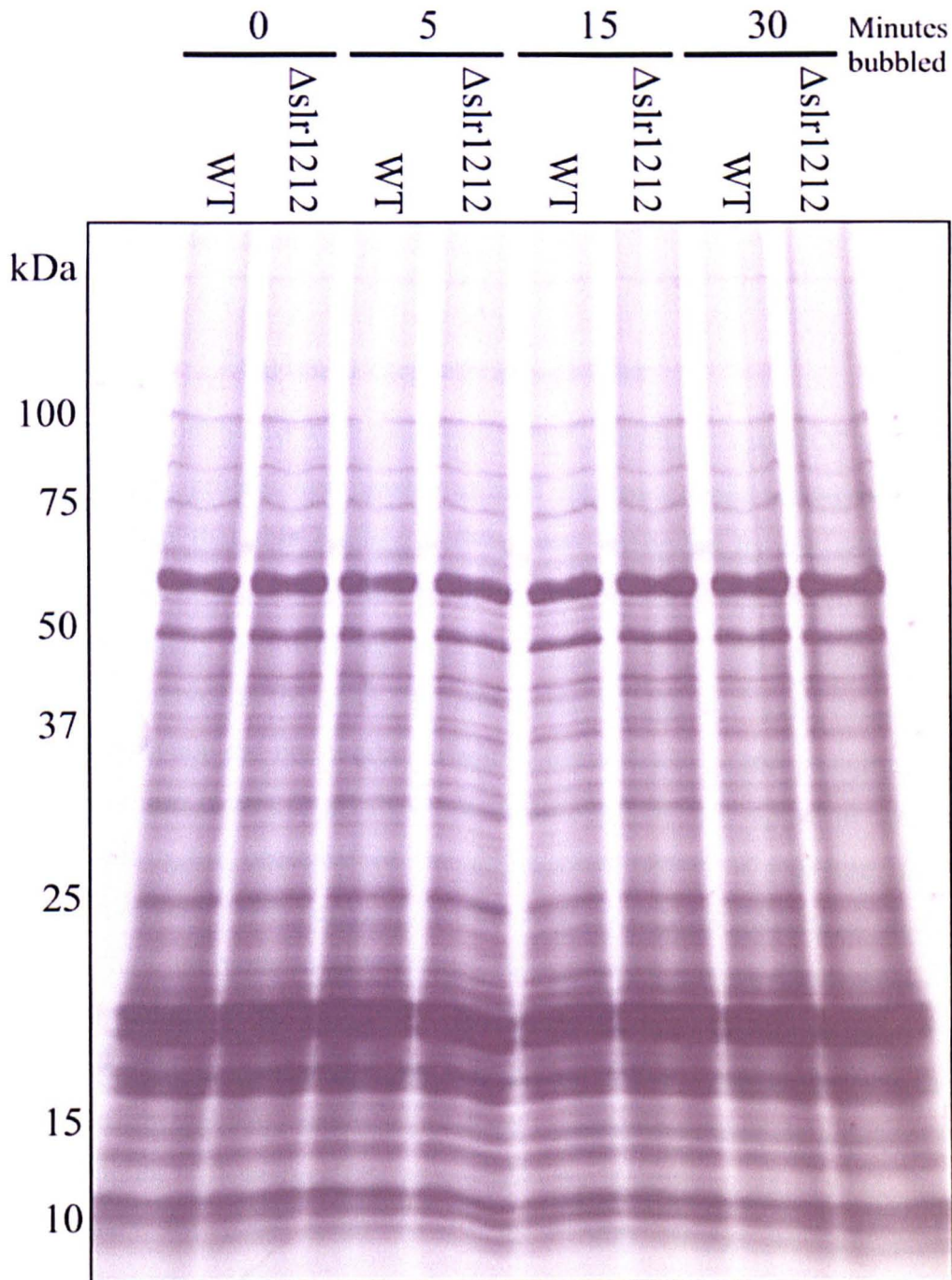
Figure 5-16 :- Autoradiography of a 6-24% (w/v) SDS polyacrylamide gel showing the effects of bubbling *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells with 99.5% (v/v) ethylene for 0-30 minutes before a 2 hour labelling period with 0.037 MBq / ml ^{35}S-methionine . An equivalent of 3 ml OD$_{750}$ 0.5 liquid culture was loaded per lane.

5.7. Discussion

This chapter describes the experiments that were designed and carried out to characterise a phenotype for Δslr1212, Δslr1213 and Δslr1212 Δslr1213 mutations in *Synechocystis* sp. PCC 6803 cells. The experiments conducted were aimed at elucidating a possible role for Slr1212 and Slr1213 in acclimation to light and/or ethylene signalling in this organism.

5.7.1. Ethylene receptors and copper

Previously published work had shown that *Synechocystis* sp. PCC 6803 cells lacking the Slr1212 protein were unable to bind ethylene, whilst wild-type cells could. It was shown that this binding activity was enhanced by the addition of copper (Rodriguez *et al.*, 1999), and reported that Δslr1212 cells were more resistant to copper toxicity than wild-type cells (Esch *et al.*, 1998). We attempted to confirm the latter phenotype in the Δslr1212 mutant generated in this study, and it was observed that our mutant did not exhibit enhanced resistance to copper toxicity. In fact, upon investigation it was found that this phenotype was only apparent when cells were cultured in the presence of the appropriate selective pressure, the antibiotic spectinomycin. This suggests that the copper resistant phenotype results from the addition of antibiotic to the growth medium, rather than an effect of the mutation on the physiology of the cell. This contradicts the findings of Esch *et al.* (1998), and implies that the phenotype observed may result from kanamycin and copper complexing together. Published findings have demonstrated the ability of kanamycin to complex with copper (II) ions (Jezowska-Bojczuk *et al.*, 1998). It would be necessary for Esch *et al.* to duplicate their copper resistant phenotype in *Synechocystis* sp. PCC 6803 Δslr1212 cells in the absence of kanamycin to prove that disruption of ORF slr1212 causes this alteration. The presence of kanamycin in the growth medium of cultured Δslr1213 mutant cells did not impact upon copper resistance however.

As it was initially concluded that disruption of ORF slr1212 did cause a copper-resistant phenotype, the proposed two-component system comprising ORFs slr1212 and slr1213 became candidates as components of the regulatory system controlling the expression of *pacS*, a gene encoding a copper exporter (Robinson, 1999). However, in such a case, it would be expected that Δslr1212 cells would

share a common phenotype with ΔpacS cells i.e. loss of control of the genetic switch controlling plastocyanin and cytochrome c_{553} expression. However, studies of cells cultured in copper replete and deplete conditions using laser flash photolysis did not substantiate this hypothesis.

The role of copper ions at the binding sites of ethylene receptors as originally hypothesised in 1967 (Burg and Burg, 1967) has been well studied. The hypothesis is based upon the interactions between olefins and transition metals. The phenomenon of small gaseous molecules binding reversibly with a protein-based receptor through interactions with a transition metal co-factor is well established: FixL in bacteria (Gilles-Gonzalez *et al.*, 1991), and the nitric oxide receptor in animals (Ignarro, 1991) are good examples. Recent studies have demonstrated a requirement for copper in ethylene signalling *in planta* and identified RAN1, a copper transporter that acts by delivering copper to a post-golgi compartment to form functional ethylene receptors (Hirayama *et al.*, 1999). It has further been shown that the addition of 300 µM exogenous copper sulphate to yeast expressing the *etr1* gene from *Arabidopsis thaliana* leads to a 10 to 20-fold increase in ethylene-binding activity (Rodriguez *et al.*, 1999).

The presence of a functional ethylene-binding domain in a protein found in cyanobacteria supports the hypothesis that ethylene receptors of higher plants may have a common ancestor in cyanobacteria (as may phytochromes). However, the findings in this study that disruption of the ORF slr1212 does not cause an increased resistance to copper toxicity contradicts other findings. The data from this study does not support the hypothesis by Esch *et al.* (1998) that Slr1212 'may have originally functioned strictly as a copper-binding domain that was recruited as an ethylene sensor because it serendipitously created a unique a unique chemical environment that allowed ethylene to bind copper with exceptional stability'. The requirement for copper ions in ethylene-binding by *Arabidopsis thaliana* ETR1 has been clearly demonstrated, and it is speculated that Slr1212 also requires copper ions for efficient ethylene-binding (Rodriguez *et al.*, 1999).

5.7.2. Slr1212 and light

Analysis of the predicted protein sequence of Slr1212 reveals a protein that could have a role in ethylene or light signalling, possibly both. This chapter described a detailed characterisation of the Δslr1212 mutant in order to test the hypothesis that this protein plays a role in light sensing. Experiments analysed the growth of the mutant in light of different spectral quality, and a characterisation of the photosynthetic machinery was carried out, alongside analysis of the cellular response to an altering fluence rate.

Slr1212 has a GAF domain (Aravind and Ponting, 1997), exhibiting homology to the chromophore binding domain of phytochromes, and contains the conserved cysteine residue required for binding of a chromophore (Bleecker, 1999), implicating Slr1212 as a protein that can sense both light and ethylene signals. Growth of the mutants generated in this study in different light qualities did not implicate Slr1212 or Slr1213 in the acclimation of cells to these environments. This data differs from that generated from studies of other phytochrome-like proteins identified from the *Synechocystis* sp. PCC 6803 genome. It has been shown that *cph1* and *cph2* autocatalytically attach linear tetrapyrroles to form spectrally active holoprotein (Hughes *et al.*, 1997; Yeh *et al.*, 1997). Mutants with disrupted *cph1* and *cph2* genes have a pleiotropic phenotype under high light conditions. Under such conditions it was observed that the chlorophyll content per cell was reduced in the *cph1* mutant, and that the growth rate is decreased. *cph2* mutants have a reduced growth rate in red light conditions. It has been suggested that these genes may function in adjusting metabolism to higher irradiances, as well as in motility and pili formation (Fiedler *et al.*, 2000). *plpA* mutants have been shown to be unable to grow photoautotrophically under blue light (Wilde *et al.*, 1997). Despite Δslr1212 cells being able to maintain a normal growth rate in different light qualities, a role for this protein in light sensing/acclimation to the light environment cannot be ruled out, as their may be overlapping sensory roles by the different phytochrome-like proteins (see section 5.2.1 on page 129). Thus, growth of the Δslr1212 strain under far-red light still remains to be investigated. If Slr1212 were to act as a phytochrome-like receptor of higher plants, the Δslr1212 strain may grow poorly under these conditions in comparison to wild-type.

An analysis of the photosynthetic machinery in *Synechocystis* sp. PCC 6803 wild-type and mutant strains was carried out to determine if Slr1212 and/or Slr1213 have a role in the regulation of these cellular components. SDS-PAGE analysis of cell fractions initially suggested a reduction in the cellular content of PSI reaction centres when cultured under standard conditions. Although western blotting using an antibody against two of the constituent polypeptides did not support this, analysis of cells by 77K fluorescence spectroscopy revealed some alterations in the reaction centres of Δslr1212 cells. Under normal and high light conditions, alterations in the structure of PSI reaction centres were implicated, and Δslr1212 cells displayed an enhanced response to high light by reducing the number of PSI reaction centres per cell. Further study of this is required, and PSI content per cell needs to measured empirically. Despite these alterations however, whole chain electron transfer rates in photosynthesis were unaffected.

The acclimation of *Synechocystis* sp. PCC 6803 to a high light environment was also studied using a ^{35}S-methionine pulse-chase experiment. A similar method has already been used successfully in *Synechocystis* sp. PCC 6803 for the identification of proteins induced by salt shock, with induced proteins being observed within one hour (Fulda and Hagemann, 1995; Hagemann *et al.*, 1994), proteins induced by salt, heat and light stresses (Fulda *et al.*, 1999), and shifts in inorganic carbon regimes (Maestri *et al.*, 1998). Interestingly following a light shock of 2000 μmolm^{-2}s^{-1}, there was no observable induction of stress proteins in *Synechocystis* sp. PCC 6803, unlike the response of both *Synechococcus* sp. PCC 7942 and *Synechococcus* sp. PCC 7418. Intermittently, the synthesis of a few proteins was found to be weakly enhanced in light-shocked *Synechocystis* sp. PCC 6803, although in general no significant differences were observed between control and light-shocked cells (Fulda *et al.*, 1999). In this study, when cells were exposed to a light stress of 850 μmol m^{-2} s^{-1} a protein of approximately 33.5 kDa was induced, which may be a stress protein. However no differences in the protein profiles of wild-type and Δslr1212 cells was observed, which does not provide any evidence of a role for Slr1212 in the acclimation of cells to a high light environment.

The phototactic responses of *Synechocystis* sp. PCC 6803 wild-type and mutant cells were also studied. Previous research had implicated a phytochrome red/far-red like photoreceptor in positive phototactic responses (Choi *et al.*, 1999). It

was observed that the wild-type and mutant strains showed an equal positive phototactic response in a light gradient. This result does not therefore implicate either Slr1212 or Slr1213 in the light sensing or regulatory mechanisms of *Synechocystis* sp. PCC 6803 involved with the phototactic responses. A recent study of phototaxis in *Synechocystis* sp. PCC 6803 (Yoshihara *et al.*, 2000) found that distinct colonies isolated from a PCC strain reproducibly exhibited either positive or negative phototaxis in lateral illumination. Disruption of a series of ORFs identified from the genome database with similarity to chemotaxis genes of flagellated bacteria caused negative phototactic responses in previously positive phototactic isolates. Interestingly one of the ORFs, sll0041, encodes a protein with the highly conserved signalling domain of the methyl-accepting chemotaxis protein, Tsr, as well as two GAF domains. It is tempting to speculate that the positive phototactic response may be regulated by phytochrome-like photoreceptors, and it is possible that sll0041 may act in conjunction with other sensors. However, no evidence was obtained that might suggest a role for the Slr1212 and Slr1213 proteins in this. Furthermore, analysis of the phototactic movements of Δ*cph1*, Δ*cph2*, Δ*plpA*, Δslr1393 and Δsll1473 cells by Fiedler *et al.* (2000) found no evidence to suggest a role for these phytochrome-like proteins in phototaxis. However, the results gained in this study, and also by Fiedler *et al.* (2000) and Choi *et al.* (1999) will have been confused by the presence of distinct cell types within the population. The identification of positively and negatively acting phototaxis cell types should allow further dissection of the phototaxis responses of *Synechocystis* sp. PCC 6803.

It was also observed that when a Δslr1212 mutant in a glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was generated, there was no difference between the growth characteristics of wild-type and mutant cells. This does not concur with the findings of Wilde (Pers. Comm., 2000), who observed that a Δslr1212 mutation in a glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was very difficult to maintain in any growth condition. It would be interesting to transform the ATCC strain of *Synechocystis* with the ORF slr1212 knockout construct, p1b, to test if this phenotype is strain dependent.

5.7.3. Slr1212 and ethylene signalling

This chapter also describes pulse-chase experiments carried out using ^{35}S-methionine to identify components of both the ethylene biosynthetic and ethylene signalling pathways.

The laser photoacoustic studies undertaken in this study (see Chapter 4) demonstrated that the addition of ACC to *Synechocystis* sp. PCC 6803 cultures resulted in the release of ethylene. This response is identical to that observed with higher plants, such as that observed with *Cymbidium* flowers (Woltering *et al.*, 1991), as ACC is the immediate precursor of ethylene, a step catalysed by the enzyme ACC oxidase (Yang and Hoffman, 1984). However, analysis of the *Synechocystis* sp. PCC 6803 genome did not reveal any homologues of the ACC oxidase enzyme. In an effort to identify components of the ethylene biosynthetic pathway, a pulse-chase experiment was carried out following the addition of 5 mM ACC to cells (this concentration resulted in a marked release of ethylene by cells). It was established that at this concentration, ACC halted protein synthesis, and following incubation for 3 days caused cell death. A possible explanation is that the ethylene release observed results not from a physiological process, but is the result of membrane damage to the cells caused directly by ACC. Alternatively, the release of ethylene following ACC addition may be due to ACC being a component of an intact ethylene biosynthetic pathway in *Synechocystis* sp. PCC 6803. The conversion of ACC to ethylene is an oxidation step which could possibly be catalysed by a host of oxidase enzymes. Despite the lack of an ACC oxidase enzyme, the fact that ACC results in ethylene release could indicate the presence of a plant-like biosynthetic pathway. All vascular plants analysed to date synthesise ethylene via the Yang cycle, where AdoMet is converted to ACC by ACC synthase (Johnson and Ecker, 1998). However, ethylene production following incubation of *Synechocystis* sp. PCC 6803 with L-methionine was found not to result in ethylene production. It would be interesting to determine the effect of AdoMet addition on ethylene production by *Synechocystis* sp. PCC 6803. A final possibility is that ethylene is produced and released by cells following exposure to ACC as a response to a non-specific stress. The production of ethylene in plants is induced both at specific stages of development and by stress. It has been shown that a relationship exists between mechanical stresses such as wounding (Bouquin *et al.*, 1997), other stresses such as

flooding and pathogen attack (Johnson and Ecker, 1998) and the release of ethylene. It is therefore possible that ethylene is produced in similar circumstances in *Synechocystis* sp. PCC 6803, eliciting a response appropriate to the original stimulus. However, to test this hypothesis further studies using laser photoacoustics would need to be carried out, examining the effects of other stresses such as nutrient, temperature, and light stresses on ethylene release. It is intriguing to speculate that ethylene may function as a signalling molecule in *Synechocystis* sp. PCC 6803, perhaps acting to prime cells in the surrounding population against an impending stress. To the best of the investigators knowledge, there is no precedent for such a mechanism in another prokaryotic organism.

Further pulse-chase experiments with ^{35}S-methionine were carried out following treatment with ethylene to attempt to identify a cellular response. However, although a novel 21 kDa polypeptide was induced in wild-type cells compared to Δslr1212 cells, this response was irreproducible. Further experimentation suggested that ethylene has no effect upon protein synthesis that could be identified by SDS-PAGE analysis and autoradiography. It is possible however that changes in protein synthesis may require a longer time period to become apparent than that used in the experiments in this study.

A recent review commented 'the biological function of Slr1212 remains obscure given that *Synechocystis* makes no detectable ethylene and has no known response to applied ethylene' (Bleecker, 1999). Although this study has shown the capability of *Synechocystis* sp. PCC 6803 to produce ethylene (although it remains unknown if this is the result of a physiological process) it remains difficult to determine the biological function of this protein. It is likely that the use of ethylene antagonists such as 1-methylcyclopropene (1-MCP) (Hall *et al.*, 2000; Sisler *et al.*, 1999) will prove to be invaluable tools in discerning a role for Slr1212 of *Synechocystis* sp. PCC 6803 in ethylene signalling. 1-MCP acts at very low concentrations and was found to inhibit ethylene-induced ripening and senescence (Sisler *et al.*, 1999). It was shown that 1-MCP acts as an effective inhibitor of ethylene-binding in transgenic yeast expressing either the *ETR1* or *ERS1* genes (Hall *et al.*, 2000).

5.7.4. Concluding remarks

This chapter has discussed the work carried out to phenotypically characterise the Δslr1212, Δslr1213 and Δslr1212 Δslr1213 mutants of *Synechocystis* sp. PCC 6803 generated in this study. It has been shown that Slr1212 and Slr1213 have no discernible roles in copper binding, contrary to earlier reports. Furthermore, it has been shown that Slr1212 may be involved in the acclimation of cells to their light environment. No evidence was gathered to support the hypotheses that Slr1212 is involved in either the phototactic responses of *Synechocystis* sp. PCC 6803 or in ethylene signalling.

Chapter 6

Expression analysis of slr1212

6. Expression analysis of slr1212

6.1. Introduction

Analysis of the genome sequence around ORF slr1212 led to the supposition that ORFs slr1212 and slr1213 would constitute the components of a two-component sensor kinase system, and would be co-expressed as part of an operon structure. The expression of these ORFs is of particular interest. If ORFs slr1212 and slr1213 were shown to be co-expressed, it would provide strong evidence supporting the hypothesis that they acted together in a signal transduction system. Although previous analysis has shown that histidine kinases and response regulator genes are spread throughout the genome of *Synechocystis* sp. PCC 6803 (Kotani and Tabata, 1998), the close proximity of ORFs slr1212 and slr1213 suggests that they may work in conjunction with one another. Further transcriptional studies could be used to determine the environmental conditions in which ORFs slr1212 and slr1213 are up- or down-regulated.

Studies of the expression of ethylene receptors in tomato found that expression was regulated, and whilst undetected in unripe fruit or pre-senescent flowers, expression was induced in ripening, flower senescence, and in abscission zones (Payton *et al.*, 1996). A different study, also in tomato, showed that eTAE1 (cDNA homologue of *Arabidopsis thaliana* ETR1) was constitutively expressed in vegetative and reproductive tissues (Zhou *et al.*, 1996). A subsequent study identified two homologues of *Arabidopsis thaliana* *ETR1* in tomato, *LeETR1* and *LeETR2* (*Lycopersicon esculentum ETR1* and *ETR2*). Both were constitutively expressed in all tissues, but *LeETR2* was expressed at lower levels, and induced prior to seed germination, and down-regulated in elongating seedlings (Lashbrook *et al.*, 1998). Most recently, research in tomato has identified five ETR1 homologues. Despite LeETR5 lacking a conserved histidine in the histidine kinase domain, mutants still conferred dominant ethylene insensitivity in transgenic *Arabidopsis* plants, indicating that histidine kinase activity is not required for an ethylene response. Both *LeETR4* and *LeETR5* are also constitutively expressed, although expression was much higher in reproductive tissues (Tieman and Klee, 1999). ETR2 of *Arabidopsis thaliana* has

also been shown to be constitutively expressed, with a higher expression in flowers and leaves (Sakai *et al.*, 1998).

The ability to detect the expression of ethylene receptor homologues in tomato plants in most tissues and conditions, and in *Arabidopsis thaliana* led the investigator to attempt such studies with *Synechocystis* sp. PCC 6803. In this study two techniques have been used, Northern blotting and RT-PCR, to examine the expression of the ORFs slr1212 and slr1213 to establish if they are expressed, and if so, if they are co-regulated as part of an operon.

6.2. Northern blotting

6.2.1. Analysis of gel systems

For the analysis of mRNA by Northern blotting to be successful, it was important to use a gel system that denatured the RNA, but did not lead to its degradation. In this study, two gel systems were evaluated for their use with RNA isolated from *Synechocystis* sp. PCC 6803.

Total RNA was isolated as described in section 2.15.3 on page 78. Although several techniques were evaluated, including the use of commercially available reagents and kits such as TRIzol® reagent (GibcoBRL, USA), SV total RNA isolation system (Promega, USA) and QIAGEN RNeasy miniprep kit (Qiagen, UK), none were found to isolate a large enough quantity of intact RNA for analysis. As a result, acid phenol extractions were implemented (see section 2.15.3 on page 78).

Initial evaluation of the integrity of the isolated RNA involved sample analysis on 1% (w/v) agarose gels and examination of the ribosomal RNA bands. As rRNA comprises approximately 70% of a cells total RNA, gross degradation of a sample is easily observed by viewing the integrity of the rRNA bands; these being 23S (2.95, 2.5 and 0.5 kb) and 16S rRNA (1.5 kb) (Alley, 1987; Mulligan *et al.*, 1984). The 5S subunit tends to co-migrate with tRNAs at the bottom of the gel. In most cases, this proved to be suitable for provisional analysis of RNA. However, the best test of the integrity of RNA is to observe discrete bands on a Northern blot.

Further analysis of the integrity of the RNA samples used two different denaturing gel systems. The first system, described by Sambrook *et al.* (1989), contained MOPS, sodium acetate, EDTA and formaldehyde as constituents of the

gel, whilst samples were denatured in formaldehyde and formamide (see section 2.15.4). The second system contained HEPES, EDTA and formaldehyde as constituents of the gel and samples were denatured in deionised formamide and formaldehyde (see section 2.15.5).

Figure 6-1 shows the effectiveness of the gel systems tested for the separation of total RNA samples isolated from *Synechocystis* sp. PCC 6803. It can be seen that non-denaturing agarose gel electrophoresis was sufficient to quickly check the integrity of the RNA.

Of the two denaturing gel systems, the formaldehyde-HEPES system was significantly better at preventing RNA degradation than the formaldehyde-MOPS system. Following these preliminary investigations, all RNA for analysis by Northern blotting hybridisation was separated using the formaldehyde-HEPES denaturing agarose gel system.

6.2.2. Analysis of ORF slr1212 expression using [α-^{32}P]-dCTP labelled DNA probes

Initial attempts to examine the expression of ORF slr1212 in *Synechocystis* sp. PCC 6803 used [α-^{32}P]-dCTP labelled DNA probes. Although not as sensitive in this application as labelled RNA probes, DNA probes are easier to produce and are not subject to degradation by RNase contamination. As only the presence of discrete bands on a blot (from highly expressed transcripts) could categorically demonstrate the presence of intact RNA, samples were also hybridised with probes against *cpcB*, the gene encoding for the beta subunit of phycocyanin (Plank and Anderson, 1995). The probes for hybridisation were generated using the primer pairs Etr1 F/Etr1 R and CpcB F/CpcB R (see section 2.12), yielding PCR products of 2535 and 353 bp respectively. These PCR products were then labelled by random priming with [α-^{32}P]-dCTP as described in section 2.17.2.

The blots shown in Figure 6-2 show the analysis of RNA isolated from *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells in the late-exponential phase of growth, cultured at 30°C with white light at a fluence rate of 35 μmol m^{-2} s^{-1}. It was observed that when RNA was hybridised with the Cpc B F/CpcB R probe, a transcript of
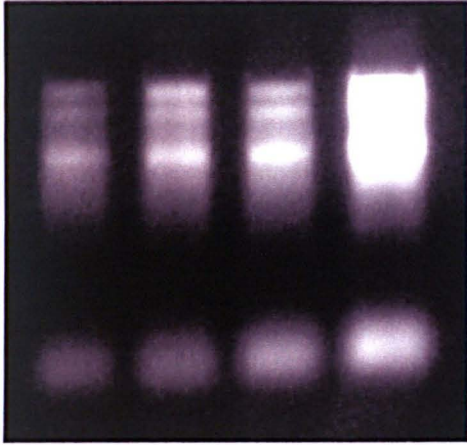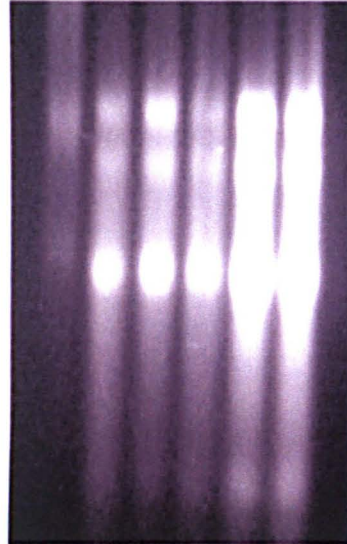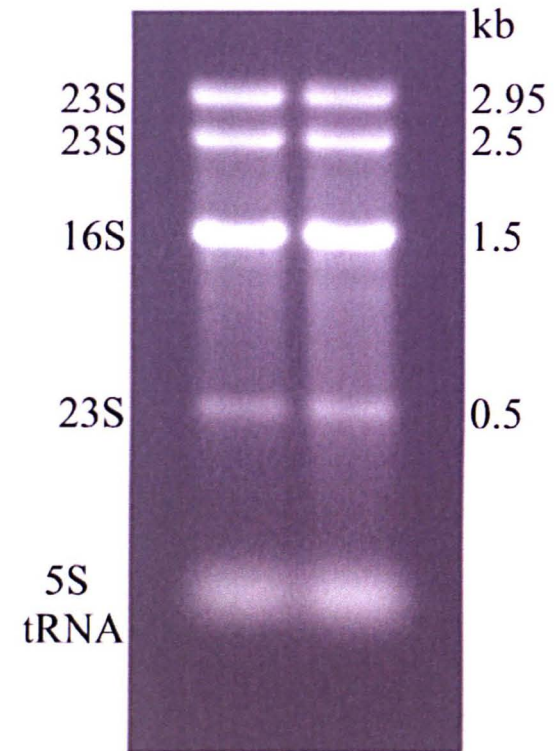
Figure 6-1 :- Total RNA isolated from *Synechocystis* sp. PCC 6803 wild-type and mutant strains and analysed on A. a 1% (w/v) agarose gel; B. a formaldehyde-MOPS denaturing gel; C. a formaldehyde-HEPES denaturing gel. The RNA has degraded in B., but the ribosomal subunits are clear in C., and the RNA is intact. 20 μg RNA was loaded per lane.
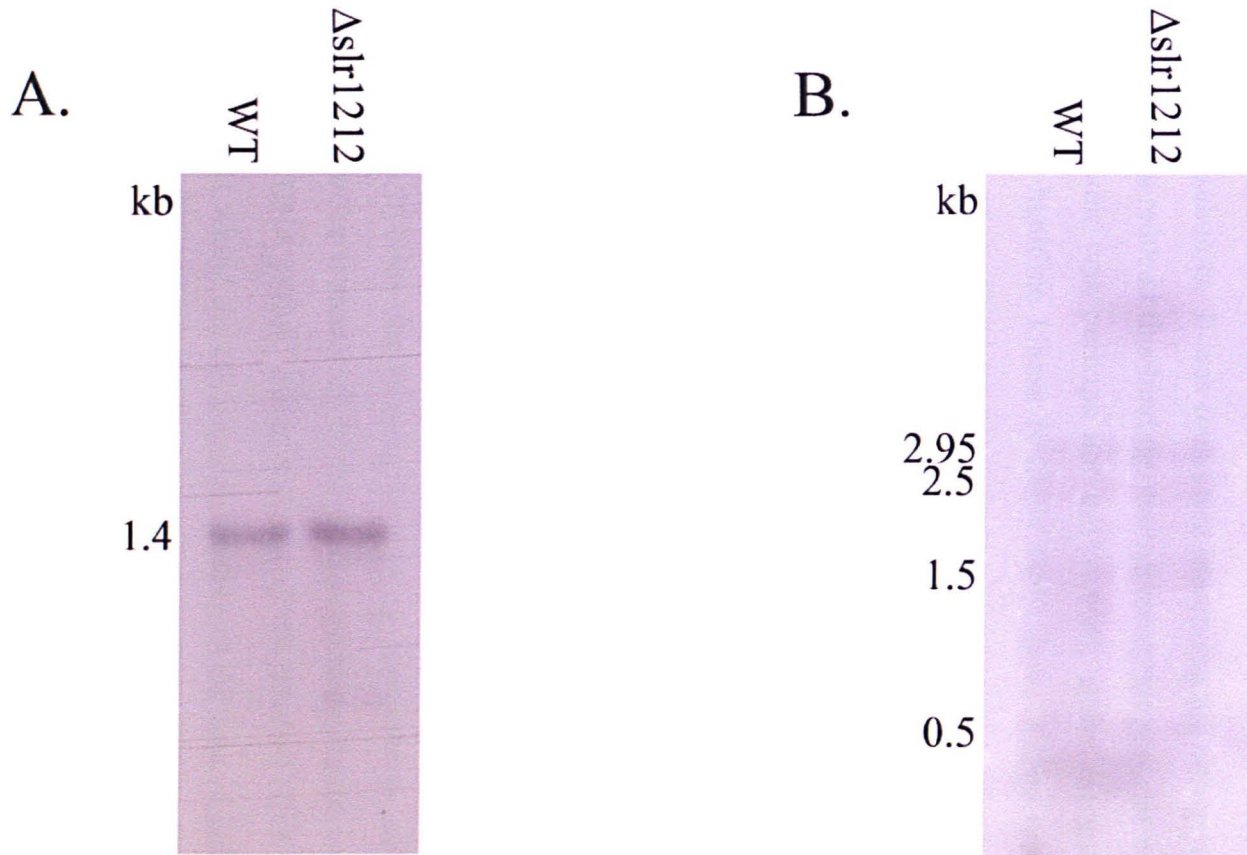
Figure 6-2 :- Northern analysis of total RNA isolated from *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells in the exponential growth phase, cultured with illumination from a white fluorescent lamp at a fluence rate of 35 μmol m^{-2} s^{-1}. A. Northern blot probed with a [α-^{32}P]-dCTP labelled cpcB F/cpcB R DNA probe. B. Northern blot probed with a [α-^{32}P]-dCTP labelled etr1 F/etr1 R DNA probe. 30μg RNA was loaded per lane.

approximately 1.4 kb was detected. The size of this transcript is consistent with the *cpcBA* genes (encoding the alpha and beta subunits of phycocyanin) being co-transcribed, as previously reported by Plank and Anderson (1995). This result demonstrated that the isolated RNA was intact, and had not been degraded at any stage. This also demonstrated the suitability of Northern blotting to examine the co-regulation of genes in operons.

However, when total RNA was hybridised with a probe homologous to ORF slr1212, the only bands visible on the blot were due to non-specific binding to the ribosomal subunits. It was found that increasing the stringency of the hybridisation and washes reduced this binding, but no transcript was detectable with this system. Such a result was perhaps not unexpected, since expression of a membrane-associated histidine kinase is likely to be low. Other research has shown that expression of an *ETR1* homologue in tomato was low, and could not always be detected reliably by Northern hybridisation (Payton *et al.*, 1996).

6.2.3. Analysis of ORF slr1212 expression using $[\alpha\text{-}^{32}P]$-UTP labelled RNA probes

In order to increase the sensitivity of the Northern hybridisation system, the construction of RNA probes was planned. Although technically more demanding to produce, the use of RNA probes in Northern hybridisation experiments should increase the overall sensitivity by 8-10 times (Melton *et al.*, 1984; Srivastava and Schonfeld, 1991). This is because RNA:RNA duplexes tend to be more stable than RNA:DNA hybrids due to a higher melting temperature and binding affinity, and when the probe is made, all of the product is homologous to the target. This is in contrast to DNA probes, where only one of the labelled DNA strands is homologous to the target whilst the other is not, leading to the loss of half of the original label. However, there are disadvantages associated with the use of RNA probes for Northern hybridisation. The probes can be subject to degradation due to RNase contamination, and there can be an increase in cross-hybridisation (Ambion Technical Bulletin).

RNA probes against ORF slr1212 and 16S rRNA transcripts were produced by incorporating T7 phage RNA polymerase promoters onto the 5' end of PCR primers, which were then used to amplify *Synechocystis* sp. PCC 6803 genomic

DNA as described in section 2.17.4 on page 83. The resultant PCR products had a core T7 phage promoter incorporated downstream of the gene, which could then be used for *in vitro* transcription reactions to yield radiolabelled antisense RNA probes. This approach was superior to the more traditional approach which involves cloning the gene (or portion thereof) of interest into the multiple cloning site (MCS) of a cloning vector such as pBLUESCRIPT II KS, flanked on each side by promoters for different polymerases (which can include SP6, T7, and T3 phage RNA polymerases). Sense and antisense transcripts could then be produced by linearising the vector to allow run-off RNA transcripts to be produced. However, such a strategy is more labour-intensive, and the transcripts produced will contain some homologous sequence to the MCS.

If the hypothesis about ORFs slr1212 and slr1213 being co-transcribed is correct, the transcript would be at least 3.3 kb, and up to 8.3 kb in length (if ORFs slr1211 through to slr1214 are included). It is likely that the ORFs are co-transcribed, as the majority of transcripts in bacteria are polycistronic (Lewin, 1994). Such a large transcript could have a short half-life, and perhaps mRNA degradation would begin following translation at the 5' end before transcription has completed synthesis of the 3' end (Lewin, 1994). Therefore, the use of probes short in length, such as those RNA probes designed here would increase the likelihood of the probe hybridising to a homologous target.

An *in vitro* transcription reaction with a T7 Etr1 F/Etr1 R template yielded a 280 bp antisense RNA probe homologous to the 5'-portion of ORF slr1212 transcripts. The T7 16S F/T7 16S R template yielded a 107 bp antisense RNA probe homologous to the 5' portion of 16S rRNA. Figure 6-3 A shows the PCR products used as template in *in vitro* transcription reactions to yield antisense RNA probes. A T7 Etr1 F/T7 Etr1 R PCR product was subsequently used to generate an $[\alpha\text{-}^{32}P]$-dCTP labelled DNA probe (see section 2.17.2) and an $[\alpha\text{-}^{32}P]$-UTP labelled RNA probe (see section 2.17.4). The sensitivity of these probes was compared by hybridising them with T7 Etr1 F/T7 Etr1 R PCR products on a dot blot (see Figure 6-3 B). It was observed that the DNA and RNA probes were equally sensitive in this application, detecting 8 ng dsDNA following 4.5 hours exposure. Following a 5-day exposure, 200 pg of

A.

T7 Etr1 R
T7 Etr1 F/
T7 16S R
T7 16S F/

bp

303

130

B. [α-^{32}P]-UTP labelled
T7 Etr1 F/Etr1 R RNA
probe. 4.5 hrs exposure.

ng   500 80 40 8 800 400   pg

[α-^{32}P]-dCTP labelled
T7 Etr1 F/Etr1 R DNA
probe. 4.5 hrs exposure.

C.

200   pg

[α-^{32}P]-UTP labelled
T7 Etr1 F/Etr1 R RNA
probe. 5 day exposure.
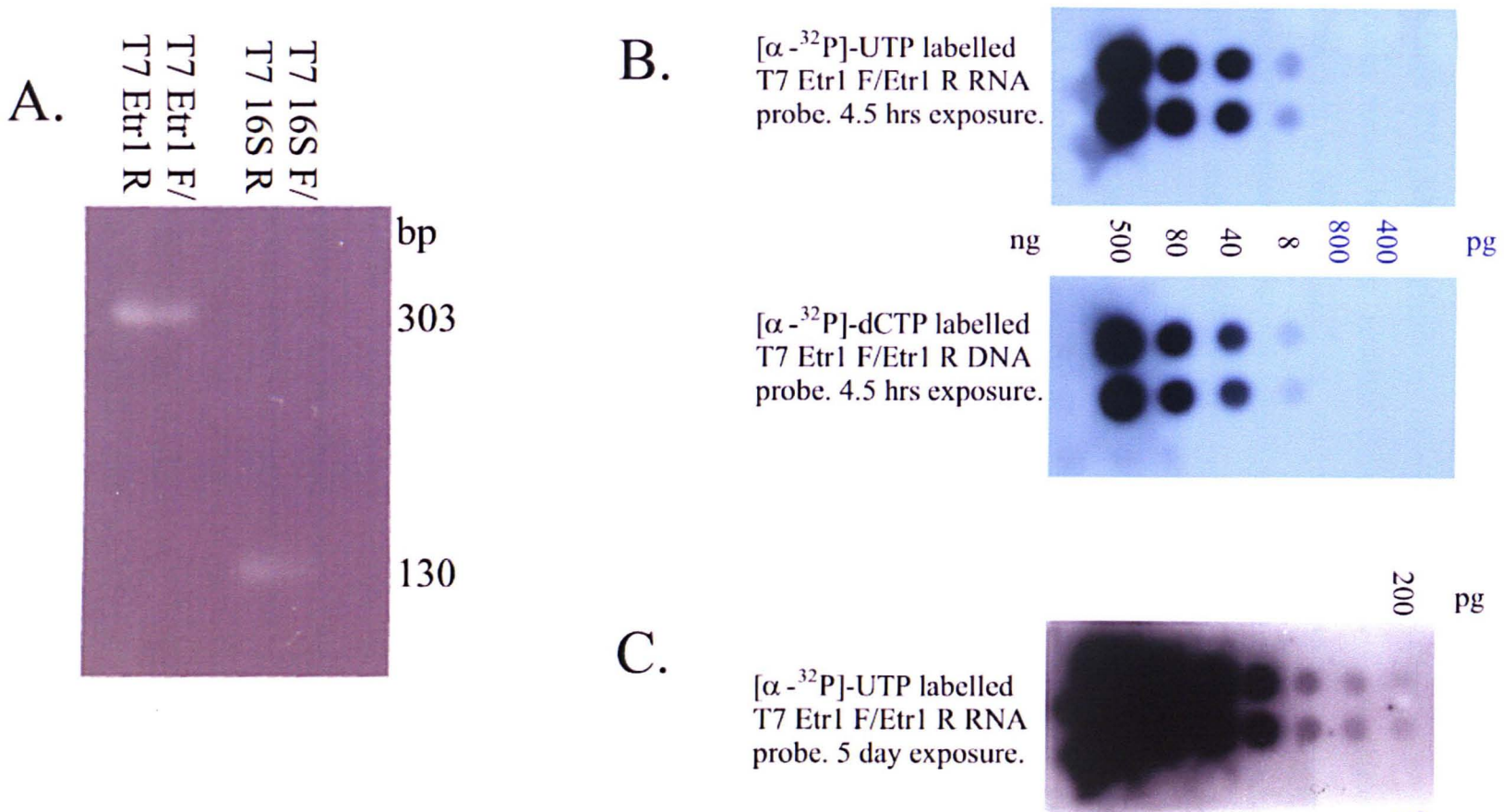
Figure 6-3 :-Evaluation of RNA probes for the detection of ORF slr1212 transcripts by Northern analysis. A. PCR products incorporating portions of the slr1212 and 16S rDNA ORFs and core T7 phage RNA polymerase promoters analysed on a 2% (w/v) agarose gel. B. Comparison of radiolabelled RNA and DNA probes for the detection of T7 Etr1 F/Etr1 R PCR product. C. Following a 5-day exposure, the detection limit of a radiolabelled RNA probe against a homologous DNA target is 200 pg.

dsDNA was detected using the RNA probe (see Figure 6-3 C). This implied that when hybridised with a homologous RNA target, the detection limit would be lower than 100 pg transcript. This is because in this experiment, the RNA probe could only bind to half the total amount of DNA present, coding for sense RNA, and also because RNA:RNA duplexes are more stable. It was therefore concluded that RNA probes would be more suitable than DNA probes for detecting ORF slr1212 transcripts by Northern analysis. As a result of problems encountered intermittently, a positive control dot blot was carried out with each blot to ensure that the probe was not degraded and could effectively detect its target.

An analysis of the expression of ORF slr1212 and 16S rDNA was carried out with RNA extracted from *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells cultured at 30°C with illumination from a white fluorescent lamp at a fluence rate of 35 μmol m^{-2} s^{-1} using [α-^{32}P]-UTP labelled RNA probes. The use of the Δslr1212 strain provides a negative control for ORF slr1212 expression. The expression of 16S rDNA was used as an internal control as its expression tends to remain constant (Bhaya *et al.*, 1999; Bustos *et al.*, 1990). This RNA therefore serves as an internal standard against which other RNA values can be normalised. Such a standard is required due to inconsistencies which can be introduced from the procedures used to isolate and analyse the RNA samples (Smith, 1995). Figure 6-4 shows the Northern analysis carried out. Hybridisation of the T7 16S F/T7 16S R probe with 16S rDNA transcripts was very efficient, and exposure of the blot for a period of longer than 15 minutes at room temperature resulted in over-exposure. It is likely that due to the high abundance of 16S rRNA in the cell, a probe of lower specific activity would have been more suitable. Also, loading a much lower concentration of RNA on the denaturing gel for analysis would significantly reduce the signal leading to cleaner signals. When the total RNA was hybridised with the T7 Etr1 F/T7 Etr1 R RNA probe, cross hybridisation was observed with all of the ribosomal subunits. Increasing the stringency of the hybridisation conditions (up to 65°C) and washes (65°C, 0.5x SSC 0.1% (w/v) SDS) did not noticeably reduce this. However, a transcript of approximately 1.95 kb was detected in wild-type RNA but not in RNA isolated from Δslr1212 cells. This indicated that this could be a transcript from ORF slr1212. However, an mRNA of this size does not provide any additional
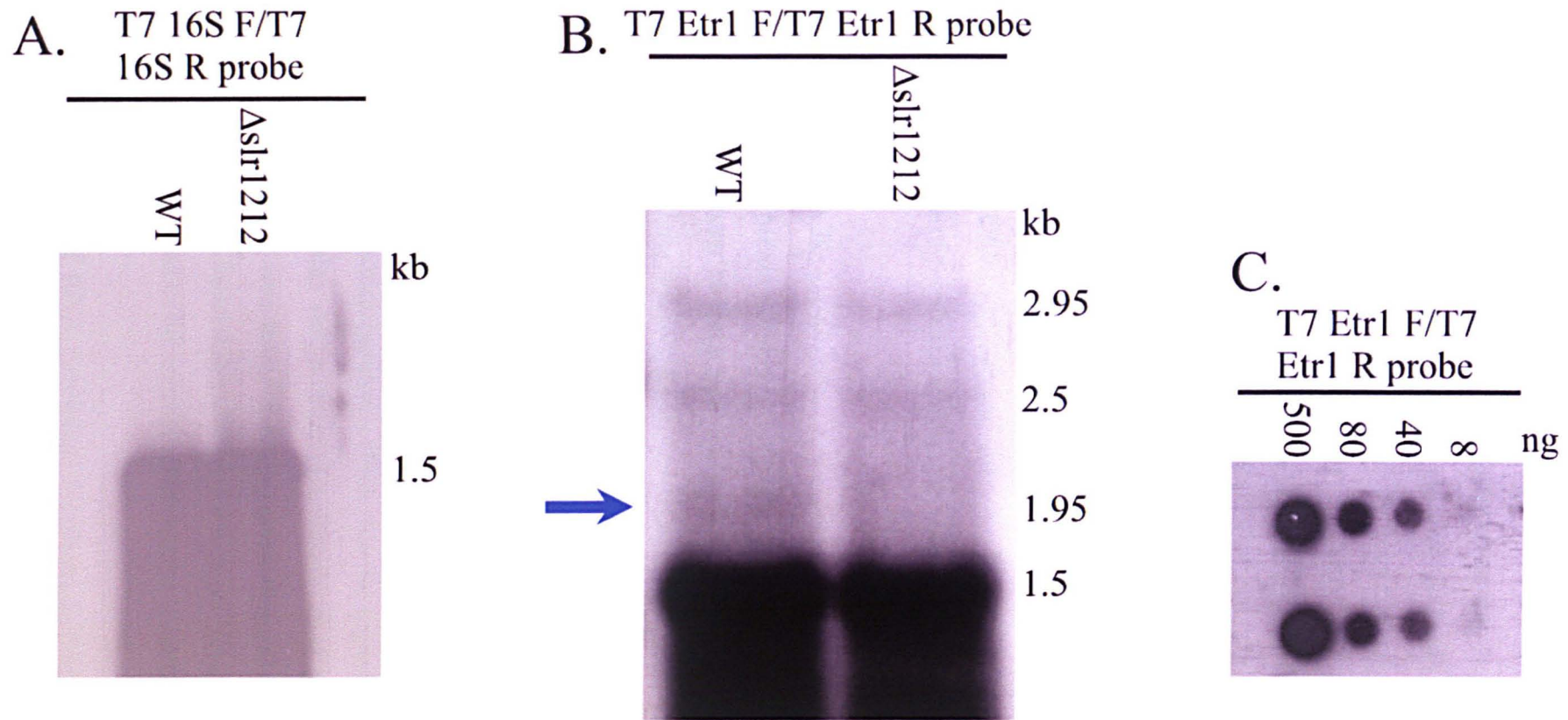
Figure 6-4 :- Northern analysis of the expression of ORF slr1212 in *Synechocystis* sp. PCC 6803 wild-type and Δslr1212 cells cultured at 30°C with illumination from a white fluorescent lamp at a fluence rate of 35 μmol m^{-2} s^{-1}. 30 μg total RNA hybridised with A. a labelled T7 16S F/T7 16S R RNA probe (10 minute exposure), and B. a labelled T7 Etr1 F/T7 Etr1 R RNA probe (22 hour exposure). The blue arrow highlights a possible ORF slr1212 transcript. C. Dot blot analysis of T7 Etr1 F/T7 Etr1 R RNA probe hybridised with T7 Etr1 F/T7 Etr1 R PCR product (22 hour exposure).

information regarding the co-regulation of the ORFs including and surrounding slr1212 as the ORF itself is 2.5 kb in length. This result could be due to the process discussed earlier, where degradation of the mRNA begins following translation at the ribosome, before transcription has finished at the 3' end. Hence, a full-length transcript would never be present in the cell to detect. This result does indicate however, that ORF slr1212 is potentially expressed in cells cultured in standard growth conditions. This complements research with tomato ETR1 homologues, which demonstrated constitutive expression (Lashbrook *et al.*, 1998). The dot blot illustrated that the T7 Etr1 F/T7 Etr1 R probe had not degraded and was effective to a satisfactory level of sensitivity.

Since a polycistronic mRNA could not be detected containing slr1212 transcripts, it was decided not to attempt further Northern analysis using probes directed against transcripts from ORFs slr1212 and slr1213. Instead, efforts were directed towards RT-PCR which is more sensitive, and less technically demanding.

6.3. RT-PCR

Since it did not prove possible to confirm the co-regulation of ORFs slr1212 and slr1213 by Northern blotting techniques, RT-PCR was carried out using sequence specific primers designed to span the intercistronic regions between ORFs.

Despite the increased sensitivity of this technique which is useful for detecting rare messages, a commonly associated technical difficulty is the contamination of RNA samples with genomic DNA. Most RNA isolation techniques yield RNA with significant amounts of genomic DNA contamination. Although the isolation technique used in this study (see section 2.15.3 on page 78) utilised acidic phenol extractions (which partitions DNA into the organic phase) and an RNase-free DNase I step, samples still contained contaminating genomic DNA. Following extensive testing, it was found that contaminating DNA could be removed from small RNA samples following passage through an RNA binding column and DNA digestion whilst bound to the column as described in section 2.15.6.

RT-PCR analysis of RNA confirmed Northern hybridisation experiments which showed that ORF slr1212 is transcribed under standard growth conditions (see Figure

6-5 A). Primers were also designed spanning the intercistronic regions to examine the co-regulation of genes around ORF slr1212, and as a control additional primers were designed to span between *cpcB* and *cpcA* (RT CpcBA F/RT CpcBA R). Results obtained from RNA analysis with these primers showed that this was a suitable strategy to prove the existence of adjacent ORFs on a polycistronic mRNA. As expected, the genes coding for the phycocyanin subunits were expressed normally in Δslr1212 cells. These results are displayed in Figure 6-5 B. However, no evidence was found of the co-regulation of ORFs slr1212, slr1212, and slr1213 using primers to span between them (data not shown). This may be because the half-life of the mRNA is too short to detect (the slr1212 transcript was not consistently detected). There may also be slight degradation of the RNA samples as a whole, although this was not revealed following analysis by denaturing gel electrophoresis. Therefore, this does not preclude the possibility that these ORFs are part of an operon.

6.4. Discussion

The experiments presented in this chapter were carried out to examine the expression of ORF slr1212, and to specifically determine whether slr1212 was expressed as part of a polycistronic mRNA along with ORF slr1213, and perhaps ORF slr1211.

Using two separate techniques it was possible to detect ORF slr1212 transcripts, but no evidence of gene co-regulation was obtained. The detection of a transcript shows that under standard growth conditions, *Synechocystis* sp. PCC 6803 wild-type cells transcribe ORF slr1212 at a low rate. As expected this was not the case in the Δslr1212 strain, which shows that the allelic replacement strategy described in Chapter 3 to disrupt ORF slr1212 and prevent transcription and translation was successful.

RT-PCR appears to be a much more sensitive technique than Northern blotting, and the strategy designed to use primers that span between genes to detect polycistronic mRNA was shown to work with *cpcBA*. The tested hypothesis was that ORFs slr1212 and slr1213 would be co-transcribed as part of an operon. Proving this would have provided further evidence that these two proteins together formed a two-component signal transduction system.

A.

RT + - + -

WT

Δslr1212

bp
500
400
300

RT Etr1 F/
RT Etr1 R

B.

+ - + - RT

WT

Δslr1212

bp
500
400
300
200

RT CpcB F/
RT CpcB R

C.

bp + - + - RT

500
400
300
200
100

RT CpcB F/   RT CpcBA F/
RT CpcB R   RT CpcBA R
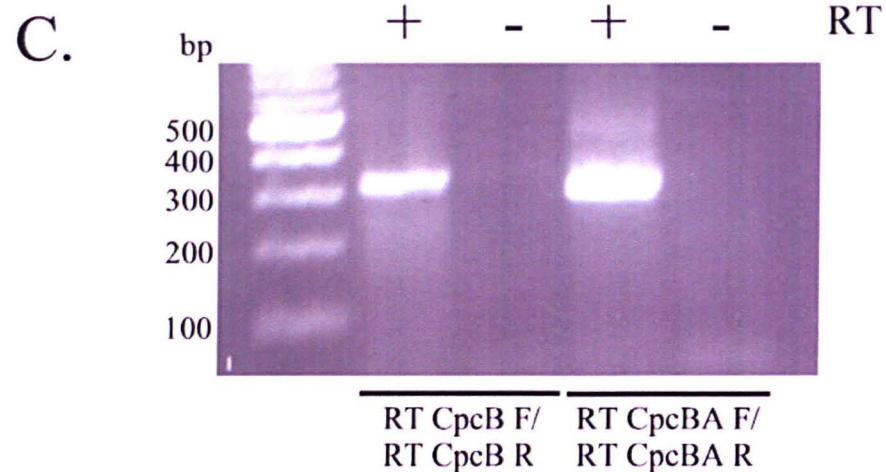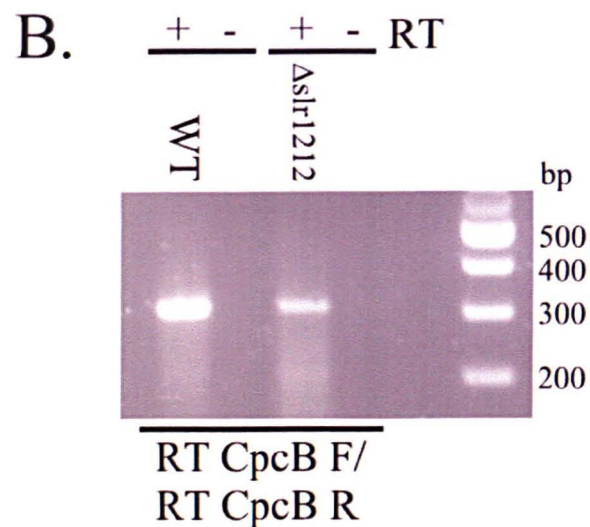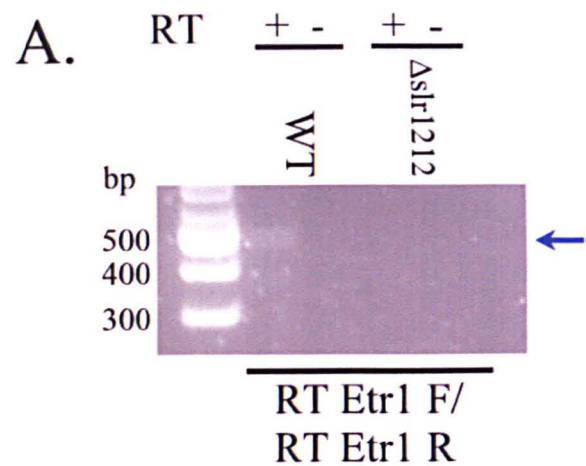
Figure 6-5 :- RT-PCR analysis of total RNA isolated from cells cultured at $30°C$ with white light illumination at a fluence rate of 35 µmol $m^{-2} s^{-1}$.
A. Analysis of total RNA from wild-type and mutant cells using sequence specific primers to amplify ORF slr1212 transcripts. The blue arrow highlights a transcript. B. Analysis of total RNA from wild-type and mutant cells using sequence specific primers to amplify *cpcB* transcripts. C. RT-PCR analysis of total RNA isolated from wild-type cells using sequence specific primers to amplify *cpcB* and *cpcBA* transcripts.

RT-PCR analysis has been used previously to demonstrate that two ORFs putatively encoding for a two-component signal transduction system are transcribed as a polycistronic message. *hpuA* and *hpuB* encode proteins in a two-component signal transduction pathway from *Neisseria meningitides* involved with binding to haemoglobin. As in this study, using primers designed to span between the ORFs it was possible to prove that the message was polycistronic (Lewis *et al.*, 1997). A similar approach was used to demonstrate that *silRS*, a two-component signal transduction system from *Salmonella* involved with resistance to silver ions, is also transcribed in a polycistronic message (Gupta *et al.*, 1999).

However, such data alone would not prove the existence of a two component system. In order to show this, it would be necessary to show a direct interaction between the two proteins. Two methods exist to do this, phospho-transfer and yeast two-hybrid assays. Yeast two-hybrid assays have been used previously to examine the interactions between NtrB and NtrC (Martinez-Argudo *et al.*, 2001). Although planning for the yeast two-hybrid assays was carried out, time constraints ruled those experiments out. Clearly, this is an area for further study by a future investigator.

The data gained showed that ORF slr1212 was transcribed under standard growth conditions, albeit at a rate so low as to be difficult to detect. As the slr1212 transcripts were detected only inconsistently, even using RT-PCR analysis, it is possible that ORF slr1213 transcripts were also present, but at levels too low to detect. A large transcript would also have a short half-life. Indeed, as discussed there is the possibility that a full-length transcript would never be present in the cell as transcription and translation are closely coupled in the same cellular compartment. Ribosomes begin translation of mRNA in the 5' to 3' direction before transcription has finished, and continue to do so whilst the mRNA persists. However, bacterial mRNA tends to be rapidly degraded in the 5' to 3' direction. Studies have shown that mRNA degradation closely follows translation, probably within 1 minute of transcription initiation, and that the 5' end of the mRNA begins degrading before the 3' has been synthesised or translated (Lewin, 1994).

As it has not proved possible to isolate an intact mRNA molecule transcribed from the ORF slr1212 region of the *Synechocystis* sp. PCC 6803 genome for analysis by Northern blotting or RT-PCR, an indirect approach could be taken which would still

provide information about the regulation of the ORFs in this region. Cloned portions of the *Synechocystis* sp. PCC 6803 genome including those around ORF slr1212 could be used as templates for *in vitro* transcription reactions, from which the labelled mRNA products are used for hybridisation experiments with DNA fragments. In this way, a detailed picture of the structure of an mRNA can be constructed without characterising the molecule directly. However, this approach is dependent on the isolation of a functional *Synechocystis* sp. PCC 6803 RNA polymerase for use in *in vitro*. Once this was available however, it would represent a very powerful means of analysing the regulation of genes in an operon, even when intact mRNA species are not available.

Despite being unable to directly identify an mRNA representative of the slr1212 region, it would also be interesting to attempt to examine the role of environmental factors on the expression of these ORFs. Perhaps a more suitable strategy to examine real-time expression of the ORFs in the slr1212 genomic region could be carried out with transcriptional fusions to reporter genes such as *lacZ* (Scanlan *et al.*, 1990) or luciferase (Aoki *et al.*, 1997). Such an approach is however dependent on an assay system sensitive enough to detect low rates of transcription.

6.4.1. Concluding remarks

This chapter has described the work that was carried out to study the expression of ORFs slr1212 and slr1213, a proposed two-component signalling pair, in *Synechocystis* sp. PCC 6803. Northern analysis identified a putative transcript expressed under white light conditions, but from its size no information was gained regarding the co-expression of this gene with others as part of an operon. A similar result was gained following RT-PCR analysis, where primers designed to span the intercistronic region between ORFs slr1212 and slr1213 did not produce a PCR product. This work did prove that the ORF slr1212 was expressed under standard growth conditions, and that the ORF therefore coded for a functional protein and was not a piece of unexpressed 'junk' DNA. This showed that Slr1212 must have a physiological role in *Synechocystis* sp. PCC 6803.

Chapter 7

Site-directed mutagenesis of Slr1213

7. Site-directed mutagenesis of Slr1213

7.1. Introduction

Two-component signal transduction systems recognise and respond to changes in environmental stimuli (Mann, 2000; Stock *et al.*, 1989). As outlined in the Introduction, such systems consist of a histidine protein kinase and a response regulator. Signalling is dependent upon autophosphorylation of a conserved histidine residue within the histidine protein kinase, and transfer of the phosphate to an aspartyl residue in the response regulator.

Modification of the conserved aspartyl residue in the response regulator to mimic the phosphorylated state can lead to constitutive activation of the signalling pathway. For example, NtrC of enteric bacteria is an enhancer-binding protein that activates transcription in nitrogen limiting conditions via the σ^{54}-holoenzyme. This protein is the response regulator of a two-component signal transduction system, and site directed mutagenesis studies of the *Salmonella typhimurium* protein found that a D54E substitution (the sole site of phosphorylation) caused constitutive activation (Klose *et al.*, 1993; Nohaile *et al.*, 1997). Subsequent studies with RcsB of *E. coli* K-12 (Gupte *et al.*, 1997) also showed that this aspartate-to-glutamate substitution caused constitutive activation of the response regulator.

The work in this study has been carried out with wild-type and mutant strains to identify a role for Slr1212 and Slr1213 in the physiology of *Synechocystis* sp. PCC 6803. Whilst this has provided some intriguing insights into the functioning of these proteins, no definitive role can currently be assigned. In an effort to further elucidate the roles of these proteins, it was decided to carry out an aspartate-to-glutamate activating substitution of Slr1213. The constitutive activation of this protein may allow the study of this two-component signal transduction system from a different point of view. Assuming that when ORF slr1212 is interrupted there is no activation of Slr1213 (as opposed to Slr1213 being activated in the absence of signal perception by Slr1212), constitutive activation of Slr1213 would allow the investigator to study the effects on cell physiology of the system always being turned 'on'.

This chapter describes the approach used to engineer the aspartate-to-glutamate substitution into ORF slr1213, and the subsequent strategy used to transfer this mutation onto the *Synechocystis* sp. PCC 6803 genome.

7.2. Methods

The sequence of events involved in the site-directed mutagenesis of Slr1213 involved identification of the conserved aspartate, cloning, site-directed mutagenesis, sequencing, deletion of ORF slr1213 and subsequent transformations.

A sequence alignment was carried out to identify the conserved aspartate residue of Slr1213. The alignment is shown in Figure 7-1 A on page 196. The residues that correspond to aspartate-13, aspartate-57, and lysine-109 of the *E. coli* response regulator CheY tend to be conserved among all sequences (Stock *et al.*, 1989). The conservation of the aspartate-13 and aspartate-57 residues is highlighted in the sequence alignment and a more detailed protein analysis is included in the Introduction. Aspartate-57 has been confirmed as the site of phosphorylation in CheY (Appleby and Bourret, 1999), and aspartate-54 in NtrC of *Salmonella typhimurium* (Nohaile *et al.*, 1997). Aspartate-54 of Slr1213 aligns with aspartate-57 of CheY and aspartate-54 of NtrC, and is the presumptive site of phosphorylation, and is therefore the target for an aspartate-to-glutamate substitution in this work.

ORF slr1213 was cloned from *Synechocystis* sp. PCC 6803 genomic DNA using the LumQ Del1 F/LumQ Del1 R primer set (see section 2.12.5 on page 75) designed from the sequence contained in the CyanoBase website (see Figure 7-1 B on page 196). The PCR reaction was carried out with *pwo* DNA polymerase. This polymerase has 3'-5' exonuclease 'proofreading' activity, and was used to ensure that the PCR product did not contain any errors. PCR products were identified following restriction analysis with *Nco*I and *Stu*I. As a consequence of the proofreading activity of *pwo*, PCR products were blunt-ended and not immediately suitable for cloning with the Invitrogen® TOPO™ TA cloning® kit. Products were treated with *Taq* DNA polymerase as described in section 2.12.4 on page 74 to add 3' adenosines prior to cloning. This plasmid was designated pMUT1, and was used as the template in a site-directed mutagenesis reaction as described in section 2.13 on page 76. The mutagenic primers used in the reaction were LumQ Mut F/LumQ Mut R, containing two base substitutions as detailed in Figure 7-1 C. The first base substitution causes a GAT to GAA codon change, resulting in an aspartate-to-glutamate alteration in Slr1213. The second alteration inserts a unique *Sna*BI restriction site adjacent to the first change without altering the amino acid sequence of Slr1213. The restriction site

was inserted so that selected clones could be quickly screened without sequencing. The alterations made were based on a codon usage table compiled for *Synechocystis* sp. PCC 6803 by analysis of 10781 codons by Dr. Nigel Silman. The control reaction for the site-directed mutagenesis, which involves an amino acid substitution that converts a β-galactosidase gene from an inactive to an active form, gave a mutagenesis efficiency of 92.6%. Positive colonies were analysed using a *Sna*BI-*Stu*I restriction digest, and all clones were observed to contain the unique *Sna*BI site (see Figure 7-2 A on page 197). The construct was designated SD pMUT1.

Before further cloning was carried out, it was necessary to sequence the mutated LumQ Del1 F/LumQ Del1 R insert of SD pMUT1 to ensure that the required mutations had been incorporated and that no others were present. To do this the insert was amplified by PCR with *pwo* DNA polymerase and the LumQ Del1 F/LumQ Del1 R primer set and used as sequencing template. The primers used for sequencing are detailed in section 2.14.1 on page 76, and were designed from sequence obtained from the CyanoBase website. Sequences were assembled using the DNA star program, SeqMan. In this fashion the whole of the PCR product was sequenced and the data assembled into one contiguous sequence. Any gaps that existed that prevented the contig being read in one continuous sequence were addressed at this point. Figure 7-3 highlights the coverage of the region with the sequencing runs, and it can be seen that sequence was gained for the whole PCR product apart from the first 13 bp and the last 6 bp. The region encompassing the site-directed alterations was sequenced twice independently on one strand, and once on the other. All three sequencing runs confirmed that the correct alterations had occurred. The contiguous sequence was aligned with the expected sequence for the LumQ Del1 F/LumQ Del1 R PCR product and the sequence around the site-directed mutations is shown in Figure 7-4.

In order to transfer the mutation onto the *Synechocystis* sp. PCC 6803 genome, a selective pressure was required. To provide this, the omega cassette from pHP45Ω (Prentki and Krisch, 1984) was excised with *Sma*I. The cassette, which carries a selectable marker (*aadA*[+]; Sm[R]/Spc[R]), was cloned into the *Stu*I site (thus disrupting both the *Sma*I and *Stu*I restriction sites) of SD pMUT1 creating plasmid SD pMUT1[R] (see Figure 7-2 B for full restriction map). It can be seen from Figure 7-1

B that the insertion site lies between ORFs slr1213 and slr1214. Unfortunately, it is possible that an insertion of the omega cassette may interfere with the expression of ORF slr1214. However, the only way to confirm this is to create the mutant and analyse expression of this gene.

The strategy by which the ORF slr1213 site-directed mutation on SD pMUT1[R] was transferred onto the *Synechocystis* sp. PCC 6803 chromosome is shown in Figure 7-5. It can be seen that if wild-type cells are transformed with SD pMUT1[R], whilst recombination would occur downstream of the omega cassette, it could happen anywhere upstream, including downstream of the site-directed mutation. As a result, a large number of clones may require screening by PCR and restriction analysis with *Sna*BI. Whilst this was attempted, as a contingency a construct that would delete ORF slr1213 was created. When SD pMUT1[R] was transformed into this pMUT3 background, recombination would occur upstream of the site-directed mutation, carrying it onto the *Synechocystis* sp. PCC 6803 genome.

The strategy by which the construct used to delete ORF slr1213 from the *Synechocystis* sp. PCC 6803 chromosome was generated is shown in Figure 7-6. To make the deletion, the LumQ Del 1/LumQ Del1 R amplified genomic DNA was excised from pMUT1 with *Eco*RI and sub cloned into the unique *Eco*RI site of pBR322 to form pMUT2. This was carried out, as the pCR®2.1-TOPO® vector contains a *Nco*I restriction site, intended for use in the gene deletion. Also, the vector already carries a kanamycin cassette, which was used to make the deletion. The kanamycin cassette was removed from pUIDK1 (Bardonnet and Blanco, 1992) using *Nco*I and *Stu*I and cloned into ORF slr1213 in pMUT2, with the deletion of a 968 bp coding fragment which includes the whole of ORF slr1213 (see Figure 7-1 B). This completed the construction of pMUT3.

Synechocystis* sp. PCC 6803 cells were transformed with both SD pMUT1[R] and pMUT3. Following selection with the appropriate antibiotics, cells were repeatedly patched onto fresh media to achieve segregation of the D54E mutation of Slr1213 and the deletion of ORF slr1213 respectively.

A.
```
NtrC     MQRG--IVWVVDDDSSIRWVLERALAGAGLTC-TTFENGNEVLAALASKTPDVLLSDIRM  57
PhoB     MAR---RILVVEDEAPIREMVCFVLEQNGFQP-VEAEDYDSAVNQLNEPWPDLILLDWML  56
OmpR     MQEN-YKILVVDDDMRLRALLERYLTEQGFQV-RSVANAEQMDRLLTRESFHLMVLDLML  58
CheY     MADKELKFLVVDDFSTMRRIVRNLLKELGFNNVEEAEDGVDALNKLQAGGYGFVISDW--  58
Slr1213  MVT---KILIVEDERLVAQHIAQLLKSDGYEICVIASDGATALKKIAEFYPDLVLLDIRI  57
```

B.

Presumptive site of phosphorylation
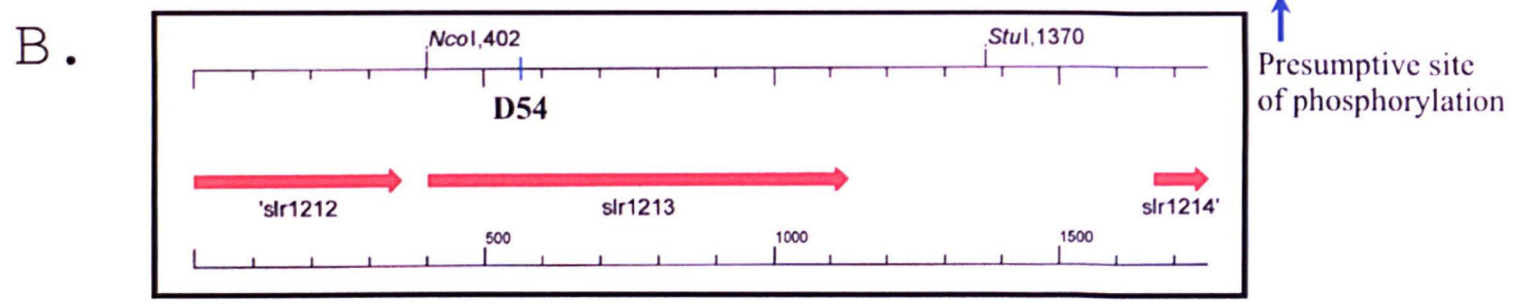
C.

```
5'  cca  gat  cta  gtt  tta  cta  gat  att  cgt  att  aaa  gga  gag  3'
3'  ggt  cta  gat  caa  aat  gat  cta  taa  gca  taa  ttt  cct  ctc  5'
          p    d    l    v    l    l    d    i    r    i    k    g    e
```

▼ SnaBI

```
5'  cca  gat  cta  gtt  tta  cta  gaa  ata  cgt  att  aaa  gga  gag  3'
3'  ggt  cta  gat  caa  aat  gat  ctt  tat  gca  taa  ttt  cct  ctc  5'
          p    d    l    v    l    l    e    i    r    i    k    g    e
```

Figure 7-1 :- A. CLUSTAL W sequence alignment of response regulator proteins highlighting characteristic features B. Scale diagram of LumQ Del1 F/LumQ Del1 R PCR product (1755 bp) used for the site-directed mutagenesis of Slr1213. C. Two base substitutions were planned. The first causes an activating D54E amino acid alteration, and the second introduces a unique *Sna*BI restriction enzyme site.
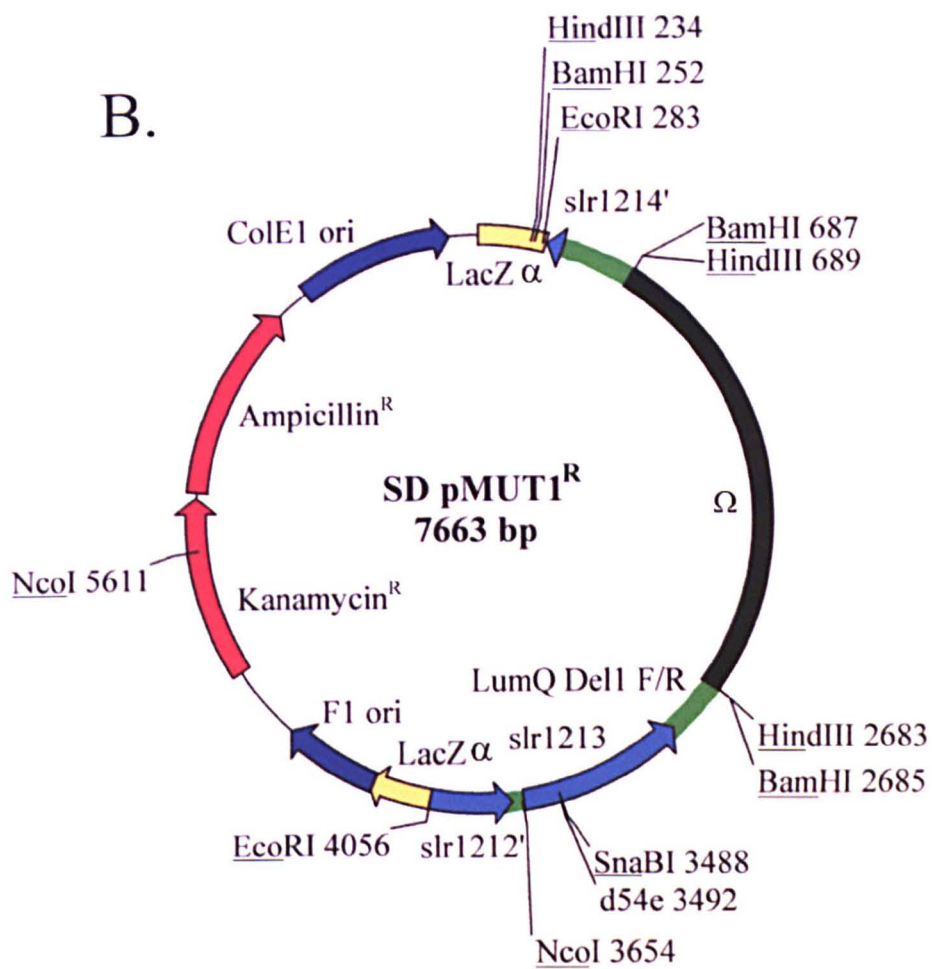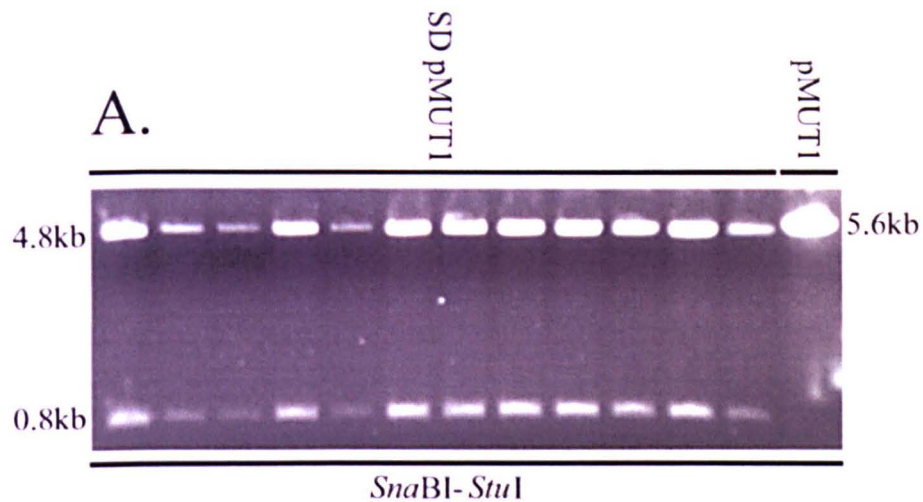
Figure 7-2 :- A. *Sna*BI-*Stu*I analysis of putative SD pMUT1 clones from
E. coli MC1061. All clones tested contained the unique *Sna*BI restriction
site. B. Detailed restriction map of the construct used for the site-directed
mutagenesis of ORF slr1213, SD pMUT1[R].
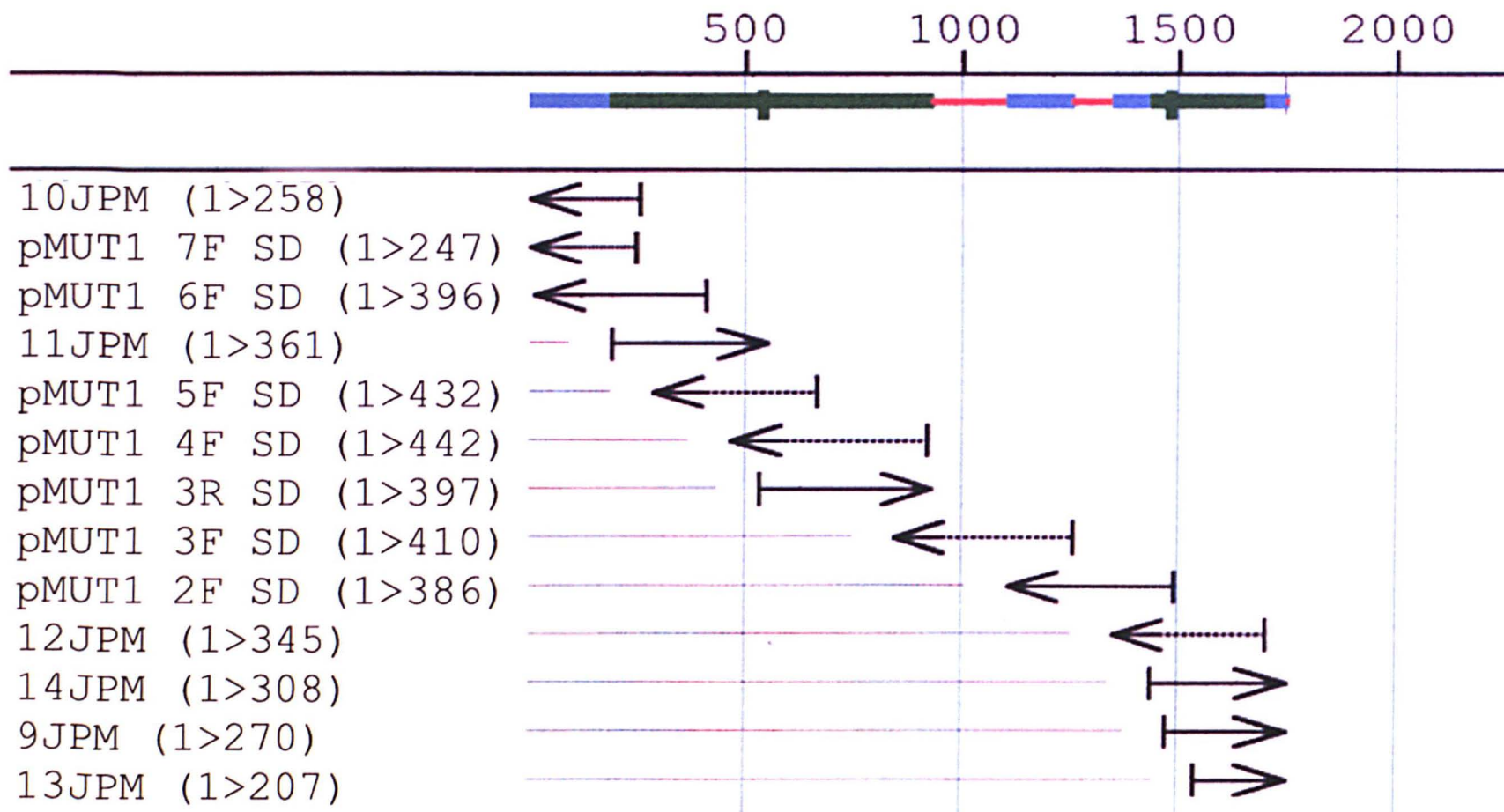
Figure 7-3 :- Summary of the sequences used to compile the contiguous alignment of the mutated region of SD pMUT1, the LumQ Del1 F/LumQ Del1 R PCR product. Sequences are represented conventionally with the 5'-end on the left of the diagram. The direction of the arrow indicates which strand is being sequenced (left to right is sense strand sequencing and right to left is antisense strand sequencing) and the label refers to the primer used. A thin red line represents a region sequenced only once. A medium blue line represents regions sequenced on one strand only. Green lines represent regions sequenced on both strands.

```
Contig_1   TCCTTTAGAGCAGTCCATTCCTTTGGGATTAATTATTCAGGAATTAGTTTCCAATGCTTT 107
LumQ       TCCTTTAGAGCAGTCCATTCCTTTGGGATTAATTATTCAGGAATTAGTTTCCAATGCTTT 120
           ************************************************************


Contig_1   AAAACATGCTTTCCCAACGACGGAAGGGGAAATTTCGATTAAATTCACTTCAATGAACAG 167
LumQ       AAAACATGCTTTCCCAACGACGGAAGGGGAAATTTCGATTAAATTCACTTCAATGAACAG 180
           ************************************************************


Contig_1   TCACTATAGTTTACAGGTGTGGGATAACGGAGTTGGAATTTCCAGGGATATAGATTTGGA 227
LumQ       TCACTATAGTTTACAGGTGTGGGATAACGGAGTTGGAATTTCCAGGGATATAGATTTGGA 240
           ************************************************************


Contig_1   AAATACAGATAGTTTGGGCATGCAACTTATCTACAGCCTAACTGAACAACTCCAGGGAGA 287
LumQ       AAATACAGATAGTTTGGGCATGCAACTTATCTACAGCCTAACTGAACAACTCCAGGGAGA 300
           ************************************************************

                                                     ORF slr1212 STOP
Contig_1   ATTGCACTATGAATATGTCGGTGGCGCTCAATTTGGGTTGGAATTTTCACTTTAGCTAGC 347
LumQ       ATTGCACTATGAATATGTCGGTGGCGCTCAATTTGGGTTGGAATTTTCACTTTAGCTAGC 360
           ************************************************************

                                         ORF slr1213 START
Contig_1   TCCATCAAAAATCAACGAAAATCAACATTATTTGACTGCAACCATGGTAACCAAAATACT 407
LumQ       TCCATCAAAAATCAACGAAAATCAACATTATTTGACTGCAACCATGGTAACCAAAATACT 420
           ************************************************************


Contig_1   GATTGTAGAGGATGAAAGGCTAGTGGCCCAACACATTGCCCAATTATTAAAAAGTGATGG 467
LumQ       GATTGTAGAGGATGAAAGGCTAGTGGCCCAACACATTGCCCAATTATTAAAAAGTGATGG 480
           ************************************************************


Contig_1   CTATGAAATTTGTGTGATCGCCAGTGATGGAGCAACGGCGCTGAAAAAAATTGCTGAGTT 527
LumQ       CTATGAAATTTGTGTGATCGCCAGTGATGGAGCAACGGCGCTGAAAAAAATTGCTGAGTT 540
           ************************************************************

                            Site-directed alterations
Contig_1   TTATCCAGATCTAGTTTTACTAGAAATACGTATTAAAGGAGAGATCGACGGGATAGAGGT 587
LumQ       TTATCCAGATCTAGTTTTACTAGATATTCGTATTAAAGGAGAGATCGACGGGATAGAGGT 600
           ************************ **  ********************************


Contig_1   GGCGGAACGGATAAAATCTCTTTACTCCATCCCCATTGTTTATCTCACAGCTTTTTCTGA 647
LumQ       GGCGGAACGGATAAAATCTCTTTACTCCATCCCCATTGTTTATCTCACAGCTTTTTCTGA 660
           ************************************************************


Contig_1   TGGGGAAACCCTGGAGCGGGCCCAGAAAACCAATCCCCAGGGCTATGTGATTAAGCCTTT 707
LumQ       TGGGGAAACCCTGGAGCGGGCCCAGAAAACCAATCCCCAGGGCTATGTGATTAAGCCTTT 720
           ************************************************************
```

Figure 7-4 :- CLUSTAL W sequence alignment of the insert carrying the site-directed mutation of ORF slr1213 from SD pMUT1 (contig_1) with the genome of *Synechocystis* sp. PCC 6803 (LumQ). Gene start and stop codons, along with the two base alterations are highlighted.
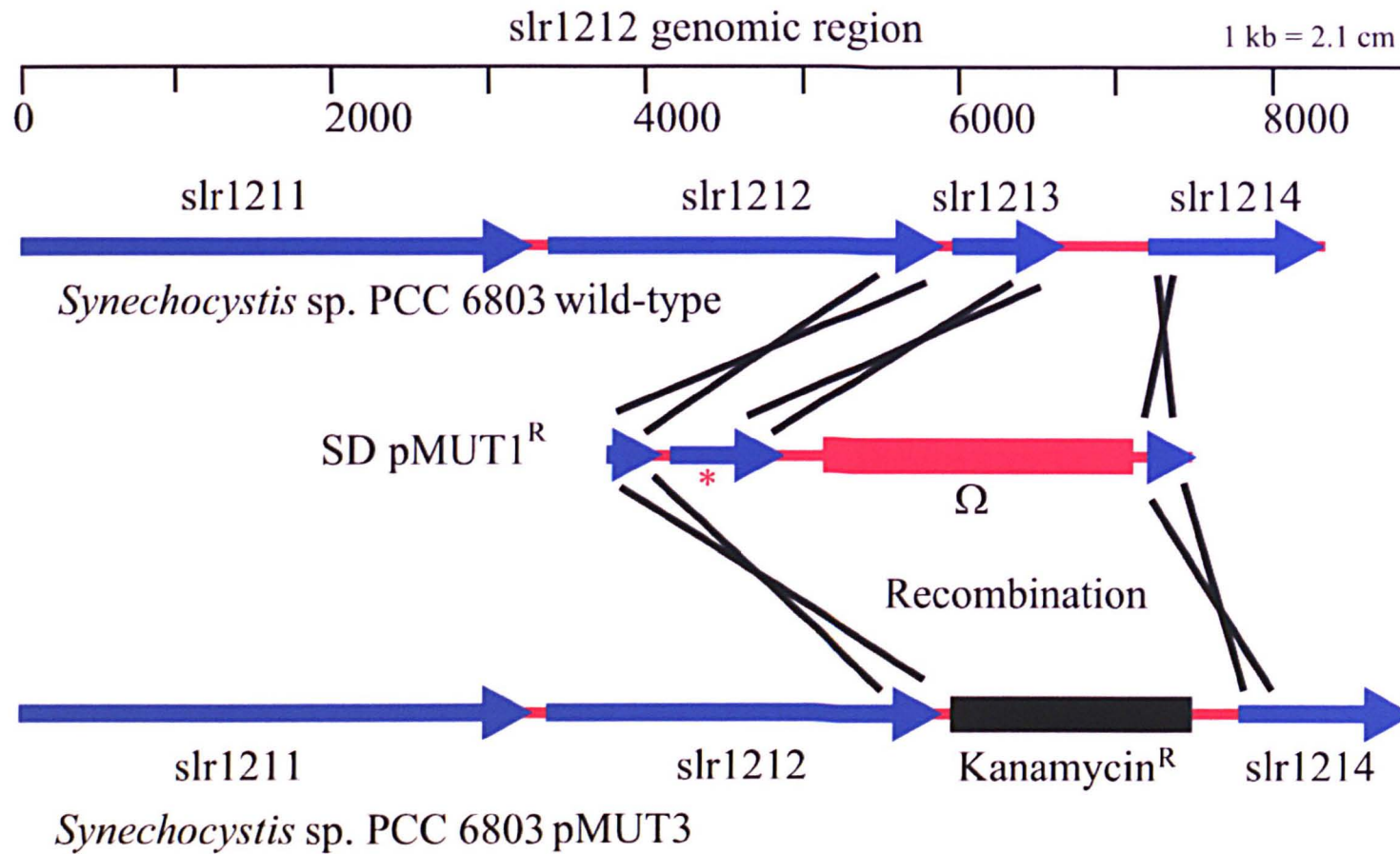
Figure 7-5 :- Scale diagram showing the strategy used to transfer the Slr1213 D54E mutation onto the chromosome. The red star shows the position of the D54E substitution. If SD pMUT1[R] is transformed into a wild-type background, recombination may occur without transfer of the D54E mutation to the chromosome. However, if the plasmid is transformed into a pMUT3 background, the D54E substitution must have been transferred to the chromosome in spectinomycin resistant colonies.
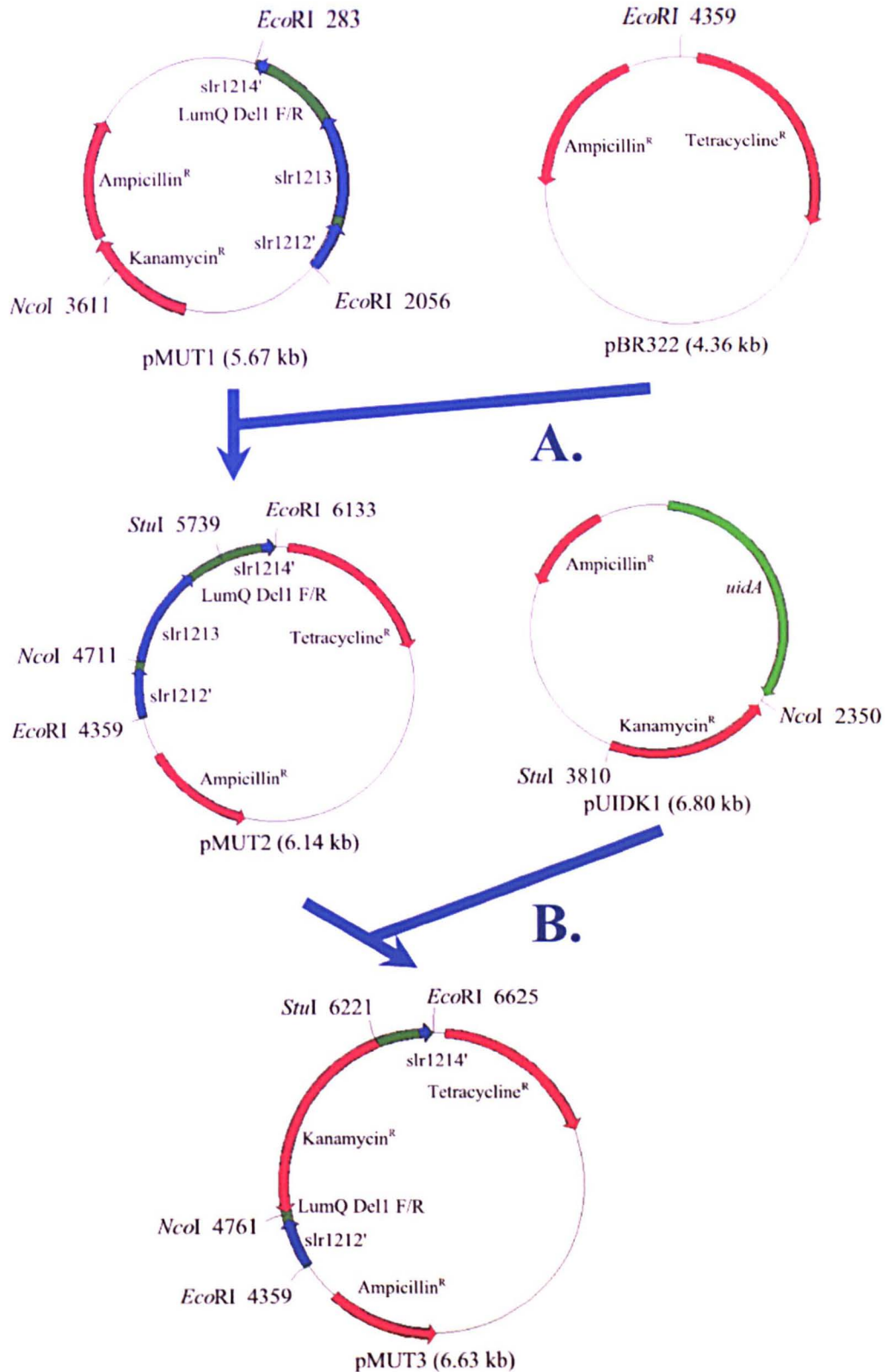
Figure 7-6 :- Scale diagram of the strategy used to generate pMUT3, for the allelic replacement of ORF slr1213. A. The LumQ Del1 F/R PCR product was excised from pMUT1 and inserted into the unique *Eco*RI restriction site of pBR322. B. ORF slr1213 was replaced with a kanamycin resistance cassette from pUIDK1.

7.3. Results

The transformation of *Synechocystis* sp. PCC 6803 with SD pMUT1^R and pMUT3 produced many spectinomycin and kanamycin resistant colonies respectively. Following selection, 25 colonies from each transformation were picked for further analysis.

Cells were patched six times onto fresh solid medium containing the appropriate antibiotics to segregate the mutations. A PCR based method was employed to screen colonies for the mutation and segregation. Primer set LumQ Del1 F/LumQ Del1 R was used to amplify DNA directly from cell patches. In the generation of both mutant constructs additional DNA (antibiotic resistance cassettes) was added (and in the case of pMUT3 removed), and as a result the size of the PCR amplicon over each region was altered accordingly. The expected product sizes are shown in Table 7-1.

Primer set	Strain		
	Wild-type	SD pMUT1^R	pMUT3
LumQ Del1 F/LumQ Del1 R	1755	3755	2337

Table 7-1 :- Expected sizes of PCR amplicons (bp) in the mutant screen.

As can be seen from this screen (see Figure 7-7), the expected PCR product sizes were observed from *Synechocystis* sp. PCC 6803 pMUT3 colonies, indicating that complete segregation had occurred. However when SD pMUT1^R colonies were analysed, PCR products of two different sizes were observed. These products were consistent with both wild-type and mutant copies of the genes being present in the genome, indicating that incomplete segregation i.e. merodiploid formation had occurred. This result also indicates that a PCR-based screen is sensitive enough to detect incomplete segregation from whole cell templates and is unaffected by bias to the most abundant template, as hypothesised in section 3.3.1 on page 104. Repeated patching of cells onto selective medium did not promote further segregation, as judged by further PCR analysis.
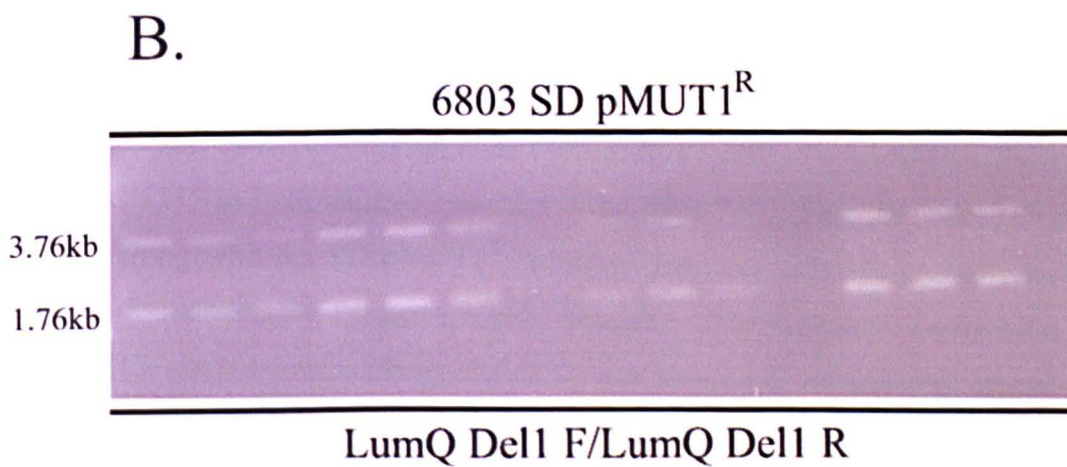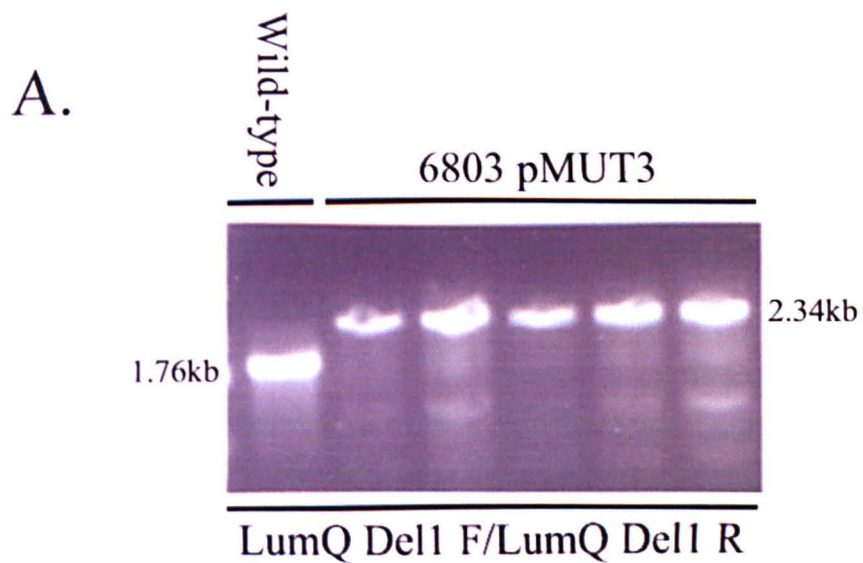
Figure 7-7 :- PCR analysis of strains to confirm the presence of mutations and segregation based on size differnces caused by cloning. A. PCR analysis of *Synechocystis* sp. PCC 6803 pMUT3 colonies . B. Analysis of *Synechocystis* sp. PCC 6803 SD pMUT1[R] colonies.

7.4. Discussion

This chapter has described the work carried out towards making a D54E mutation in the putative response regulator Slr1213, a change known to cause constitutive activation in other response regulators (Gupte *et al.*, 1997; Nohaile *et al.*, 1997). Upon transformation of *Synechocystis* sp. PCC 6803 wild-type cells with the SD pMUT1^R construct, colonies were selected and analysed. It was observed that the omega cassette inserted between ORFs slr1213 and slr1214 would not segregate onto every copy of the cells chromosome. A concern of transforming this construct directly into a wild-type background was that recombination could occur upstream of the antibiotic cassette but downstream of the site-directed mutation. It was not expected that the insertion of the omega cassette upstream of ORF slr1214 would have a lethal phenotype. The implication therefore is that in all the clones analysed recombination had occurred upstream of the site-directed mutation carrying it onto the chromosome, and that this mutation had a lethal phenotype. To be certain of this however, the PCR reaction would need to yield enough of the 3.76kb product to allow analysis by restriction digest with *Sna*BI. The presence of the *Sna*BI would indicate that the D54E substitution had also been carried on to the chromosome and could be confirmed by sequencing. In order to force the D54E mutation on to the chromosome, *Synechocystis* sp. PCC 6803 pMUT3 cells should be transformed with SD pMUT1^R as outlined in Figure 7-5 A on page 200. This ORF slr1213 deletant strain along with the SD pMUT1^R construct provides a future investigator with a strong foundation for further study in this area. The complete segregation of the Δslr1213 total deletion (from pMUT3) was expected, as this had already been achieved with the Δslr1213 strain from pJIM7.

It was interesting to observe that the D54E activating substitution of Slr1213 had an apparently lethal phenotype. As discussed though, the insertion of an omega cassette upstream of ORF slr1214 may disrupt transcription of this gene. As outlined in the Introduction, Slr1214 appears to be a cytosolic protein (analysed by PSORT) containing a response regulator receiver domain and having similarity to PatA of *Anabaena* sp. PCC7120, a response regulator involved in regulating heterocyst pattern formation (Liang *et al.*, 1992; Wolk, 1996). Before conclusions can be drawn about the effects of the aspartate-to-glutamate substitution in Slr1213, it should be

confirmed in the mutant strain that the expression of ORF slr1214 is unaffected. If the expression of ORF slr1214 is affected by the presence of the upstream omega insertion, a separate Δslr1214 strain should be constructed to confirm that this gene product is not essential for cell survival. If Δslr1214 cells were viable this would demonstrate that the activating substitution of Slr1213 has a lethal phenotype.

From the current data available, it is difficult to draw a conclusion about why constitutive activation of Slr1213 would be lethal. Work in this study has highlighted a role for Slr1212 in the acclimation of cells to high light environments. It has also been shown that incubation with ACC causes a large release of ethylene from cells, but that the ACC itself is lethal after a few days. This led to the hypothesis that ethylene release could be a stress response, perhaps signalling to surrounding cells and instigating an avoidance response. All of this evidence suggests a pleiotropic role for Slr1212. Assuming that Slr1212 does act in conjunction with Slr1213 to form a two component signalling pathway, constitutive activation could have far reaching consequences. As an example, if a cell is placed in a stressful environment such as a light or temperature extreme, it may release ethylene as a stress response. Ethylene-binding by Slr1212 (as demonstrated by Rodriguez *et al.* (1999)) would result in phospho-transfer to Slr1213 causing a conformational alteration. This in turn would either allow or prevent DNA binding by Slr1213 resulting in transcriptional activation or repression of gene(s) unknown. Perhaps a result of this could be for the cell to enter an extended lag phase, in an effort to avoid exposure to the stress of the environment. In such a situation, it might be expected to be difficult to isolate mutants with constitutively active (/inactive) Slr1213. If such a situation were to exist though, it might therefore also be expected that a Δslr1213 strain such as the one created in this study would be more susceptible to extreme stresses.

As outlined in section 7.1 on page 192, this strategy was based on the work of others who identified that substituting the conserved aspartate of a response regulator with a glutamate residue caused constitutive activation of the protein. In these cases, it was possible for the investigators to show that aspartate-to-glutamate substitutions had an activating effect because of knowledge concerning the regulatory roles of the

proteins. In a study of capsule production in *E. coli* K-12 for example, it was already known that there was an absolute requirement for RcsB (a response regulator protein) for capsule expression. Aspartate-to-glutamate substitutions of RcsB caused constitutive capsule expression (Gupte *et al.*, 1997). Similarly, an aspartate-to-glutamate substitution in NtrC resulted in constitutive transcription from a *glnA* promoter (Klose *et al.*, 1993).

The production of a constitutively active Slr1213 could provide key insights into the role of this protein in cell physiology, and if this can be shown to act in conjunction with the histidine kinase protein Slr1212, provide key insights into the environmental stimuli perceived and responded to by this two-component sensor kinase system. Despite being unable to fully segregate the Slr1213 site-directed mutation, merodiploids may still provide a phenotype.

Interestingly, a method of mimicking the phosphorylation of a response regulator protein *in-vitro* has now been identified. Complexing a response regulator protein with beryllofluoride (BeF$_x$) forms an acyl phosphate analogue of each of the proteins tested (NtrC, OmpR, NarL, CheY, SpoOF and DctD) (Yan *et al.*, 1999). Such work should allow the study of the interactions of response regulators with their corresponding kinases and other components in their signal transduction pathways.

7.4.1. Concluding remarks

This chapter describes the work carried out towards generating a strain of *Synechocystis* sp. PCC 6803 containing a constitutively active Slr1213 protein. The necessary constructs were produced, but it did not prove possible to select for complete segregation of the mutation. Indeed, currently it is uncertain if the site-directed mutation was carried onto the genome by recombination. In order to be sure this occurs, a strain with a complete deletion of ORF slr1213 has been constructed which can be transformed with the site-directed construct SD pMUT1^R. If the inability to segregate a site-directed mutation fully is correct it suggests that constitutive activation of Slr1213 has a lethal phenotype.

Chapter 8

Concluding remarks and future work

8. Concluding remarks and future work

This short chapter will draw together the major findings of this thesis and suggest directions for future work.

The aim of this study was to determine a physiological role for the slr1212 and slr1213 ORFs of *Synechocystis* sp. PCC 6803, identified from the genome sequence as having possible roles as a two-component signal transduction system involved with adaptive responses to environmental change. Sequence analysis of the predicted proteins implied possible sensing roles involved with either light sensing or ethylene signalling mechanisms, possibly both. Experiments were therefore designed, in conjunction with mutant analysis, to assess the role of Slr1212 and Slr1213 in these sensing mechanisms. Generation of *Synechocystis* sp. PCC 6803 mutants with interruptions in ORFs slr1212 and slr1213 using antibiotic resistance cassettes allowed comparison with the wild-type strain of the physiological response of these mutants to variety of environmental conditions. Such an approach is therefore extremely powerful in assessing the physiological role of proteins, and has been used widely in *Synechocystis* sp. PCC 6803 and other organisms for which genome sequence data is available as a means of assigning function.

It was relatively straightforward to generate Δslr1212 and Δslr1213 mutant strains, and following repeated exposure to the correct selective pressure it was demonstrated that the mutations could be segregated onto every copy of the chromosome. It therefore seems likely that the Slr1212 and Slr1213 proteins do not perform any essential functions *in vivo* when cells are cultured in standard conditions.

No evidence was gained from gene expression studies to suggest co-transcription of ORFs slr1212 and slr1213, an observation that might have suggested that the proteins acted together in a two-component signal transduction system. A future direction for this work could involve the direct determination of this through the use of a yeast two-hybrid screen.

Anthony Bleecker's research group has previously demonstrated the ability of *Synechocystis* sp. PCC 6803 cells to bind ethylene. Furthermore, this activity was completely disrupted in a Δslr1212 background (Rodriguez *et al.*, 1999). This observation suggested that *Synechocystis* sp. PCC 6803 cells might be able to

produce ethylene. However, no homologues of enzymes known to be involved in ethylene biosynthesis in other organisms could be found in the genome of *Synechocystis* sp. PCC 6803. Even so, despite a report that ethylene biosynthesis could not be detected in cultures of *Synechocystis* sp. PCC 6803 (Bleecker and Kende, 2000), by using sensitive LPA techniques it proved possible to demonstrate ACC-dependent ethylene biosynthesis. This was interesting, as a homologue of ACC oxidase from higher plants could not be identified from the *Synechocystis* sp. PCC 6803 genome. This ACC induced ethylene production could be the result of different mechanisms. The first is that ACC could be converted to ethylene by an enzyme other than ACC oxidase. Secondly, since ACC was shown to be lethal to *Synechocystis* sp. PCC 6803 cells over a long period of exposure, ethylene release could be produced via a biosynthetic mechanism induced as a consequence of cellular stress (release was also observed following incubation with 2-oxoglutarate). Finally, ethylene production may be a result of a non-physiological release due to membrane degradation. Given that ethylene release may be stimulated by a non-specific stress, it would be pertinent to examine the effect of other cellular stresses such as heat shock, salt shock, exposure to high light irradiance or changes in redox on ethylene production in *Synechocystis* sp. PCC 6803. It was also observed that cultures of both *Gloeotrichia* and *Nostoc* sp. PCC 7118 produced ethylene in an L-methionine dependent manner, and this was not observed with *Synechocystis* sp. PCC 6803 cells. This could imply the presence of distinct ethylene biosynthetic routes within freshwater cyanobacteria.

In addition, since ethylene has been shown to be produced in a circadian manner in some higher plants including Sorghum (Finlayson *et al.*, 1998) and *Arabidopsis thaliana* (Dr. Andrew Millar, pers. comm.), and since *Synechocystis* sp. PCC 6803 possesses a circadian clock (Aoki *et al.*, 1997), it would also be interesting to entrain cultures to a 12:12 (hour) light/dark cycle and examine ethylene production in the presence and absence of substrates such as ACC.

Whatever the mechanism of ethylene production, be it a specific biosynthetic pathway or via a non-physiological route such as membrane degradation, given that ethylene-binding activity has been demonstrated in *Synechocystis* sp. PCC 6803, this suggests that an ethylene signalling mechanism exists. Following the identification of putative ethylene receptors in both *Anabaena* sp. PCC 7120 and *Nostoc*

punctiforme ATCC 29133, it would be very interesting to examine both ethylene-binding activity and ethylene biosynthesis in these organisms. The presence of similar receptors in filamentous strains suggests that a conserved ethylene signalling mechanisms may be a common feature of cyanobacteria, and from which the ethylene signal transduction mechanisms of higher plants may have evolved.

Protein sequence analysis of Slr1212 also suggested a role for this protein in light signalling mechanisms in *Synechocystis* sp. PCC 6803. Numerous experiments were carried out to evaluate the role of Slr1212 in light sensing, and although Δslr1212 mutations were not found to affect the growth of cells in different light qualities, 77K fluorescence spectroscopy indicated a role for Slr1212 in acclimation to a high light environment. This could be further characterised by empirical measurement of the cellular content of PSI and PSII reaction centres following acclimation to different light irradiances. 77K fluorescence data also suggested structural alterations in the PSI reaction centres. Even though western blotting against two PSI polypeptides did not reveal this, future work using a wider range of antibodies could be carried out to characterise these structural differences further.

Whilst a role for Slr1212 was implicated in acclimation to high light irradiance, no indication of roles in sensing light quality or direction (as evaluated through phototaxis assays) were found. To definitively evaluate the role of Slr1212 as a light sensor, it should be determined whether the apoprotein can autocatalytically associate with a chromophore molecule.

Site-directed mutagenesis of Slr1213 that potentially simulated constitutive activation of the protein by substituting a conserved aspartate residue with a glutamate residue was found to have a lethal effect on *Synechocystis* sp. PCC 6803 cells, as the mutation could not be segregated onto every chromosomal copy despite repeated selection. In order to conclusively confirm this however, it would be necessary to transform a *Synechocystis* sp. PCC 6803 slr1213 deletant strain (constructed using pMUT3) with the site-directed construct (SD pMUT1^R) to ensure chromosomal transfer of the site-directed mutation.

If this phenotype could by confirmed, it would be revealing to identify targets for transcriptional regulation by this protein.

8.1. Summary of suggestions for future work

- Analyse the interactions between Slr1212 and Slr1213 using a yeast two-hybrid screen to demonstrate whether they act together in a two-component signal transduction system. Phosphorylation assays could also be performed.

- Measure the ethylene-binding activity of the mutant strains generated in this study, as well as ethylene-binding in *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* ATCC 29133 and any ethylene receptor mutants that are constructed. Ethylene-binding activity can be assayed using the method originally developed by Sisler (1979) and modified by Rodriguez *et al.* (1999). In this method, whole cells are incubated with ^{14}C-labelled ethylene, and aired. Released ethylene is then trapped in mercuric perchlorate in a scintillation vial, and subject to scintillation counting.

- Determine the effect of other cellular stresses e.g. temperature shock, salt shock, on ethylene biosynthesis by *Synechocystis* sp. PCC 6803. Also examine ethylene biosynthesis in *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* ATCC 29133. Entrain cultures to a 12:12 light:dark cycle and examine circadian regulation of ethylene biosynthesis.

- Examine the growth rate of *Synechocystis* sp. PCC 6803 Δslr1212 cells in far-red light.

- Characterise the structural differences observed in PSI reaction centres by western blotting.

- Overexpress full-length and truncated Slr1212. Following purification, assay for the ability to associate with a chromophore molecule such as phycocyanobilin.

- If Slr1212 does bind a chromophore, analyse photoreversibility of the holoprotein.

- Determine the cellular content of PSI and PSII reaction centres empirically following acclimation to different light irradiances.

- Transform *Synechocystis* sp. PCC 6803 pMUT3 strain with SD pMUT1^{R} to confirm that a D54E mutation in Slr1213 has a lethal phenotype.

- Identify targets for transcriptional regulation by Slr1213. This could be carried out using DNA chips produced from a wild-type and Δslr1213 strain, and analysing gene expression by hybridising the chips with total RNA

isolated from cells cultured in conditions in which Slr1213 is thought to be active.

8.2. Conclusions

In conclusion, *Synechocystis* sp. PCC 6803 was found to release ethylene following incubation with ACC, the ethylene precursor in vascular plants, and 2-oxoglutarate, an ethylene precursor in many microorganisms. *Nostoc* sp. PCC 71218 and *Gloeotrichia* were both found to release ethylene following incubation with L-methionine and 2-oxoglutarate. Following the identification of putative ethylene-binding proteins in both *Nostoc punctiforme* ATCC 29133 and *Anabaena* sp. PCC 7120, it seems possible that cyanobacteria possess an intact ethylene signal transduction pathway from which the plant system may have evolved.

Study of the role of Slr1212 in light signalling mechanisms revealed that the protein is involved with the acclimation to high light irradiance, and could possess a role in the regulation of photosystem stoichiometry. It therefore seems that Slr1212 could have a role in both ethylene and light signalling mechanisms.

Constitutive activation of Slr1213 appears to have a lethal effect in *Synechocystis* sp. PCC 6803 cells. However, analysis of merodiploid cells could provide insights into the potential regulatory role of this protein.

This work will undoubtedly form the basis for some fascinating future insights into the function of these two proteins in cyanobacterial signalling mechanisms.

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