University of Otago

DEPARTMENT OF BIOCHEMISTRY

RESEARCH REPORT

The role of Universal Stress Proteins 1 and 2 in *Synechocystis* sp. PCC 6803

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Abbreviations

OD: Optical Density

PSII: Photosystem II

USP/Usp: Universal Stress Protein

DMBQ: 2,5-Dichloro-p-benzoquinone

DTT: Dithiothreitol

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Introduction

1.1 Universal Stress Proteins

Universal Stress Proteins (USPs), found in all domains of life, are named as such because their production is upregulated in response to a wide range of environmental stress and starvation conditions, as well as during cellular stationary phase (reviewed in Kvint *et al.,* 2003). These include chemical stresses, such as exposure to antibiotics, uncouplers, reactive oxygen species, and DNA-damaging agents, as well as physical stresses such as temperature extremes and UV irradiation. It should be noted that some conditions, such as extreme temperatures and exposure to tetracycline repress rather than induce the production of USPs (reviewed in Kvint *et al.,* 2003). Through a variety of mechanisms, USPs confer upon an organism the ability to resist stress by reprogramming it towards defense and escape (Nachin *et al.,* 2005). Knockout studies in *E. coli* involving deletions of USP-encoding genes have resulted in increased sensitivity to stresses (and in some cases, cell death). USP-containing organisms usually have multiple copies of *usp* genes coding for a variety of paralogues, that have similar and sometimes overlapping functions (Nachin *et al.,* 2005). *E. coli* for example contains six *usp* genes, divided into four classes based upon protein sequence similarity. USPs present themselves as independent proteins (13-14 kDa), as two proteins in tandem, as well as being fused to other proteins, known as a Usp-like domain (Diez *et al.,* 2000).

As shown in studies by Nachin *et al.,* USPs divided into classes by sequence similarity were also shown to have class-specific roles, as was documented by knockout studies in *E. coli* with strains lacking a particular class. A Class I (UspA, UspC, and UspD) triple knockout displayed a much higher degree of sensitivity to oxidative stress. Single knockouts of the USPs in this class showed less sensitivity than the triple, but more than wild type, suggesting that the individual proteins augment each others function (Gustavsson *et al.,* 2002). Iron uptake into cells has

been shown to be reduced by UspA, by inhibiting the synthesis of iron siderophore uptake proteins (Nachin *et al.,* 2005). Iron magnifies the effect of reactive oxygen species (ROS), hence the role of UspA is prevent accumulation of intracellular Iron, and subsequently decrease the associated damage from ROS. The lack of this protein would result in high Iron concentrations - a factor that increases the toxicity of spectinonigrin. *E. coli* mutants lacking UspD (Class I), and the Class II proteins UspF and UspG also display a higher sensitivity to streptinonigrin, shown by diminished rate of growth. Furthermore, knockouts in class I and III/V resulted in decreased ability of fimbria-mediated adhesion, as well as inhibited motility. Other knockout studies have shown that the *E. coli* lacking UspC, D and E proteins display greater sensitivity to UV irradiation, suggesting that these proteins help confer protection against DNA-damaging agents(Nachin *et al.,* 2005).

Heerman *et. al* (2009) has proposed a mechanism to describe how USPs fulfill their role as agents of stress resistance. In *E. coli*, K^+ limitation and osmotic stress imposed by salt activates the KdpD/KdpE phosphorylation cascade, which results in the production of KdpFABC to restore the cell to normal salt concentrations. KdpD autophosphorylates in response to salt stress conditions, and subsequently phosphorylates KdpE, thereby increasing its affinity for the *KdpFABC* promoter, triggering transcription. UspC is induced under salt stress, and interacts with a Usp domain in the N-terminal stimulus perception domain of KdpD. This important interaction "scaffolds" the KdpD/KdpE/DNA complex, allowing KdpD to phosphorylate KdpE and therefore induce production of KdpFABC (Heerman *et al.,*2009).

1.3 Cyanobacteria

Cyanobacteria are gram-negative prokaryotes, that obtain energy from photosynthesis. They are credited with playing an important role in the creation of an oxygenic atmosphere, as well as being the precursor to chloroplasts (Olson, 2006; Smith, 2000)

The cyanobacteria *Synechocystis* sp. PCC 6803 provides an effective model for photosynthesis research. This is because it is able to take up foreign DNA and assimilate it into its genome by homologous recombination, making the generation of knockout strains a straightforward process. This particular strain is also glucose tolerant, and can grow heterotrophically in the presence of glucose, meaning that the cell can use glucose as a source of energy if a mutation knocks out its ability to perform photosynthesis.

1.2 Photosynthesis

Oxygenic photosynthesis is the process utilized by certain organisms to generate carbohydrates. It is driven by energy from sunlight, using electrons derived from water and atmospheric carbon dioxide. The first organisms to adopt oxygenic photosynthesis appeared about 2.5 billion years ago, and are responsible for creating the oxygenic atmosphere that facilitated the explosion of oxygen-dependent life, making photosynthesis one of the most important processes in the history of life (Olson, 2006; Flores, 2008). Photosynthesis is essentially a two-stage process, which occurs in the thylakoid membranes of chloroplasts. The first stage, known as the 'Light Reactions', uses a complex array of membrane-bound proteins working in series to transform the energy from sunlight into chemical energy, as depicted in Figure 1.1. The first major reaction involves Photosystem II (PSII), a multi-subunit protein responsible for 'splitting' water and reducing plastiquinone. Initially, a photon excites an electron in the P680 chromophore (within the PSII reaction centre) to a higher energy level, where it is shuttled through a series of redox cofactors, and eventually reduces plastiquinone to plastiquinole. Electrons are supplied to the PSII reaction by the Oxygen-Evolving Complex (OEC) – a cluster of manganese ions that catalyses the splitting of water via the light energy absorbed by its reaction centre. The exited electron is then passed through a series of redox carriers, which is coupled with hydrogen ion transport across the thylakoid membrane. This generates a proton gradient that drives the production of ATP via ATP synthase. To complete electron transport to $NADP^+$, a second photon is

absorbed by photosystem I, providing the energy to transport electrons to $NADP^+$, reducing it to NADPH.The products of the light reactions, ATP and NADPH, are then used in the light-independent 'Dark reactions' to fix carbon dioxide into carbohyrates, providing a chemical source of energy for the organism (Govindjee *et al.,* 2010; Mathews *et al.,* 2000)

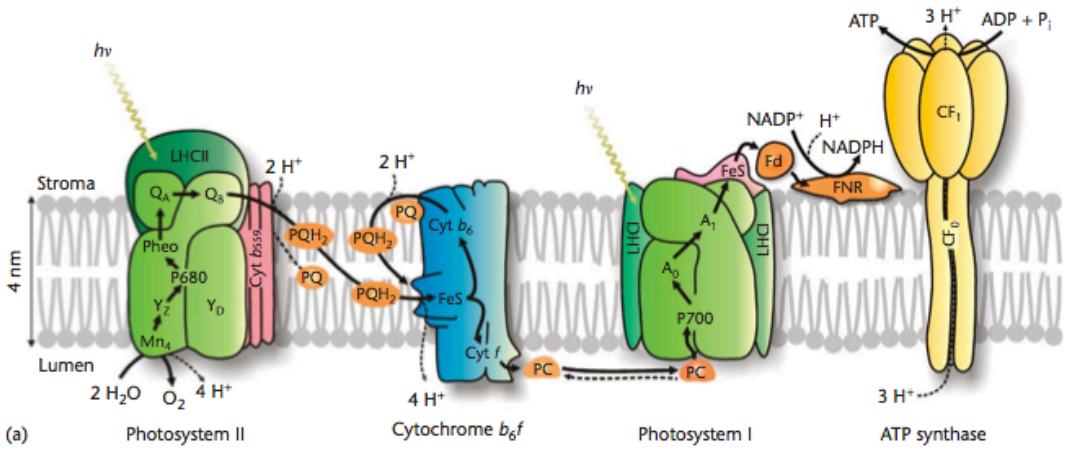


Figure 1.1 Proteins involved in the light reactions of photosynthesis (Govindjee *et al.,* (2010) *Photosystem II. In: Encyclopedia of Life Sciences (ELS),* p. 2, John Wiley & Sons, Ltd: Chichester.)

1.4 Characterisation of *Synechocystis* sp. PCC 6803 knockout strains

A common approach to studying the role of particular proteins in *Synechocystis* sp. PCC 6803 is by generating a mutant strain lacking the protein of interest. Transformation can be achieved with relative ease, by utilizing the ability of the natural competency of*Synechocystis* sp. PCC 6803. Generally, a plasmid is made whereby the gene coding for the protein to be studied is interrupted or deleted by an antibiotic-resistance cassette (commonly spectinomycin, kanamycin, and erythromycin-resistance cassettes are used). An adequate amount of flanking region is included to allow for homologous recombination. The construct is then used to transform *Synechocystis* sp. PCC 6803, resulting in a knockout strain.

1.4.1 Photoautotrophic growth curve

This experiment measures photoautotrophic growth. Liquid cultures of mutant/s and wild type are started with the same OD, and grown in the same media and under the same conditions. OD measurements are subsequently taken every 12 hours. A decrease in the rate in growth in a mutant strain normally indicates that a crucial element of photosynthesis has been disabled. Nutrient-stress growth curves can also be performed, where relative growth of strains is analysed under stress. This is achieved by growing the cells in media lacking a certain nutrient.

1.4.2 Whole cell absorbance spectra

This assay analyses the pigments associated with photosynthesis, by measuring absorbance over the range of 400 – 800 nm using scattered light. Each pigment absorbs light of a particular wavelength, which is represented by a peak. The size of the peak indicates the amount of pigment.

1.4.3 Oxygen evolution of whole cells

The purpose of this assay is to determine if PSII is functioning normally, by measuring oxygen evolution. If the electron flow through PSII is impaired in any way, its ability to produce oxygen will be stifled, reflected by a drop in oxygen evolution. Electron acceptors are used to take electrons directly from PSII, preventing them from being passed to the electron transport chain. This essentially isolates PSII, allowing for PSII-specific analysis.

1.5 Aims of this study

To date, little work has been done to clarify the role of USPs in photosynthetic bacteria. The aim of this study was to therefore investigate the role of two such proteins, Usp1 and Usp2, in *Synechocystis* sp. PCC 6803. To investigate this, a knockout strain lacking Usp2 was generated (a Usp1-deficient strain existed in the lab prior to the commencement of the project). Further, a double knockout strain was generated lacking both USPs. These strains were characterised by measuring

photoautrophic growth rates, absorption spectra, and the ability to evolve oxygen. Furthermore, response to stress was investigated by measuring *usp1* and *usp2* epxression and cellular growth under stress condtions.

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Reagents used in this project were of analytical grade, and obtained from either AJAX chemicals (Sydney, NSW, Australia), AppliChem (Gatersleben, Germany), BioRad (Hercules, CA, USA) DBH Chemicals Ltd. (Poole, England, UK), ChemService (West Chester, PA, USA), Roche (Penzberg, Germany), Scharlau Chemie (Barcelona, Spain), Global Science and Technology (Auckland, New Zealand), Serva (Heidelberg, Germany), or Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.1.2 Oligonucleotides

Oligonucleotides used in this project were obtained from Sigma-Aldrich, Australia. The following primers were used to amplify genes of interest and gene regions from *Synechocystis* sp. PCC 6803 using PCR.

Table 2.1 Primers for PCR used in this project

Gene	Primer sequence (5' to 3')	Reference
slr0670	*f:* GTGGATGATATGTTGGTGGT *r:* AATTACTACGTCCAATCTGG	This study
slr0670 (RT)	*f:* CCTATCTGGAGTCCTTGGTGGGTCAG *r:*CAGCACTTACACCGTTGAAGAAATGGC	This study
slr0244 (RT)	*f:* CCAGTAAGGTTTCCACCGTGTTGCG *r:* CATGGTGGCCCTAGATAAGTCAGCG	This study

2.1.3 *Escherichia coli* strain

The DH5α *E. coli* strain was obtained from Invitrogen (CA, USA). *E. coli* was grown in liquid LB media and on LB-containing agar plates, and incubated at 37°C.

2.1.4 *Synechocystis* sp. PCC 6803

The *Synechocystis* sp. PCC 6803 strain used in this project was derived from the Pasteur Culture Collection, and was the glucose tolerant strain as described in Williams (1988). *Synechocystis* sp. PCC 6803 cultures were grown on agar plates in the presence of BG-11, 5 mM glucose, 20 μM atrazine, and appropriate antibiotics. Liquid cultures were grown in BG-11 with antibiotics when necessary, with and without glucose. These cultures were grown under a constant source of light (30 $\mu E.m^{-2}.s^{-1}$) and air, supplied by aquarium pumps.

2.1.5 Growth Media

2.1.5.1 Lysogeny Broth

Lysogeny Broth (LB media) was used as a medium for *E. coli* growth. LB media consisted of 1% bactotryptone, 0.5% yeast extract, and 1% NaCl, with the addition of 1.5% agar for plates. Antibiotics were included when necessary, added after the media was autoclaved and cooled to a temperature of 55°C. In this project, ampicillin and spectinomycin were added to give a final concentration of 50 $\mu g.ml^{-1}$.

2.1.5.2 BG-11 (100x without Iron, Phosphate, or Carbonate)

BG-11 media used in this project to grow *Synechocystis* sp. PCC 6803 included 1.76 M $NaNO_3$; 30.4 mM $MgSO_4.7H_2O$; 24.5 mM $CaCl_2.2H_2O$; 2.86 mM $C_6H_8O_7$ (citric acid); 0.22 mM EDTA, (pH 8.0); 10% (v/v) trace minerals. Trace minerals included 42.26 mM H_3BO_3; 8.9 mM $MnCl_2.4H_2O$; 0.77mM $ZnSO_4.7H_2O$; 0.32 mM $CuSO_4.5H_2O$; 0.17 mM $Co(NO_3)_2.6H_2O$.

BG-11 agar plates were buffered with 10 mM TES-NaOH (pH 8.2), as well as containing 0.3 % sodium thiosulfate and 1.5% agar.

Liquid BG-11 media was supplemented with 6 $\mu g.ml^{-1}$ ferric ammonium citrate, 20 µg.ml Na_2CO_3, and 30.5 $\mu g.ml^{-1}$ K_2HPO_4.
Antibiotics were added when necessary after autoclaving, giving a final concentration of 25 $\mu g.ml^{-1}$ for spectinomycin and kanamycin. Glucose was added when required prior to autoclaving, giving a final concentration of 5 mM. Atrazine was added to agar plates only, to a final concentration of 20 µM.
BG-11 buffered to pH 7.5 was made by supplementing regular BG-11 with 5.96$g.l^{-1}$ HEPES (4-(2-Hydroxyethyl)-1-piperazine-1-ethanesulfonic acid), and calibrating the pH using NaOH.

2.1.6 Buffers and Solutions

2.1.6.1 Sodium Boric Acid Buffer

Sodium Boric Acid (SB) Buffer (Brody and Kern, 2004) was used both as a component in gels, and as a buffer for gel electrophoresis. A 20 times stock solution contained 8$g.l^{-1}$ of NaOH, adjusted to a pH of 8 by addition of 45 $g.l^{-1}$ of Boric Acid.

2.1.6.2 Solutions for Alkaline Lysis Miniprep

Solutions used for plasmid extraction using Alkaline lysis miniprep:
Solution 1 – 50 mM glucose, 25 mM Tris pH 8, and 10 mM EDTA
Solution 2 – 0.2 M NaOH and 1% SDS
Solution 3 – 3 M potassium acetate, and 11.5% acetic acid
All three solutions were stored at 4°C.

2.1.6.3 DEPC-treated water

Water was treated with DEPC (Diethylpyrocarbonate) to inactivate RNase, thereby making it suitable for use in RNA extraction. 1 ml of DEPC was added to 1 l of mili-Q water, and stirred with a stirring bar at room temperature for 3 hours. The water was incubated overnight at 37°C, and then autoclaved at 15 psi for 20 minutes.

2.1.6.4 MOPS buffer

A pre-existing stock of 10 times MOPS (3-(N-morpholino)propanesulfonic acid) was used for this project, containing 0.2 M MOPS, 0.05 M sodium acetate (ph 5), 0.01 M EDTA (pH 8), and buffered to pH 7 with NaOH. This buffer was stored in a dark glass bottle at room temperature.

2.1.6.5

RNA Loading buffer

Loading buffer for RNA analysis (on a formeldahyde gel) contained 500 µl formamide, 160 µl formeldahyde, 100 µl of 10 times MOPS, 100 µl glycerol, 105 µl sterile DEPC-treated water, 25 µl bromophenol blue (10 $mg.ml^{-1}$), 10 µl ethidium bromide (10 $mg.ml^{-1}$).

2.2 Methods

2.2.1 Aseptic technique

Aseptic technique was used for the duration of this project. This included use of laminar flow cabinets when working with cultures, as well as sterilizing liquid media and water used to grow and maintain cultures (by autoclaving at for 20 minutes at 15 psi, at 250°C).

2.2.2 Polymerase Chain Reaction

2.2.2.1 Polymerase Chain Reaction for Molecular Cloning

Polymerase Chain Reaction (PCR) was used to amplify regions of genomic DNA from *Synechocystis* sp. PCC 6803. A master reaction mix of 50 µl was made with 5x buffer (Phusion HF buffer, Thermo Scientific, Sweden), 100 µM dNTPs, 10 µM of forward and reverse primers, 400 ng of genomic DNA, 0.5 µl of Phusion DNA polymerase (Phusion Hot Start II High-Fidelity DNA polymerase, Thermo Scientific, Sweden) and distillied, deionized water (ddH_2O). PCR was performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following protocol: Initial denaturation at 98°C for 30 seconds, followed by a second denaturation at 98°C for 10 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 60 seconds. The previous 3 steps were repeated for 30 cycles. A final incubation at 72°C for 5 minutes was included at the end of the reaction. Amplification of target region was verified by gel electrophoresis, and the PCR product was subsequently ligated into a pGEM-T Easy vector.

2.2.2.2 Colony PCR applied to *Synechocystis* sp. PCC 6803

Colony PCR was used to confirm segregation of a mutant gene throughout all copies of the *Synechocystis* sp. PCC 6803 genome. A 100 µl master mix was made, including 10x buffer, 50mM $MgCl_2$, 200µM dNTPs, 10µM of forward and reverse primers, 1µl of Taq polymerase (Platinum Taq DNA Polymerase, Invitrogen) and ddH_2O. Initial denaturing was set at 95°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 4 minutes. A final incubation at 72°C for 5 minutes was included at the end of the reaction. The PCR products were subsequently analysed using gel electrophoresis.

2.2.2.3 Reverse Transcription PCR (RT-PCR)

RT-PCR was used to determine expression levels of particular genes. A master mix was made as per section 1.1.2.2, and subsequently divided into four 40 µl aliquots, to each of which was added 10 µl of cDNA. Initial denaturing was set at 95°C for 3 minutes, followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. A hold was included after every 3 cycles to allow for sample extraction from each reaction. These samples were subsequently analysed by gel electrophoresis.

2.2.3 Gel Electrophoresis for DNA sample separation and analysis

Gels used for separation and analysis of DNA samples consisted of 0.8% agarose and SB buffer, to a final volume of 25 mL. After loading samples in gel wells, electrophoresis was performed on the Horizon 58 horizontal gel system (Life Technologies, Inc., MD, USA) at 180V for 20-30 minutes, using SB solution as a running buffer. Gels were stained in 1 $mg.ml^{-1}$ ethidium bromide, and visualized with the Bio-Rad Gel Doc EQ system.

2.2.4 DNA Gel Purification

Bands corresponding to desired DNA were excised from agarose gels after visualization with UV light. The band was subsequently purified using the Purelink Quick Gel Extraction Kit (Invitrogen, CA, USA) as per the instructions provided.

2.2.5 DNA blunt-ending

Blunt ending was performed to fill in recessed 3' termini. A reaction mix was made containing 0.9 µg of DNA, 2 units of Klenow and 1 mM of dNTPs, that was incubated at room temperature for 15 minutes, then heat inactivated at 75°C for 10 minutes.

2.2.6 Vector dephosphorylation

To increase the likelihood of successful ligation, vector blunt-ends were dephosphorylated. To achieve this, 17µl of vector DNA was incubated with 1 µl of alkaline phosphatase and 2 µl of 10x alkaline phosphatase buffer, at 37°C for 10 minutes. The reaction was then heat deactivated at 75°C for 2 minutes.

2.2.7 Ligation of PCR product into plasmid vector

Addition of an adenosine base to the ends of the PCR product (A-tailing) was achieved by T using a mix of 10mM $MgCl_2$, 20mM dATP, 1 µL of Taq Polymerase, 6.7 µl of PCR product, and ddH_2O, which was incubated at 70°C for 30 minutes. The PCR product was then ligated into a pGEM-T Easy vector in a 10 µl reaction containing 5 µl 1x ligation buffer, 0.5 µl of pGEM-T Easy vector, 2 µl PCR product, 1 µl DNA ligase, and 1.5 µl ddH_2O. This reaction was incubated at room temperature for 60 minutes.

2.2.8 *E. coli* transformation

Pre-existing stocks of competent DH5α cells were used for transformation. 300 µl aliquots of DH5α cells were thawed on ice for 10 minutes, having previously been frozen at -80°C. Once thawed, the entire 10 µl ligation reaction mixture was added to the cells. After gentle mixing, the cells were incubated on ice for 30 minutes, followed by a 'Heat-Shock' incubation at 37°C for 2 minutes, then returned to ice for a further 3 minutes. The cells were then added to 2 mL of LB media, and incubated on a shaker at 37°C for 90 minutes. Following this, the cells were concentrated by centrifugation at 12000g for 30 seconds, and resuspended in 200 µl of LB media, then introduced to agar LB media plates containing 50µg.ml^{-1} of ampicillin and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) to select for transformed cells. The plates were incubated overnight at 37°C.

2.2.9 Isolation of plasmids from *E. coli* using Alkaline Lysis Miniprep

White colonies from transformation plates were picked and introduced to 5 ml of LB media and appropriate antibiotics. These samples were incubated overnight at 37°C.

Each 5 mL sample was centrifuged at 12,000g for 30 seconds in microfuge tubes. After the supernatant was removed by aspiration, the cells were resuspended in100 µl of Solution 1, followed by a 5 minute standing period at room temperature and a further 5 minutes on ice. To precipitate nucleic acids, 400 µl of Solution 2 was added to the sample, then shook vigorously. After 5 minutes on ice, 300 µl of Solution 3 was added, followed by 6 inversions of the microfuge tube. The samples were placed on ice for 5 minutes, then centrifuged at 12,000g for 5 minutes. 800 µl of supernatant was removed and transferred to a new microfuge tube, to which was also added 4 µl of RNAase. Nucleic acids were extracted by introducing a 200 µl 1:1 mix of chloroform and TES-equilibrated phenol to the sample. This was vortexed, left for 2 minutes, then vortexed again. The samples were centrifuged for 5 minutes at 12,000g, after which 750 µl of supernatant was removed and transferred to a new microfuge tube. The samples were mixed with 400 µl of 100% isopropanol, left to stand for 10 minutes, then centrifuged at 12,000g for 10 minutes. The supernatant was removed by aspiration, and the remaining pellet washed with 200 µl of cold 70% ethanol. To retrieve the pellet, the samples was centrifuged for 1 minute. Ethanol was aspirated, and the samples were left to to dry at 37°C. Once dry, the pellet was resuspended in 50 µl of ddH_2O. Samples were digested and analysed by gel electrophoresis.

2.2.10 Transformation of *Synechocystis* sp. PCC 6803

A liquid culture of wild-type *Synechocystis* sp. PCC 6803 was grown photoautotrophically in BG-11 media to an optical density of 0.8-1.0. The liquid culture was transferred to a 50 ml falcon tube, and centrifuged at 4000g for 7 minutes at 25°C. The supernatant was removed, and the cells were resuspended in 2 ml of BG-11. A solution of cells and media was made to a volume of 0.5 ml and an

optical density of 2.5, to which was added 5 µl of plasmid DNA. This was incubated at 30°C for 6 hours under optimal light, including a shake of the sample at 3 hours. After the incubation period, 200 µl of cells were spread on Whatman filters, placed on BG-11 agar media with glucose and without antibiotics. After an overnight incubation at 30°C, the filters were removed and placed on BG-11 agar media containing glucose and appropriate antibiotics. Colonies appeared after 10 days, which were restreaked for a further 3 generations to allow for segregation. Segregation was confirmed by colony PCR.

2.2.11 Studying photoautotrophic growth of *Synechocystis* sp. PCC 6803

Starter liquid cultures of *Synechocystis* sp. PCC 6803 strains to be analysed were grown in the presence of glucose and antibiotics, to an optical density of 0.8-1.0. Since photoautrophic growth was being measured, glucose had to be removed from the cells by a series of washes. This involved transferring the cells to 50 ml Falcon tubes, followed by centrifugation at 4000g for 7 minutes at 25 °C. The supernatants were removed, and the cells were then resuspended in 5 ml of BG-11. After resuspension, BG-11 was added to each sample, giving a final volume of 50 ml. This was centrifuged as above. After repeating these washes 3 times, the cells were finally resuspended in 5 ml of BG-11. New liquid cultures were then started, using 150 ml of BG-11, appropriate antibiotics, and washed cells from the starter cultures, giving an optical density of 0.05. The optical density of the liquid cultures was subsequently measured every 12 hours (for 60 hours) then every 24 hours (for 80 hours).

2.2.12 Determining chlorophyll *a* concentrations in samples of *Synechocystis* sp. PCC 6803

Assays analysing photosynthetic ability require determination of chlorophyll a concentration. To find this, 200 µl of resuspended *Synechocystis* sp. PCC 6803 cells were centrifuged at 16,000g for 2 minutes. The supernatant was removed, and the pellet was resuspended in 1 ml of methanol. This was then centrifuged at 16,000g for 5 minutes. The optical density of the chlorophyll-containing supernatant was

then measured at a wavelength of 663 nm. Chlorophyll a concentration in $mg.ml^{-1}$ was determined by multiplying the absorbance of each sample by 5 (the dilution factor), and dividing by 82.

2.2.13 Measuring Oxygen Evolution

Liquid cultures of *Synechocystis* sp. PCC 6803 were grown heterotrophically in the presence of antibiotics. These cultures were centrifuged and washed as in section 1.1.11, however, they were resuspended after each wash in pH 7.5 HEPES BG-11 instead of BG-11. For this assay, concentrations were equalized between different samples based on chlorophyll a concentration. Samples of cells were diluted to give final chlorophyll a concentration of 5 $\mu g.ml^{-1}$.Oxygen evolution was measured using a Clarke-type oxygen electrode (Hansatech, UK). Before measuring oxygen evolution, a zero oxygen baseline needed to be set in order to calibrate the electrode. This was achieved by adding sodium dithionite to the electrode chamber, and setting the electrode to zero. Oxygen-saturated water was then added to the electrode chamber, and the resulting voltage reading was recorded.

1 ml samples of *Synechocystis* sp. PCC 6803 were added to the electrode chamber in the presence of the electron acceptor DMBQ, as well potassium ferricyanide to maintain DMBQ in its oxidized state. The assay started with a 60 second pre-recording wait period, followed by exposure to saturating actinic light of 2 $mE.m^2.s^{-1}$ for 3 minutes, during which time oxygen evolution was measured. There was an additional 60 seconds of recording without light at the end of the assay.

2.2.14 Measurement of Whole Cell Absorption Spectra

Liquid cultures of *Synechocystis* sp. PCC 6803 resuspended were also used to measure whole cell absorption spectra. Samples were diluted to an OD of 0.3, and absorbance over the range of 400 to 800 nm were measured using a Jasco v-550 UV/VIS spectrophotometer (Jasco Inc., USA). Cellotape was placed on the front and back of both cuvette holders, thereby scattering the incoming and outgoing light.

2.2.15 RNA extraction from *Synechocystis* sp. PCC 6803

300 ml liquid cultures were grown to an OD of 0.8 – 1.0, then centrifuged at 5000g for 10 minutes. The resulting pellet was resuspended in 5 ml of BG-11 and transferred to a 50 ml falcon test tube, where it was frozen using liquid nitrogen. Cells were defrosted and mixed with 2 ml of Phenol heated to 65°C, as well 2 ml of NAES extraction buffer. This was divided into four 200 µl aliquots placed in bead-beating tubes, with 100 mg of Zirconia/Silica beads (Biospec Products, Inc., USA). Cell samples were bead beat at 5000rpm for 20 seconds at 4°C using a mini-Bead beater (Biospec Products, Inc., USA). Samples were then centrifuged at 13,000g for 10 minutes at 4°C, after which the top phase was removed and added to a 500 µl, 1:1 mix of phenol and chloroform. After a vigorous mix, the samples were placed on ice, then centrifuged again at 14,000g for 10 minutes. The top phase was transferred to a new tube, upon which was performed another phenol/chloroform extraction. After a final chloroform-only extraction, nucleic acids within the samples were precipitated using 3M sodium acetate-acetate at pH 6, and 2 volumes of ethanol at -20°C. This was centrifuged at 13,000g for 10 minutes. The supernatant was removed, and the pellet was air dried on a heating block at 37°C. The pellet was resuspended in 250 µl of DEPC-treated water, and transferred to a centrifuge tube onto a O.5 ml of Caesium Chloride (5.7M) cushion. The sample was centrifuged at 90,000 rpm at 10°C for 90 minutes using a TLA ultracentrifuge, to concentrate the RNA into a pellet. After removal of the aqueous phase, the pellet was resuspended in DEPC-treated water. A chloroform wash (as described above) was used to remove insoluble contaminants, followed by precipitation of nucleic acids using 2 times volume of ethanol, as well as 1/10 volume Sodium Acetate-Acetate (pH 6). This was centrifuged at 13,000g for 10 minutes at 4°C. After removal of supernatant, the pellet was resuspended in 200 µl DEPC-treated water.

2.2.16 Synthesis of cDNA

cDNA was synthesized using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlbad, CA, USA). A reaction mix containing 0.5 µg of RNA, 1 µl of random hexamers, and 1 µl of 10 mM dNTPs was made, and incubated at 65°C for 10 minutes to denature, followed by 1 minute on ice. A 'synthesis mix' was made, containing 10x Reverse transcriptase buffer, 25 nM $MgCl_2$, 0.1 M DTT, RNase OUT, and SuperScript III Reverse Transcriptase. 10 µl of the RNA reaction mix was added to 10 µl of synthesis mix and incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes, and 85°C for 5 minutes. 1 µl of RNase H was added to the reaction, followed by an incubation at 37°C for 20 minutes. Samples were then stored at -20°C.

Results

3.1 Generation of Usp - deficient strains in *Synechocystis* sp. PCC 6803

The role of a protein can be determined by knocking out its corresponding gene, and observing any differences in phenotype. In this project, a strain of *Synechocystis* sp. PCC 6803 was generated lacking a functional copy of the *usp2* gene. Additionally, a double knockout lacking both Usp1 and Usp2 was generated. A Usp1-deficient strain existed in the Eaton-Rye lab prior to the commencement of this project (Peter Mabbitt). Generating these strains required making a genetic construct with a deleted *usp2* gene. This involved amplifying the *usp2* gene and ligating it into a plasmid vector. This vector was then subjected to a double restriction enzyme digest, cleaving at two points within the coding region of the gene. A spectinomycin-resistance cassette was inserted into the deleted region, allowing for selection.

The knockout plasmid Δ*usp2* was incubated with wild type *Synechocystis* sp. PCC 6803, as well as the ΔUsp1 mutant strain to achieve transformation. The resulting strains were grown on either BG-11 media containing spectinomycin (ΔUsp2) or spectinomycin and kanamycin (ΔUsp1:ΔUsp2), to select for cells containing the knockout gene. Given that *Synechocystis* sp. PCC 6803 contains approximately 10 copies of its genome, restreaking for 3 generations of plates was required for complete segregation of the knockout gene through all copies. Segregation was confirmed using colony PCR.

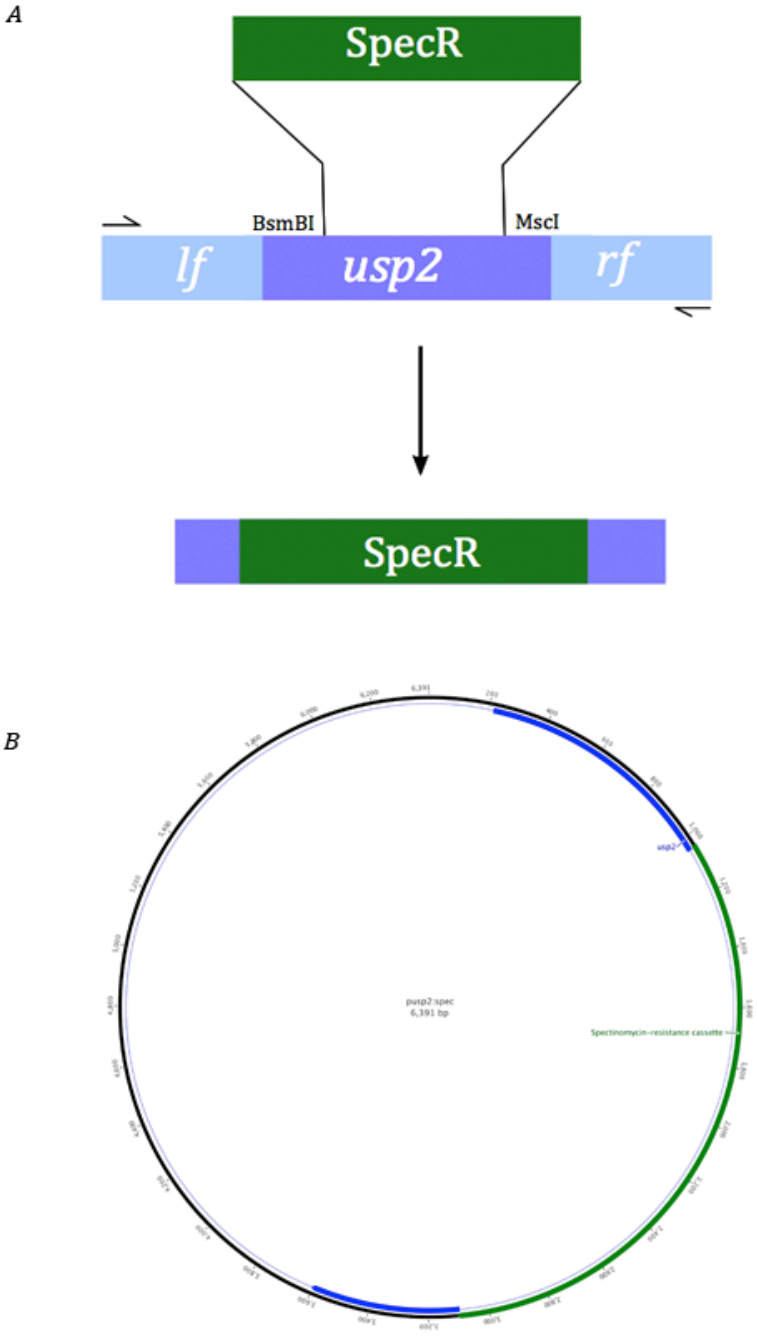


Figure 3.1 Construction of *Synechocystis* sp. PCC 6803 knockout strains. Restriction map showing the insertion of a spectinomycin-resistance cassette into a deletion within the *usp2* coding region (A). *usp2* was amplified with left and right flanking regions of 696 bp (*lf*) and 393 bp (*rf)* respectively. The amplified product was ligated into a plasmid vector. Deletion of *usp2* was generated by a double digest using the restriction enzymes BsmBI and MscI

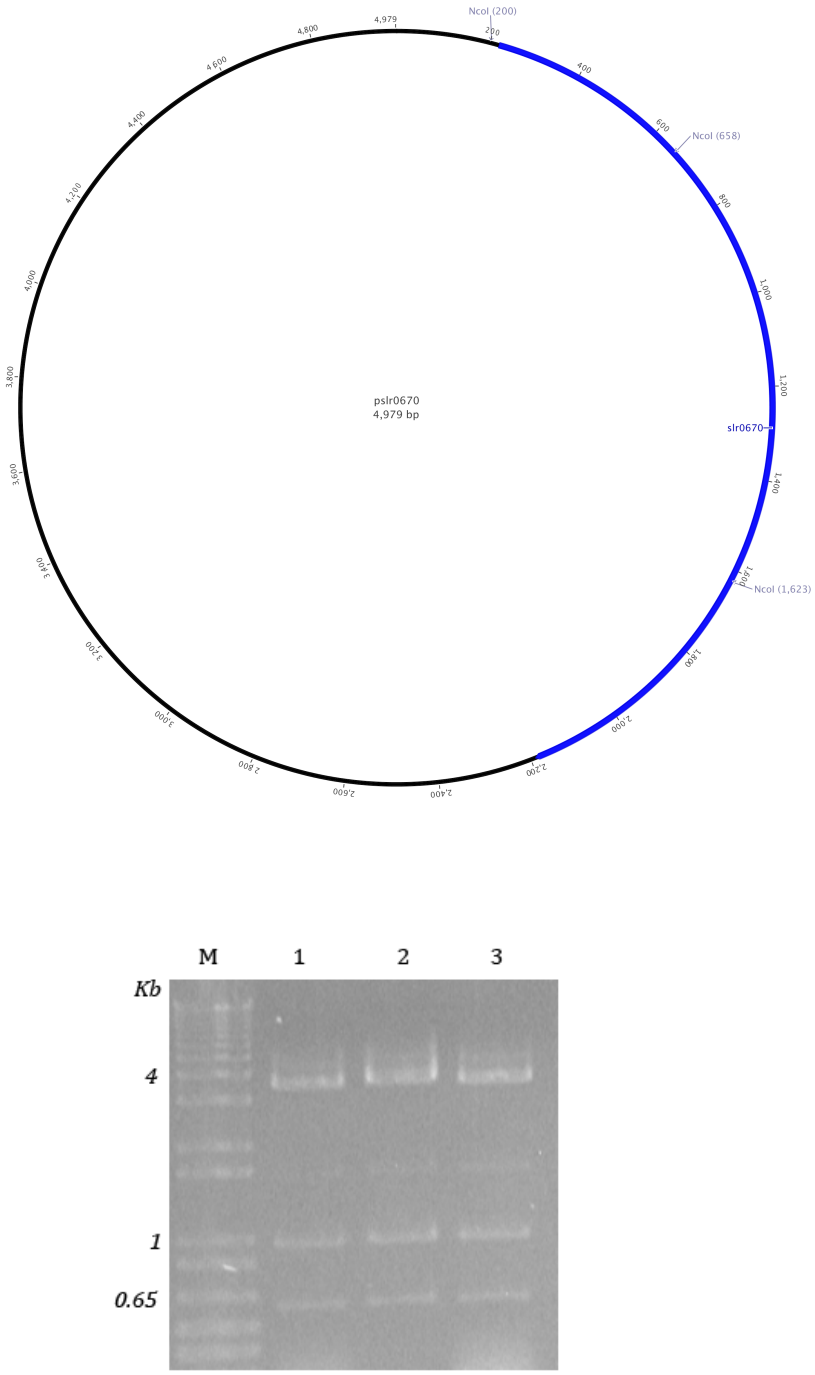
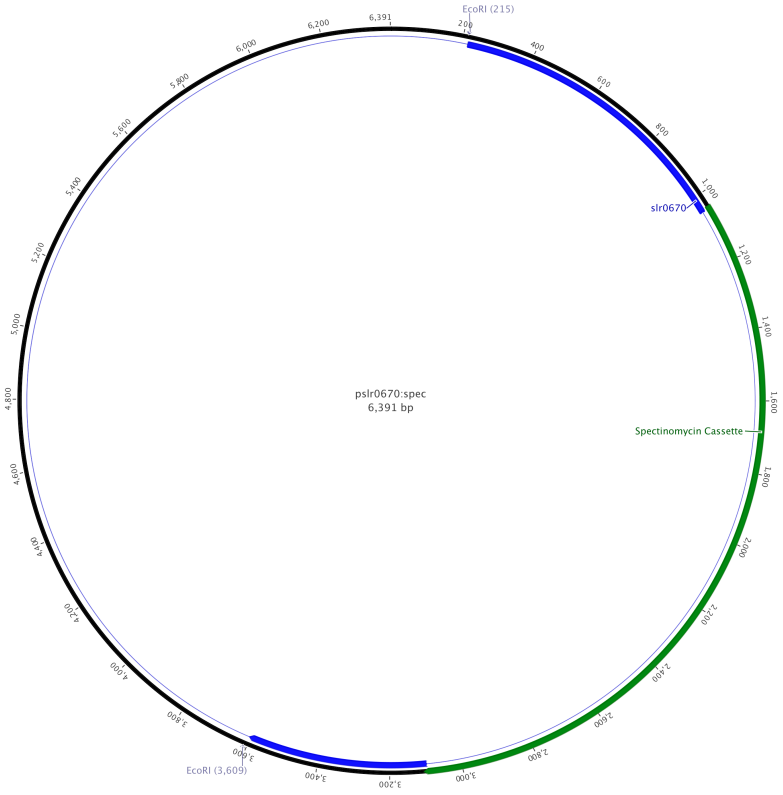


Figure 3.2 Confirming *slr0670* insertion into plasmid vector. The PCR product was cloned into a 3015 bp plasmid vector. Three miniprep samples of this were subjected to a digest with NcoI, as depicted in lanes 1,2 and 3. NcoI cleaved at two points within the *slr0670* gene and one in the multiple cloning region of the plasmid vector. This digest produced three bands of 3 kbp, 1 kbp, and 0.6 kbp, indicating the presence of the gene in the vector in a right to left orientation .



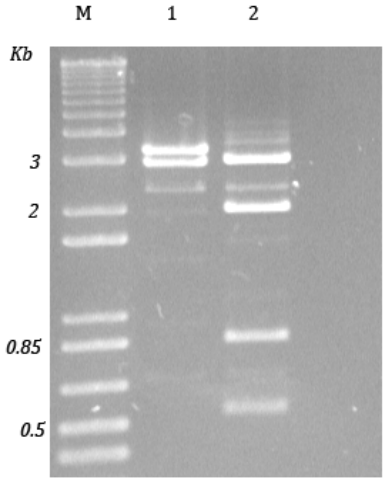


Figure 3.3 Confirming ligation of spectinomycin-resistance cassette into vector . A deletion was introduced to the *slr0670* gene, after which a spectinomycin-resistance cassette was ligated in its place by blunt ligation. This was confirmed by an EcoRI digest depicted in lane 1, yielding two bands of 3015 bp (corresponding the vector) and the 3312 bp insert (the insert consists of flanking regions, 1089 bp, undeleted portions of the *usp2* gene, 223 bp, and the spectinomysic-resistance cassette, 2 kb.

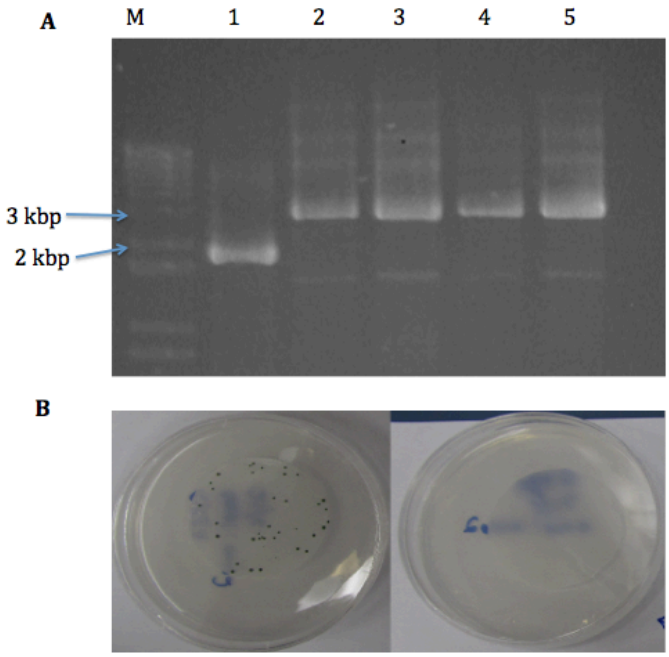


Figure 3.3 Confirmation of segregation in *Synechocystis* sp. PCC 6803. The *usp2* gene was amplified from picked colonies, using colony PCR (A). Lane 1 shows wild type, Lanes 2 and 3 ΔUsp2, and lanes 4 and 5 ΔUsp1:ΔUsp2. Wild type gave a product of just under 2000 bp, corresponding to the uninterrupted *usp2* gene (1965 bp). ΔUsp2 and ΔUsp1:ΔUsp2 gave bands of around 3000 bp, indicating presence of spectinomycin-resistance cassette. The plate in (B) depicts newly transformed colonies of ΔUsp2 before segregation. The negative control (no DNA) from the transformation is also included, showing no colonies.

3.2 Physiological characterization of Usp-deficient strains

3.2.1 Photoautotrophic growth curve

Photoautotrophic growth rates were measured for the mutant strains, to determine if the knockout strains grew grew normally under conditions of no stress. Decreased growth could be attributed to impaired photosynthetic ability, or increased susceptibility to a particular stress. Cell grown in starter cultures were added to BG-11 without glucose – the lack of an external source of carbohydrates forces the cells to grow photoautotrophically, relying solely upon photosynthesis to obtain chemical energy.All strains were able to grow photoautotrophically. Doubling times between 11 and 15 hours were recorded for all four strains (Fig. 3.4).

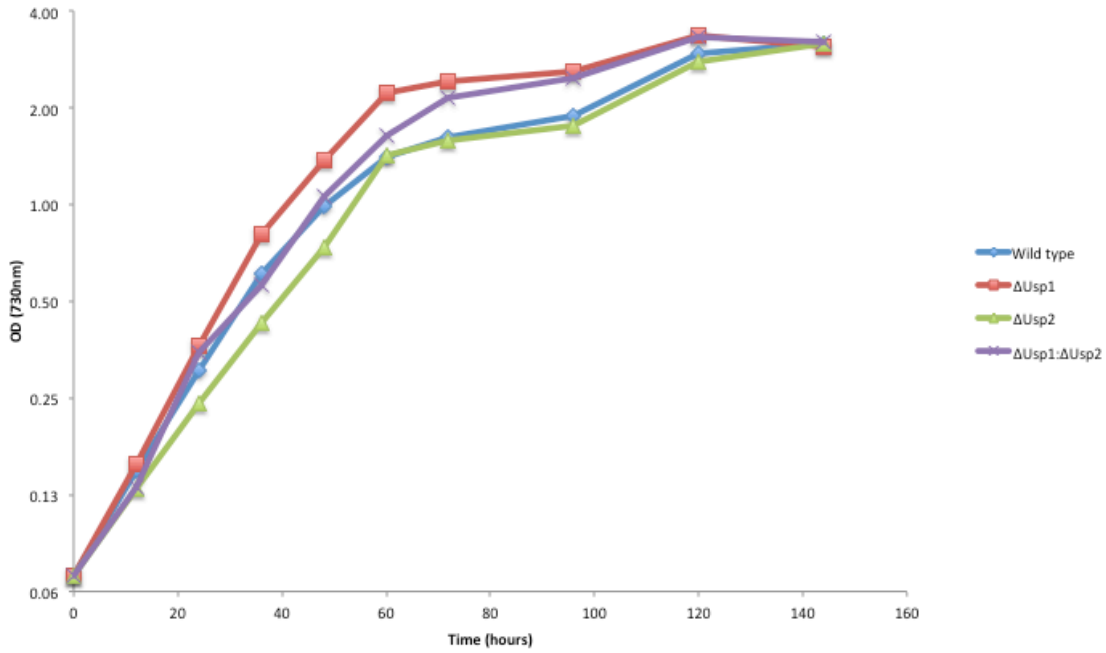
3.2.2 Oxygen evolution

Photosystem II (PSII) catalyses the splitting of water into 4 electrons, 4 protons, and molecular oxygen (O_2). The electrons produced are shuttled through a series of redox cofactors within PSII, where they eventually reduce plastinuinone to plastiquinole. Oxygen evolution is proportional to the amount of electrons passing through PSII, and is an effective way of measuring PSII activity – a knockout that impedes PSII activity is reflected in decreased ability to evolve oxygen. The artificial electron acceptor $K_3Fe(CN)_6$ was used to accept electrons straight from PSII and prevent them from being passed to the electron transport chain, allowing the specific measurement of PSII activity.

Oxygen evolution was determined for all strains in this project. The reasoning for this was that since PSII is susceptible to stress (particularly the D1 subunit REF), a possible role for USPs could be in PSII stress adaption. Oxygen evolution rates for the three knockout strains did not show a significant difference from wild type, although ΔUsp2 was slightly higher (Fig. 3.5).

3.2.3 Whole cell absorption spectra

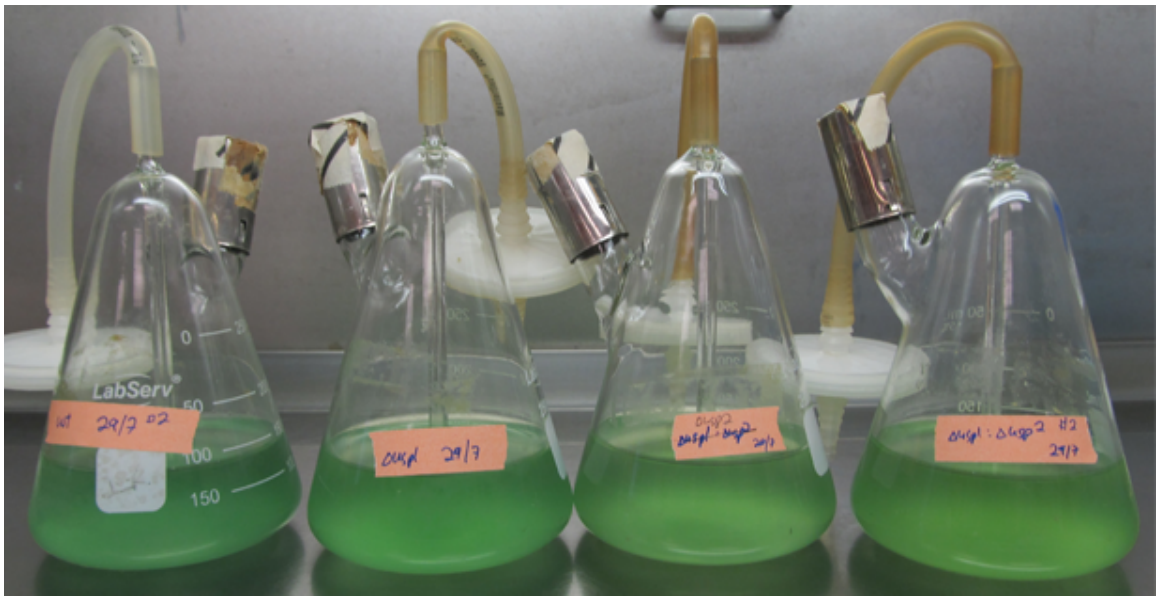
The absorption spectra of whole cells was measured to determine pigment composition, as depicted in Figure 3.6. Four distinct absorption peaks were observed, corresponding to chlorophyll *a* (440 nm and 680 nm), carotenoids (460 to 520 nm), and phycobilins (620 nm). The strains lacking Usp1 (ΔUsp1 and ΔUsp1:ΔUsp2) showed lower absorption peaks at 620 nm, implying less phycobilin pigments. Ratios of phycobilins to chlorophyll a for the Usp1-deficient strains were determined (Table 3.1) yielding a value of 1 for ΔUsp1 and 0.95 for ΔUsp1:ΔUsp2.



Strain	Doubling time (hours)
Wild type	12
ΔUsp1	11
ΔUsp2	15
ΔUsp1:ΔUsp2	14

Figure 3.4 Photoautotrophic growth curve for knockout strains of *Synechocystis* sp. PCC 6803. Cells were grown in BG-11 media without glucose, and with appropriate antibiotics. Optical density

was measured at 730 nm every 12 hours for 60 hours, then every 24 hours for 80 hours. This data is from a single experiment.



Wild type ΔUsp1 ΔUsp2 ΔUsp1:ΔUsp2

Figure 3.5 Liquid cultures of *Synechocystis* sp. PCC 6803 wild type and knockout strains used for growth curve, after 40 hours.

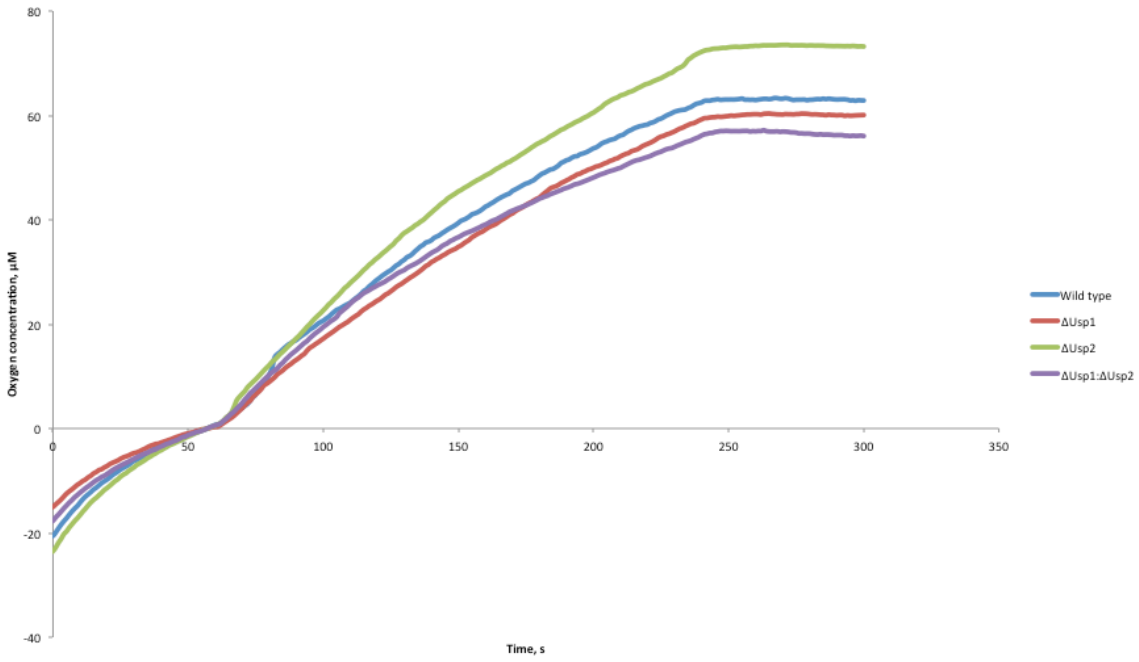


Figure 3.5 Oxygen evolution of wild type and knockout strains of *Synechocystis* sp. PCC 6803
Cells were measured for their ability to produce oxygen in the presence of the electron acceptor $K_3Fe(CN)_6$, as well as DMBQ. 1 ml samples of cells were used, at a chlorophyll concentration of 5 $\mu g.ml^{-1}$. Traces were recorded over a period of 5 min, including an initial 60 s wait period, followed by 3 min of light saturation. Recording continued for a 1 minute after the light source was turned off.

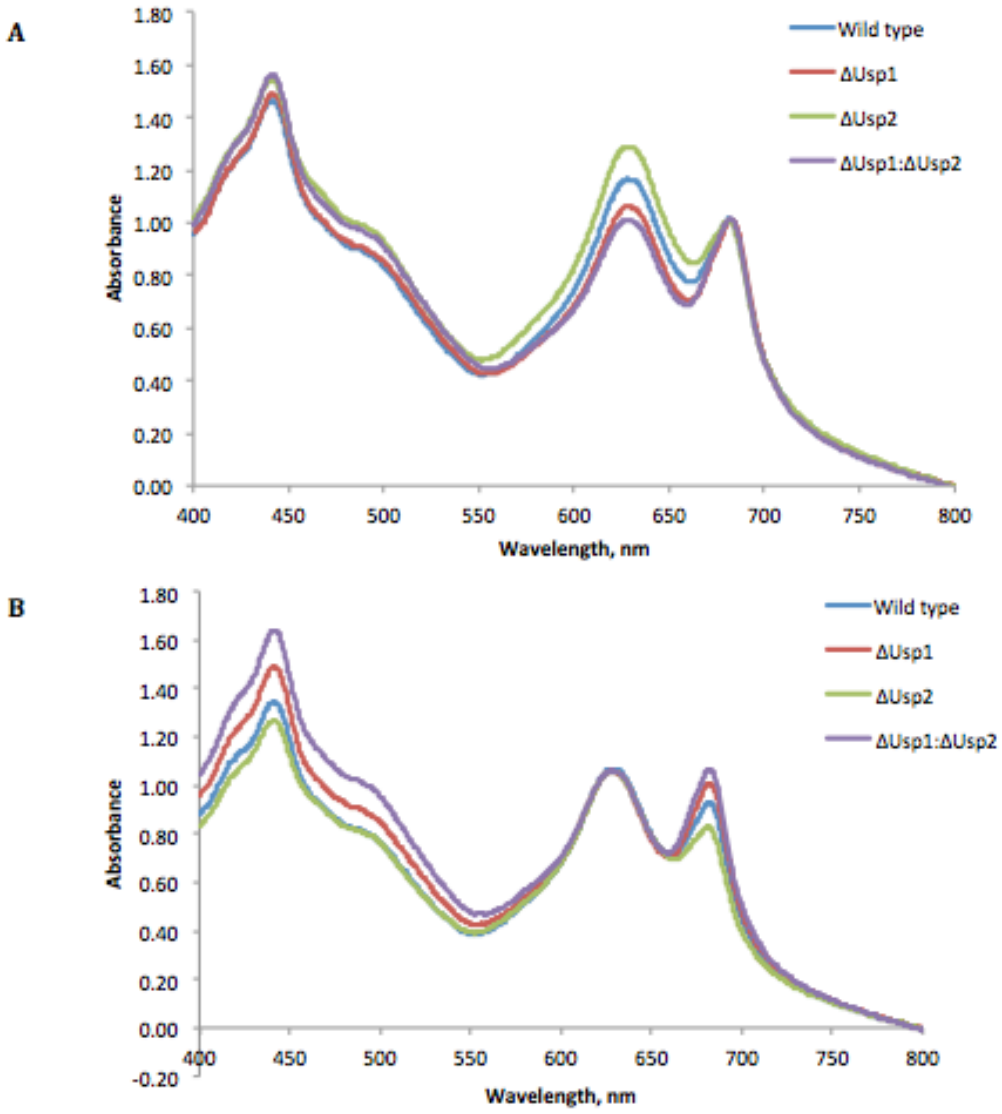


Figure 3.6 Whole cell absorption spectra for knockout strains of *Synechocystis* sp. PCC 6803. Cells were diluted to an OD_{730} of 0.3 in pH 7.5 BG-11, and absorbance was measured from 400 to 800 nm using scattered light. This data was normalized to the absorption at 680 nm (A) and 620 nm (B), and represents the average of three independent experiments.

Table 3.1 Ratio of phycobilins (620 nm) to chlorophyll *a* (680 nm)

Wild type	1.09
ΔUsp1	1.00
ΔUsp2	1.21
ΔUsp1:ΔUsp2	0.95

3.3 Stress response of wild type and USP-deficient strains

3.3.1 Nutrient and temperature stress

As a way of screening for stress reponses, wild type, ΔUsp1 and ΔUsp2 cells were grown on media lacking certain nutrients, as well as being exposed to various temperatures (at this stage, the double knockout ΔUsp1:ΔUsp2 could not be used as it had not segregated). This was achieved by making BG-11 agar plates lacking a particular nutrient, and placing them in different temperature environments (mimicking an experiment by notsureofname et. al), as seen in Figure 3.8. Plates were made either deficient of calcium, chloride, sulfate, or manganese, as well as an additional normal BG-11 plate to act as a control. Four of each kind of nutrient-deficient plate were made.

Temperature stress response

Cells grown initially at 40°C struggled to grow, and upon transferal to 42°C did not adapt well to the imposed temparture stress. Cells grown at 30°C before transferal to 42°C were more resilient and still showed signs of life after incubation at 42°C, although their growth was inhibited. Cells grown at 30°C for 5 days grew healthily, apart from the cells on nutrient-deficient plates. Cells grown at 40°C for 5 days showed less growth than at 30°C. The effect of temperature was uniform across all strains.

Nutrient stress response

All nutrient-deficient plates showed a significant decrease in cell growth. It was not possible to determine if one particular stress inhibited growth to a greater degree than the other stresses, as this experiment was a general screen and not aimed at quantitative analysis. The effect of nutrient stress was uniform across all strains.

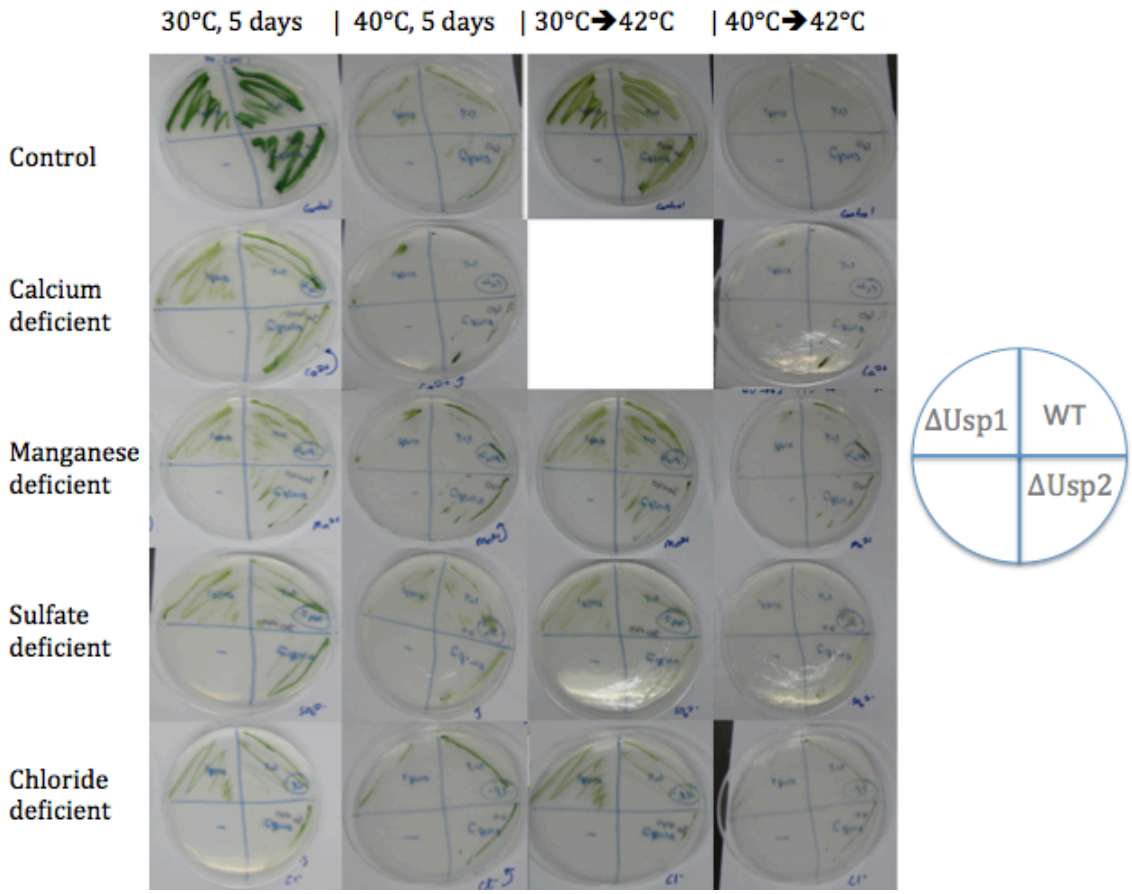


Figure 3.8 *Synechocystis* sp. PCC 6803 wild type and knockout strains grown on nutrient-deficient media, exposed to temperature stress. Each plate was divided into four quadrants, and in each quadrant was streaked a single strain . Special care was taken not to mix strains. The plates were then divided into 'sets' containing one of each type of nutrient deficiency (calcium, manganese, phosphate, sulfur, unstressed). Four 'sets' in total were made, and each set was exposed to different temperature conditions, as follows: 30°C for 5 days; 40°C for 5 days; 30°C for 5 days followed by 42°C for 2 days; 40°C for 5 days followed by 42°C for 2 days.

3.3.2 RT-PCR

Reverse transcription PCR was used to give a clearer picture of stress responses of the knockout strains. To achieve this, *usp1* and *usp2* expression was measured under stress conditions in wild type *Synechocystis* sp. PCC 6803, as detailed in table 3.1.

Stress	**Method of application**
Temperature stress	A 300 ml liquid culture of cells was grown to an OD of 1, and transferred to growth chamber, where it was incubated at 40°C for 16 h.
Iron stress	A 300 ml liquid culture of cells was grown to an OD of 1 in BG-11 media lacking iron
Phosphate stress	A 300 ml liquid culture of cells was grown to an OD of 1 in BG-11 media lacking phosphate
Oxidative stress	A 300 ml liquid culture was grown to an OD of 1, after which rose bengal was added to give a final concentration of 2 μM. Cells were exposed to this treatment for 3 h before RNA extraction

Table 3.1 Stresses applied to wild type *Synechocystis* spp. PCC 6803 for subsequent RNA extraction and analysis using RT-PCR.

RNA was extracted from cells under each stress condition, and analysed on a formeldahyde gel to check for degredation (Fig. 3.7). Under phosphate stress, cells displayed very limited growth, and subsequently no RNA was able to be extracted. RNA from each stress condition was converted into cDNA, and used as a template in RT-PCR to amplify *usp1* and *usp2*. The standard 30-35 cycles used for PCR

amplification would yield a large amount of DNA product, meaning that subsequent ethidium bromide staining would produce such intense DNA bands that differential expression would be hard to gague. In order to overcome this, pauses were included after every 3 cycles so that samples from each PCR reaction could be extracted. Samples were extracted after cycles 15,18,21,24,27, and 30, and were subsequently analysed on an agarose gel, as depicted in Figure 3.8. This figure shows that *usp1* is generally expressed more than *usp2*, based on the greater band intensities. *usp1* shows a greater degree of expression under iron, temperature, and oxidative stress relative to the unstressed control, whereas *usp2* shows uniform expression across stressed and unstressed samples.

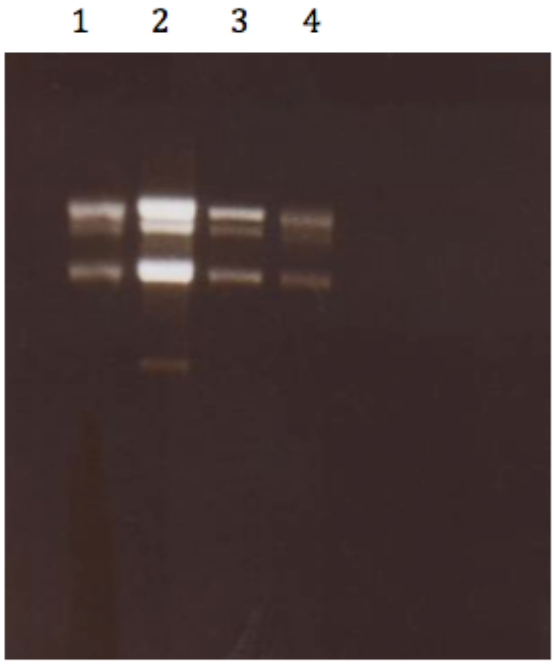


Figure 3.7 Formeldahyde gel of RNA samples extracted from stressed *Synechocystis* spp. PCC 6803 cells. Lanes correspond to 1; Oxidative stress, 2; Temperature stress, 3; Iron stress, 4; Unstressed. No RNA could be recovered from cells placed under phosphate stress.

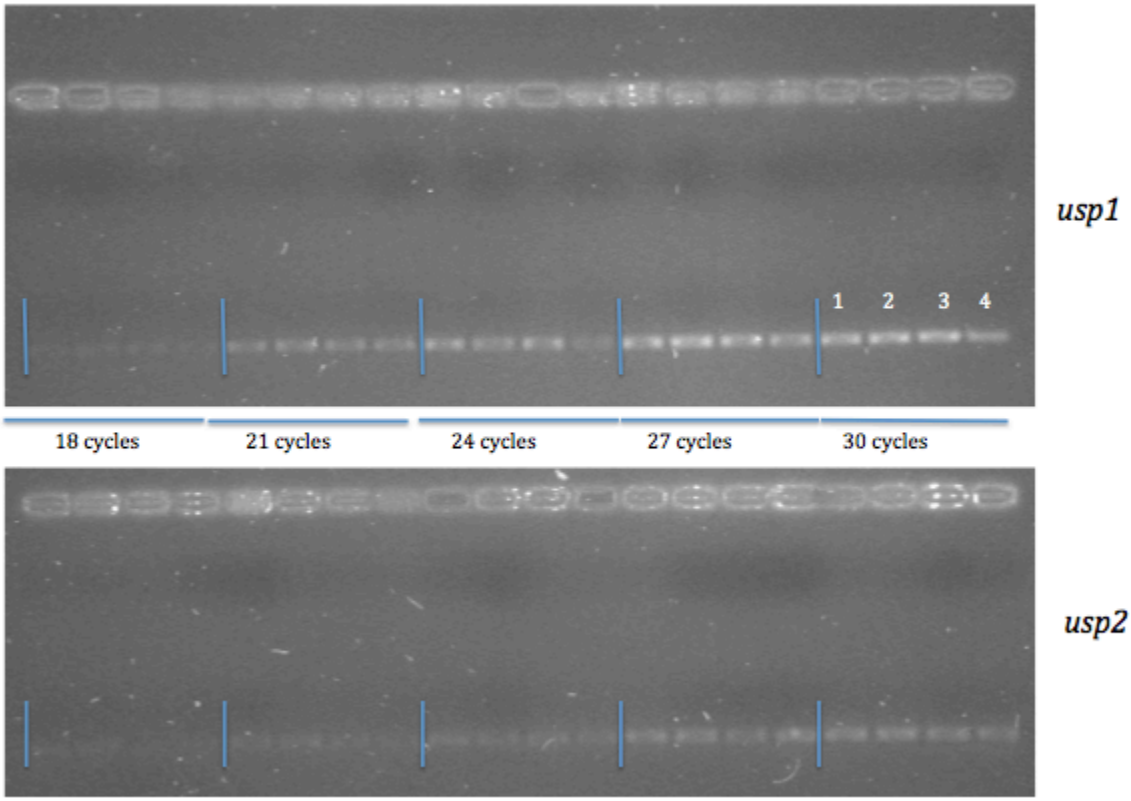


Figure 3.8 Electrophoresis gel of RT-PCR samples, extracted after every 3 cycles. RNA extracted from stressed *Synechocystis* sp. PCC 6803 was convereted into cDNA and used as a template for RT-PCR. Primers specific for *usp1* and *usp2* were used to amplify their respective genes in separate reactions. During PCR, 5 µl samples from each stress condtion were removed after every three cycles, starting at cycle 18. Lane 1 represents cDNA from iron-stressed cells, lane 2 represents oxidative stress, lane 3 represents tempertature stress, and lane 4 is the unstressed control. This order is mainted over all cycles, on both gels. The upper gel depicts *usp1*-specific expression under stress, the lower, *usp2*-specific expression under stress.

3.3.3 Photoautotropohic growth under stress

It was illustrated that *usp1* could be upregulated in response to iron, temperature, and oxidative stress, as described in section 3.3.2. The obvious experimental approach was then to determine if Usp-deficeint strains displayed a different phenotype from wild type under the previously mentioned stresses. This was investigated by measuring photoautotrophic growth of wild type and the knockout strains under temperature and iron stress (due to time constraints, growth under oxidative stress could not be measured). Additionally, growth under calcium stress was measured. Stresses were applied as described in Table 3.2.

Stress	**Method of application**
High temperature	Liquid cultures of all strains were grown at 30°C for 24 h to allow for adequate growth. After 24 h, cultures were transferred to an incubation chamber set at 40°C.
Iron deficient	Cells grown in BG-11 media lacking iron
Calcium deficient	Cells grown in BG-11 media lacking calcium, using KCl as cation source.

Table 3.2 Stresses applied to liquid cultures used in photoautotrophic growth measurements

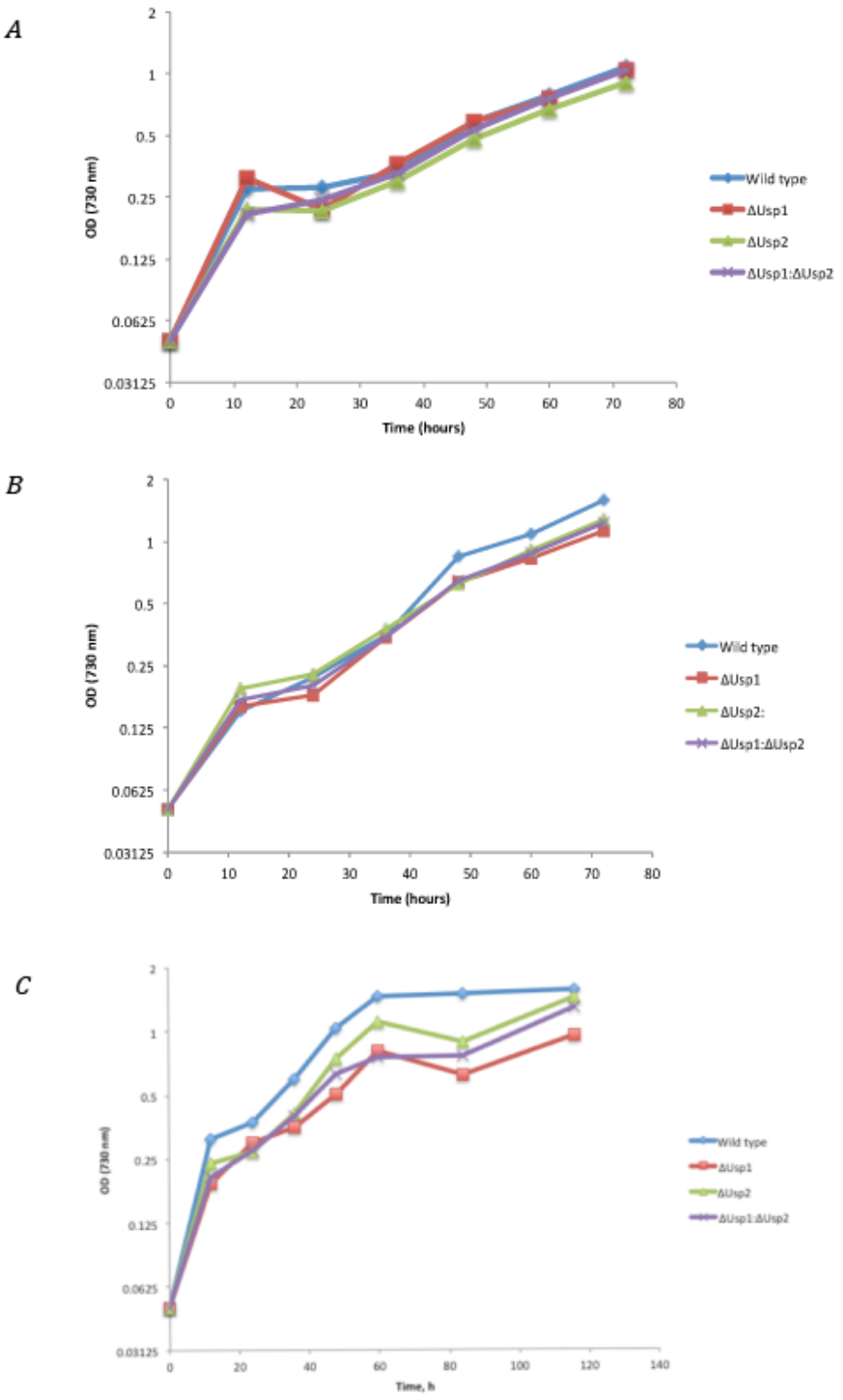


Figure 3.9 Photoautotrophic growth of wild type and knockout strains under stress

Optical density was measured every 12 hours for 72 hours (A and B) and for 120 hours (C). Stress conditions included temperature stress (A), iron stress (B), and calcium stress (C).

Discussion

4.1 Physiological characterization of ΔUsp1, ΔUsp2, and ΔUsp1:ΔUsp2

During photoautotrophic growth, an organism obtains energy solely by photosynthesis without any external source of chemical energy. The ability to grow photoautotrophically was measured in all strains under no stress, to determine if the lack of USPs affected this particular mode of growth. The growth curve depicted in Figure 3.4 suggested that there is no significant change in growth rates between wild type and the Usp-deficient strains. This was to be expected, as USPs in other organisms are induced in response to stress. It has also been found that USPs are upregulated during stationary phase, however no change was observed during this phase in the Usp-deficient strains.

Oxygen evolution measures PSII-specific activity, and was measured for all Usp-deficient strains, using unstressed cells. The rates of oxygen evolution did not show any significant difference between wild type and the Usp-deficient strains, suggesting that PSII-specific activity in unstressed conditions is unaffected by the removal of USPs. PSII is known to be stressed under high light conditions (**ref**), so a more rational approach would be to expose cells to high light before measuring oxygen evolution. The results would indicate if USPs have a role in PSII-stress adaption. Due to time constraints however, this experiment could not be performed.

Photosynthetic organisms are able to adapt to changes in light intensity and nutrient availability by altering the amount of certain proteins involved in photosynthesis (Montané et. al, 1998). The light-capturing pigment proteins are exmaples of components of photosynthesis, and include phycobiliproteins, PSII, and PSI. Phycobiloproteins contain a range of pigment molecules that can absorb light over a wide range of wavelengths, and can change their pigment composition to

adapt to the most prevalent wavelength of incident light. This is achieved by predominantly producing pigments complementary to the prevalent incident light (Riethman et, al, 1987). It has also been found that phycobiloproteins are degraded in response to nutrient deficieny, including lack of sulfate, nitrate, carbon, and iron. This is because phycobiloproteins constitute up to 45% of total soluble proteins, providing a reserve of carbon skeletons and amino acids for nutrient stressed cells (Riethman et. al, 1987). Whole cell absorption spectra was measured in this project to determine pigment composition of the Usp-knockout strains. The strains lacking Usp1 (ΔUsp1, ΔUsp1:ΔUsp2) showed a decrease in phycobilisome content, signified by a decrease in absorbance at 620 nm (Figure 3.6, A). This could indicate degredation of phycobilisomes in response to a stress that would normally be alleviated by Usp1. **Talk about usp2, and the fact that the cells were unstressed.**

4.2 Stress responses of Wild type Usp-knockout strains

Wild type, ΔUsp1 and ΔUsp2 were grown on BG-11 agar plates lacking certain nutrients, and incubated at various temperatures, to investigate growth under stress. These plates confirmed that *Synechocystis* sp. PCC 6803 struggles to grow without calcium, manganese, phosphate, and chloride. They also indicated that cell bleaching occurs around 42°C. Cell growth under all conditions was uniform between the strains; the knockout strains did not appear to show any less growth than wild type. It could be concluded that Usp1 and Usp2 in *Synechocystis* sp. PCC 6803 are not upregulated in response to these stresses. However, *Synechocystis* sp. PCC 6803 growth on plates sometimes does not give a clear or reliable picture of actual growth rate (ref), confirmed by the fact that the Usp-deficient strains showed slower growth in a liquid BG-11, but not on solid BG-11.

RT-PCR was used in this project to investigate the expression of *usp1* and *usp2* under stress conditions, in wild type *Synechocystis* sp. PCC 6803. The stress conditions included high temperature, oxidative stress, and iron stress. In all repeats of the experiments, it was found that *usp1* yielded more intense bands,

indicating that it was expressed more than *usp2*. *usp1* expression was also upregulated in response to all three stresses applied, however *usp2* did not show any apparent differential expression. These two finding suggest that Usp1 is more of a general response factor, that is upregulted in response to non-specific stress. Usp2 could be more of a specialized stress response factor, up or downregulated in response to a stress not tested for in this project.

4.3 Future directions

USPs are upregulated in response to a wide range of chemical and physical stresses, only a few of which were tested in this project. The primary aim on any future work would be to examine *usp1* and *usp2* expression under a much broader range of stresses, for example UV irradiation, osmotic stress, pH stress and other such stresses that have proven in the literature to change the expression of USPs. A more precise experimental technique for looking at expression would be Real-Time PCR, as opposed to Reverse Transcription PCR.

Furthermore, it would be useful to repeat the experiments looking at physiological characterization (oxygen evolution, whole cell absorption spectra, and photoautotrophic growth curves) after placing the cells under stress. This is because USPs are normally upregulated in response to stress, meaning phenotypes are more likely to appear under stress conditions. For example, a common experiment is to stress cells with high light prior to taking oxygen evolution measurements.
Finally, since Usp-containing organisms normally contain many *usp* genes, it would be useful to determine if there were more than two *usp* genes in *Synechocystis* sp. PCC 6803. If so, further knockouts could be generated and analysed as described in this project.

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