CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Acetic acid: BDH, England

Acetyl phosphate: Sigma, USA

Arylamide: Merck, Germany

Adenosine diphosphate (ADP): Sigma, USA

Adenosine triphosphate (ATP): Sigma, USA

Agarose: Gibco, USA

Ammonium acetate: Sigma, USA

Ammonium persulfate: Merck, Germany

Ammonium sulfate: Merck, Germany

Bacto agar: Scharlau Chemie S.A., Spain

Bactotryptone: Merck, Germany

Benzamidine: Sigma, USA

Bis-acrylamide: Merck, Germany

Bovine serum albumin: Sigma, USA

Bromophenol blue: BDH, England

Calcium chloride: Sigma, USA

Chloroform: Merck, Germany

Citric acid: Sigma, USA

Cobalt nitrate: Sigma, USA

Coomassie brilliant blue G-250: Sigma, USA

Coomassie brilliant blue R-250: Sigma, USA

Cuprous sulfate: Sigma, USA

Dipotassium hydrogen phosphate: Sigma, USA

Disodium molybdate: Sigma, USA

Dithiotreitol (DTT): Sigma, USA

Ethanol: Scharlau Chemie S.A., Spain

Ethylenedinitrolo tetraacetic acid, disodium salt (EDTA): BDH, England

Ferric ammonium citrate: Sigma, USA

Ferric chloride: Fluka, Switzerland

Glucose: Merck, Germany

Glucose-6-phosphate dehydrogenase: Sigma, USA

Glycerol: Ajax Finechem, Australia

N-[2-Hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid] (HEPES): Sigma, USA

Hexokinase: Sigma, USA

Hydrochloric acid: Lab Scan, Ireland

Hydroxylamine hydrochloride: Sigma, USA

Imidazole: BDH, England

Isoamyl alcohol: Sigma, USA

Isopropanol: Sigma, USA

N-lauryl sarcosine: Sigma, USA

Magnesium chloride: Merck, Germany

Magnesium sulfate: Scharlau Chemie S.A., Spain

Manganease chloride: Fluka, Switzerland

3-[N-Morpholino] propane sulfonic acid (MOPs): Sigma, USA

Nitro blue tetrazolium: Sigma, USA

p-nitrophenyl phospate: Sigma, USA

Phenazine methosulfate: Sigma, USA

Phenol: BDH, England

Phenyl-sepharose: Pharmacia LKB, Sweden

Potassium acetate: Merck, Germany

Potassium chloride: Merck, Germany

Polyethylene glycol 6000: Sigma, USA

Polyethylene glycol 8000: Sigma, USA

Potassium hydroxide: Fluka, Switzerland

Rubidium chloride: Sigma, USA

Sodium acetate: Merck, Germany

Sodium carbonate: BDH, England

Sodium chloride: APS, Australia

Sodium dodecyl sulfate: Sigma, USA

Sodium hydroxide: Merck, Germany

Sodium iodide: Sigma, USA

Sodium nitrate: Fluka, Switzerland

Sodium thiosulfate: BDH, England

Trichloroacetic acid: Scharlau Chemie S.A., Spain

Trihydrogen borate: Sigma, USA

Tris: USB Corporation, USA

Yeast extract: Scharlau Chemie S.A., Spain

Zinc sulfate: Sigma, USA

2.1.2 Equipment

Autoclave: Model HA-30, Hirayama Manufactering Cooperation, Japan

Centrifuge: Model J-21C, Beckman Instrument Inc, USA

Electrophoresis unit: Model Mini-protein II Cell: Bio-Rad, USA

Fraction collector: Model 2211, Pharmacia LKB, Sweden

Laminar flow: BVT-124 International Scientific Supply, Thailand

Micropipette: Pipetman, Gilson, France

PCR machine: PERKIN ELMER DNA Thermal Cycler, Japan

Peristatic pump: Pharmacia LKB, Sweden

pH meter: Model 420 A, Orion, USA

Power supply: Bio-Rad POWER PAC 1000

Spectrophotometer: Jenway: UV/VIS 6400, USA

Spectrophotometer Jasco V-550 uv/vis: Beckman USA

Waterbath: Charles Hearson, England

2.1.3 Enzymes

Klenow polymerase: Boehringer Mannheim, Germany

Lysozyme: Sigma, USA

Platinum Taq: Boehringer Mannheim, Germany

Restriction enzymes: Boehringer Mannheim, Germany

T_4 DNA ligase: Boehringer Mannheim, Germany

T_7 DNA polymerase: Boehringer Mannheim, Germany

T_4 polymerase: Boehringer Mannheim, Germany

2.1.4 Antibiotics

Ampicillin: Sigma, USA

Chloramphenical: Boehringer Mannheim, Germany

Kanamycin: Sigma, USA

Spectinomycin: Sigma, USA

2.1.5 Supplies

Hybond-N membrane: Amersham Biosciences, USA

Whatman 3MM paper: Whatman International, England

X-ray film: X-Omat XK-1, Eastman Kodak, USA

2.1.6 Kit

1 kb Plus DNA Ladder, BioLabs, New England

Muta-Gene® Phagemid *In Vitro* mutagenesis Kit: BIO-RAD, USA

Prep-A-Gene® DNA purification kit: BIO-RAD, USA

2.1.7 Organisms

The two organisms *Escherichia coli* and *Synechocystis* sp. PCC 6803 from Julian Eaton-Rye laboratory, Department of Biochemistry, University of Otago, New Zealand were used in this study.

2.1.7.1 *Escherichia coli*

Strain DH5α: F-Φ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 hsdR18(r_k^-,m_k^+)phoA supE44λ^-thi-1 gyrA96 relA1; stored at –80°C.

Strain CJ236: dut, ung, thi, rel A; pCJ105(Cm^R); glycerol stock from Bio-Rad stored at –20°C.

2.1.7.2 *Synechocystis* sp. PCC 6803

Wild-type strain: glucose tolerant and fructose resistant. The glucose tolerant strain is the Williams (1988) strain, the true wild type is not glucose tolerant.

Δsll0337 deletion mutant: a chloramphenicol-resistance cassette has replaced the sll0337 gene copies (Hirani, 2001).

Δslr0081 deletion mutant: a spectinomycin-resistance cassette has replaced the slr0081 gene copies (Cuttriss, 2000).

sll0337 mutant: a spectinomycin-resistance cassette has interrupted in sll0337 gene copies (Hirani, 2001).

2.1.8 Plasmids

Plasmids used in this study are shown in Table 1

Table 1 Plasmids were used in this study

Plasmid	Characteristics	Source/Reference
psll1299	pUC19 derivative carrying a acetate kinase gene	Hirani, 2001
pslr2132	pGEMT-easy derivative carying a phosphotransacetylase gene	Hirani, 2001
pΔPhoU	pGEMT-easy derivative carrying a *phoU* gene interrupted with a Cam^R gene cartridge	Simpson, 2003
pTZsll0337	pTZsll0337 derivative carrying a Kan^R gene cartridge downstream of sll0337	Hirani, 2001
pTZslr0081	pTZslr0081 derivative carrying a Cam^R gene cartridge downstream of slr0081	Cuttriss, 2000

Kan^R: kanamycin-resistance cartridge containing gene encoding aminoglycoside 3'-phosphotransferase, Cam^R: chloramphenicol-resistance cartridge containing the gene encoding chloramphenicol acetyl trasferase.

2.1.9 Oligonucleotides

Oligonucleotides were purchased from Genset Singapore Biotech Pte.Ltd.

2.1.9.1 Mutagenesis primers are depicted in the following tables

Table 2 Mutagenesis primers are designed to create amino acid substitutions in the histidine kinase. The amino acid substitution is underlined.

Amino acid substitution	Oligonucleotides are designed to create mutant (5'-3')
Thr-214 to Ser	AATCAAAGCCACCGCTGATAAAGGGGTTCG
Thr-214 to Ala	AATCAAAGCCACCGCTGCTAAAGGGGTTCG
Thr-214 to Arg	AATCAAAGCCACCGCTCTTAAAGGGGTTCG
Thr-214 to Gln	AATCAAAGCCACCGCTTGTAAAGGGGTTCG
Thr-214 to Gly	AATCAAAGCCACCGCTCCTAAAGGGGTTCG

Table 3 Mutagenesis primers are designed to create amino acid substitution in the response regulator. The amino acid substitution is underlined

Amino acid substitution	Oligonucleotides are designed to create mutant (5'-3')
Asp-88 to Asn	CATTCTGAACATCATGCTTC

2.1.9.2 PCR primers

Table 4 PCR primer for genomic PCR

Genes	Oligonucleotides (5'-3')
sll1299	Forward primer: CGTGGCGAGCTCCCCGGCCATTTATCAACG
	Reverse primer: CGTGTTGGCGGATCCGGACTCTAGCCAAGG
slr2132	Forward primer: GCTATCACTTAAGTAGGTGG
	Reverse primer: AGTGGTCAGCTATGCCTATGG
sll0337	Forward primer: GGAAATAATTACGTCGACGATCGGC
	Reverse primer: GCCGCAGGGCAAATTAAAAAAACCG
slr0081	Forward primer: GACCACCAAGGATCCCATGGAAATTATGG
	Reverse primer: AGTTCTACTAAGCTTCTCACCGACTCCTG
slr0741	Forward primer: GCGCAAGGAATTCTTGTTGCCATAACCCTG
	Reverse primer: CTGCCTCTCCATGTCGACCTTGTAAACTCC

Table 5 PCR primer for 5'-RACE and reverse transcription PCR

Gene	Oligonucleotides (5'-3')
sll0337	Reverse primer 1: AATCAAAGCCACCGCTGTTAAAG
	Reverse primer 2: ACTTTGCCTTCTTTGAGGGGAAATC
	Poly T primer: GACCACGCGTATCGATGTCGACTTTTTTT TTTTTTTTTT
	Anchor primer: GACCACGCGTATCGATGTCGAC
psbB	Forward primer: ACCGGTGCTATGAACAGTGG
	Reverse primer: CTTCTTTCCGGGTGGAAAGG
sll1299	Forward primer: GTCTTTATGAGCTG ACTGGCGAT
	Reverse primer: TTGAGGAATGGGATATTCG
slr2132	Forward primer: CCTAAAACTAGCCCAGACCGAGA
	Reverse primer: CTTTGCACTGCCTTGTAAGT

2.1.9.3 Sequencing primers

Table 6 PCR primer for sequencing

Genes	Oligonucleotides (5'-3')
sll1299	GTCTTTATGAGCTGACTGGCGAT
slr2132	CCTAAAACTAGCCCAGACCGAGA
sll0337	GCAATCACCGCCCACTAACA
slr0081	ACGATGGACGTGATGCCTTA
slr0741	TCTCAACAGCGGTAAGATCCTTG

2.1.9.4 Oligonucleotide probes

The intragenic probes for *sll0337* and *slr0081* were obtained by digestion psll0337 and pslr0081 with restriction enzymes. The expected bands

were cut and purifed. The purified band was used as a probe.

2.2. Method for *Escherichia coli* strains

2.2.1 General molecular biology methods

2.2.1.1 Restriction digests

Single and compatible double restriction digests were incubated at the optimum temperature of restriction enzymes for 90 min. The final volume for the digests (10 µl) was made up of the restriction enzyme and the appropriately matched 10x restriction buffer plus the DNA sample.

2.2.1.2 Gel electrophoresis

DNA samples were analysed by electrophoresis in 0.8% agarose gels. The agarose gels were run in 1x TBE buffer. One µl of DNA loading buffer was added to DNA samples, which were run at 100 volts for 50 min. The DNA samples were stained for 10 min in distilled water containing ethidium bromide at a final concentration of 1 ng/ml. A Geldoc® enabled DNA visualisation under UV light.

2.2.2 Molecular biology methods

2.2.2.1 Preparation of competent cells

A single, well-isolated colony of DH5α was inoculated into 10 ml of LB media and incubated at 37°C overnight with shaking at 200 rpm on a GIO GYROTORY® shaker. A 2 ml aliquot of the overnight culture was transferred to 100 ml of pre-warmed ΨB media and grown until the $OD_{550\ nm}$ was between 0.3-0.4. The cells were chilled for 5 min on ice followed by 4°C

centrifugation at 2,800xg for 10 min. The pellet was resuspended in 15 ml of chilled TfBI. The cells were centrifuged at 4°C at 2,800xg for 10 min and the pellet was resuspended in 2 ml of chilled TfBII. Aliquots of 200 µl were snap-frozen using dry ice and ethanol and stored immediately at –80°C

2.2.2.2 Heat-shock transformation

An aliquot of competent cells was thawed on ice for 10 min. Then DNA was added to the microfuge tube and further incubated on ice for 30 min. Cells were heat-shocked at 37°C for 2 min, then held on ice for 3 min. One ml of LB was added to this mixture and incubated at 37°C for 90 min. Cells were concentrated by centrifuging for 30 s and the pellet was resuspended in 200 µl of LB media. Cells were plated on selective media and grown at 37°C.

2.2.2.3 Ligations

Ligations were carried out at room temperature for 16 h in a total volume of 10 µl. A 6:1 insert to vector ratio was incubated at 45°C for 5 min followed by 5 min on ice to which 1 µl of 10x ligation buffer and 1 µl of ligase were added. When the mixture reached room temperature, 1 µl of 30% PEG 8000 was added for blunt ligations.

2.2.2.4 Oligonucleotide-directed mutagenesis

2.2.2.4.1 Single strand DNA

Growth of Uracil-Containing Phagemid

The CJ236 strain carrying pTZ19U containing the target gene was obtained from 80% glycerol stock solution and streaked on LB agar

containing the appropriate antibiotic and incubated at 37°C overnight. The single colony was picked and grown in 20 ml LB containing suitable a antibiotic overnight at 37°C on a GIO GYROTORY® shaker. This culture was inoculated in 50 ml of 2xYT media containing the same antibiotic and grown to an $OD_{600\ nm}$ of 0.3, then M13K07 helper phage was added to obtain a multiplicity of infection of around 20. The sample was incubated with shaking at 37°C for 1 h, then kanamycin was added to a final concentration of 70 μg/ml with continued incubation for 5-6 h. This culture was transferred to two round-bottomed centrifuge tubes and centrifuged at 17,000xg for 15 min, then the supernatant containing the phagemid particles was transferred to a fresh centrifuge tube and centrifuged again at 17,000xg for 15 min at 4°C. The second supernatant was transferred to a new centrifuge tube to which 150 μg of RNase A was added and incubated at room temperature for 30 min. To the phagemid-containing supernatant, ¼ volume of 3.5 M ammonium acetate/20% PEG-6000 was added and incubated on ice for 30 min, then the solution was centrifuged at 17,000xg for 15 min to collect phagemids. The supernatant was discarded and the pellet was drained well and resuspended in 200 μl of high salt buffer. The phagemids solution was transferred to 1.5 ml microfuge tube and chilled on ice for 30 min and centrifuged at 12,000xg to remove insolubles and the supernatant was transferred to a fresh microfuge tube.

Extraction of Phagemid DNA

The total phagemid pellet was extracted twice in an equal volume of neutralized phenol, vortexed for 1 min and centrifuged at 12,000xg

for 2 min. The upper phase was then transferred to a fresh tube and extracted once in an equal volume of phenol/chloroform (chloroform should contain 1/25 volume of isoamylalcohol). This sample was vortexed for 1 min and centrifuged at 12,000xg for 2 min. The upper phase was transferred to a fresh tube and 3-4 times extracted in chloroform/isoamylalcohol, then the upper phase was transferred to a fresh tube. The extraction was repeated by addition of 100 µl of TE buffer to each of the previous tubes to increase the yield of phagemid DNA. To the pooled aqueous phase, the 1/10 volume of 7.8 M ammonium acetate and 2.5 volumes of absolute ethanol were added and incubated at –80°C for 30 min. The sample was centrifuged at 12,000xg for 15 min at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in 20 µl of TE buffer. One µl of single-strand DNA was checked on 0.8% agarose gel.

2.2.2.4.2 Synthesis of the mutagenic strands

Phosphorylation of the oligonucleotide

The 10 µl of 20 pmol/µl mutagenic oligonucleotide, 3 µl of 1 M Tris-HCl (pH 8.0), 1.5 µl of 0.2 M $MgCl_2$, 1.5 µl of 0.1 M DTT, 13 µl of 1 mM ATP, 1 µl of sterile milli-Q water and T_4 polynucleotide kinase were added to 0.5 ml microfuge tube. This mixture was incubated at 37°C for 45 min and the reaction was stopped by heating at 65°C for 10 min.

Annealing of the mutagenic oligonucleotide

Single-stranded DNA (0.2 pmol), 6-9 pmol of mutagenic oligonucleotide, 1 µl of 10x annealing buffer and sterile milli-Q water to make

a total volume of 10 µl were added in a 0.5 ml microfuge tube. A control reaction was prepared containing all of the solutions except the mutagenic oligonucleotide. The mixture was heated to 95°C then allowed to cool at room temperature and then placed on ice.

Synthesis of the complementary strand

One µl of 10x synthesis buffer, 1 µl of T_4 DNA ligase and 1 µl of T_7 polymerase were added to the reaction mixture still on ice and further incubated on ice for 5 min followed by an incubation at 25°C for 5 min and at 37°C for 30-60 min. After incubation, 90 µl of TE stop buffer was added to the sample. Ten µl of this reaction mixture and 3 µl of the no primer control were transformed into *E. coli* strain DH5α by heat-shock transformation.

2.2.2.5 Alkaline lysis minipreparation

A single, well-isolated colony of the transformed DH5α strain was inoculated into 2 ml of LB media containing appropriate antibiotics and grown overnight with shaking at 200 rpm on GIO GYROTORY® shaker. The entire culture was centrifuged for 30 s at 12,000xg. The pellet was resuspended in 100 µl of Solution 1 to which 2 mg/ml lysozyme was added and incubated at room temperature for 5 min. The sample was placed on ice for 5 min, 200 µl of Solution 2 was added, shaken vigorously and further incubated on ice for 5 min. This was followed by the addition of 150 µl of Solution 3 and another 5 min incubation on ice. After centrifuging for 5 min at 12,000xg, approximately 350 µl of the supernatant was transferred to a fresh microfuge tube and extracted with 100 µl of a 1:1 chloroform/TES-equilibrated phenol mix

(chloroform should contain 1/25 vol. isoamyl alcohol). The sample was centrifuged for 5 min at 12,000xg, then the 300 µl upper phase was transferred to a new microfuge tube. After that 180 µl of 100% isopropanol was added to this sample and incubated at room temperature for 10 min. The sample was centrifuged for 10 min at 12,000xg then the supernatant was discarded and the pellet was washed with 800 µl of cold 70% ethanol and dried at 37°C. The final pellet was resuspended in 50 µl of TE buffer to which 20 µg/ml of RNase was added. One µl of this sample was then checked by running on a 0.8% agarose gel.

2.2.2.6 Sequencing minipreparation

A single, well-isolated colony of the transformed DH5α strain was inoculated into 4.5 ml of LB medium containing appropriate antibiotics and grown for 10 h at 37°C by shaking at 200 rpm on a GIO GYROTORY® shaker. The entire 4.5 ml culture was centrifuged in a microfuge tube. Solution1 (200 µl) was used to resuspend the pellet followed by 5 min incubation at room temperature. This sample was then incubated on ice for 5 min with 300 µl of cold Solution 2. This was followed by the addition of 300 µl of Solution 3 and kept on ice for further 5 min. After centrifuging for 5 min at 12,000xg, the approximately 700 µl of the supernatant was transferred to a fresh microfuge tube to which 2 µl of 10 mg/ml of RNase A was added. The sample was incubated at 37°C for 20 min. An equal volume of chloroform containing 1/25 isoamyl alcohol was used to extract the DNA twice. The DNA in the aqueous phase was then precipitated with 700 µl of isopropanol. The

aqueous phase was incubated for 10 min at room temperature and then centrifuged at 12,000xg for 10 min. The DNA pellet was washed by the addition of cold 70% ethanol. After drying the pellet at 37°C, the pellet was resuspended in 32 µl of sterile double-distilled water, 8 µl of 4 M NaCl and 40 µl of 13% polyethylene glycol 8000 and incubated on ice for 1 h. The sample was centrifuged at 12,000xg for 20 min. The pellet was washed by the addition of 70% ethanol and dried at 37°C for 30 min. The final pellet was resuspended in 20 µl of TE buffer. One µl of this sample was then checked by running on a 0.8% agarose gel. The DNA was then analyzed for its sequence.

2.3 Method for *Synechocystis* sp. PCC 6803

2.3.1 General methods

2.3.1.1 Growth conditions

Synechocystis sp. PCC 6803 strains were grown photoautotrophically at 30°C under constant illumination of 25 µE/m^2/s in BG-11 medium with or without phosphate as required, with the appropriate antibiotics. An aquarium pump supplied the liquid culture with filtered air. For short-term usage, the strains were maintained on BG-11 medium plates with appropriate antibiotics. For long-term usage, the strains were stored at -80°C in BG-11 liquid medium containing 15% glycerol. In some experiments acetyl phosphate was added to BG-11 without phosphate at a concentration of 20 µM.

2.3.1.2 Photoautotrophic growth curve measurements

The glassware used for phosphate-limiting experiments were rinsed with 36% HCl to remove residual phosphate. One hundred and fifty ml starter cultures were set up in phosphate-containing BG-11 and grown for 3 days. The cultures were divided into two 50 ml Falcon tubes and centrifuged at 2,760xg at 30°C for 10 min. The cell pellets were resuspended in phosphate-containing BG-11 and phosphate-limiting BG-11, respectively and centrifuged. This step was repeated once. The pellets were resuspended in 2 ml of phosphate-containing BG-11 and phosphate-limiting BG-11, respectively and the $OD_{730\ nm}$ was measured. The cultures were set up in a volume of 150 ml in phosphate-containing BG-11 and phosphate-limiting BG-11 to a starting $OD_{730\ nm}$ of 0.05. The cultures were sampled every 24 h to measure the $OD_{730\ nm}$. The cells were diluted to an $OD_{730\ nm}$ of less than 0.4 for each measurement (Hirani, 2001).

2.3.1.3 Absorption spectra

To follow absorption maxima of chlorophyll *a* and phycobilins, spectra were generated on a Jasco V550 uv/vis spectrophotometer. Whole cells were used at an $OD_{800\ nm}$ of 0.3 and scanned from 800 to 400 nm. To correct for the scattering, a piece of cellotape was put on either side of the sample cuvette holder and the reference cuvette holder such that the cellotape covered the light path. The cuvette holder was covered before setting up the reference with BG-11 (Hirani, 2001).

2.3.1.4 Alkaline phosphatase assay

Cells were grown in phosphate-containing BG-11 or phosphate-

limiting BG-11. A culture of 100 ml was harvested after 24 h and resuspended in 1 ml of Solution 1. The $OD_{730\ nm}$ was measured for each resuspended pellet. Sixty microlitres of cells were mixed with 910 µl of Solution 1. The substrate p-nitrophenyl phosphate was added to a final concentration of 3.6 mM in a total volume of 1 ml reaction mixture. The sample was incubated at 37°C for 20 min. The reaction was stopped by the addition of 150 µl of 4 M NaOH. Sample was centrifuged at 13,400xg for 5 min and the $OD_{400\ nm}$ of the p-nitrophenol in the supernatant was measured (Hirani, 2001).

2.3.2 Molecular biology methods

2.3.2.1 Transformations

The *Synechocystis* sp. PCC 6803 strains were transformed with the various plasmids by using the method of Williams (1988). The liquid cultures were grown for 3-4 days and the cells were collected by centrifuging at 2760xg for 10 min. Phosphate-containing BG-11 was used to resuspend the cells to an $OD_{730\ nm}$ of 2.5. The cells were then mixed with 2-10 µg of DNA in a sterile test-tube. The total volume was made up to 0.5 ml with BG-11 liquid media. A mixture of the cells without DNA was used as a negative control. The cells were placed under constant illumination (25 $\mu E/m^2/s$) for 6 h at 30°C and shaken regularly. Cell aliquots of 200 µl were grown on a sterile filter on a BG-11 plate for 12 h under constant illumination at 30°C. The filter membrane was then transferred to BG-11 medium plates containing appropriate antibiotics. Isolated colonies were streaked on BG-11 containing appropriate antibiotics.

2.3.2.2 DNA extraction

Total DNA was extracted from exponentially growing cultures of *Synechocystis* sp. PCC 6803 by using the method of Williams (1988). The appropriate strain was grown in a 300 ml culture for 4 days. The cell pellet was collected by centrifuging the culture for 5 min at 7,520xg. The cell pellet was resuspended in 2 ml of saturated NaI per gram of cell pellet and incubated at 37°C for 20 min. The NaI suspension was diluted with water and centrifuged at 4,000xg for 10 min. The cells were then resuspended in 8 ml of TES solution (50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0) and 50 mM NaCl) and 1.25 ml of 50 mg/ml lysozyme per gram of cells and incubated at 37°C for 20 min. The lysate was mixed gently with 1ml of 10% N-lauryl sarcosine per gram of cells and incubated at 37°C for 20 min. The lysate was twice extracted by adding an equal volume of equilibrated phenol and mixed gently for 60 min. The sample was centrifuged for 10 min at 4,000xg and the upper phase was transferred to a clean tube. An equal volume was added to this aqueous phase and mixed for 45 min before centrifuging at 4,000xg for 10 min. The upper phase was transferred to a new tube and 1/10 volume of 3 M sodium acetate (pH 5.0) and 2.5 volumes of absolute ethanol were added to precipitate the nucleic acid overnight at –20°C. The tube was centrifuged at 4,000xg for 10 min. The pellet was washed with 70% cold ethanol and centrifuged for 5 min at 12,000xg. The final pellet was dried at 37°C and resuspended in 300 µl of TE buffer.

2.3.2.3 Southern analysis

Blotting

Capillary transfer of DNA from 0.8% agarose gel to GENE-Screen® hybridization transfer membrane was carried out according to the method of Sambrook et al. (1989) with the following changes. The gel was washed in 0.25 M HCl for 10 min, rinsed in the distilled water and incubated in transfer buffer for 30 min. After transfer, the membrane was soaked in 2xSSC for 2 min and partially dried under a lamp at room temperature.

Labeling of probes

The DNA was denatured by boiling for 10 min then rapidly cooled on ice for 5 min. A 10 µl aliquot of OLB solution, 2 µl of 10 mg/ml BSA, 5 U Klenow, and 2 µl of 10 µCi/µl[α-^{32}P]CTP was made up to 50 µl with milli-Q water. The previous solution was added to the denatured probe and incubated at room temperature for 3 h. The reaction was stopped by addition of 120 µl of stop solution. The probe was centrifuged through a sephadex G-50 column at 2,760xg for 2 min to remove the unincorporate [α-^{32}P]CTP. A 1 µl aliquot of 10 mg/ml salmon sperm DNA was added to the probe prior to denaturation by boiling for 10 min then cooling on ice for 5 min.

Pre-hybridization, hybridization and washing

The membrane was placed into a plastic box containing pre-hybridization mix and incubated at 37°C for 6 h. The denatured probe was added to this solution and the membrane was incubated overnight at 37°C. The membrane was washed twice for 5 min at room temperature in 2xSSC, twice

at room temperature for 30 min each in 2x SSC with 0.2% SDS, and twice in 0.1% SSC at room temperature for 30 min each. The membrane was exposed at -80°C with Kodak Imaging Film placing in cassette.

2.3.2.4 RNA extraction

The RNA was extracted from exponentially growing cultures of *Synechocystis* sp. PCC 6803. The starter culture was grown in phosphate-containing BG-11 for 3 days. The liquid culture was centrifuged at 4,000xg for 7 min at 25°C. The cell pellet was resuspended in phosphate-containing BG-11 and phosphate-limiting BG-11, respectively. The culture was set up in a volume of 150 ml in phosphate-containing BG-11 and phosphate-limiting BG-11, respectively with the appropriate antibiotics to starting $OD_{730\ nm}$ of 0.05 and grown for 2-3 days until the culture had final $OD_{730\ nm}$ of 0.6. The cell pellet was collected by centrifugation and frozen in the liquid nitrogen and stored at –80°C. The frozen pellet was resuspended in 2 ml 65°C phenol then NAES (50 mM Sodium acetate (pH 5.1), 10 mM EDTA, 1% SDS) was added. All the following steps were done on ice, the 4 ml cell suspension was aliquoted to 4 tubes containing glass beads. The tubes were agitated in a bead beater for 3x20 sec at 5,000 rpm. The samples were centrifuged at 4°C for 10 min at 14,000xg to separate phases. The top phase was transferred to a new microfuge tube and subjected to two phenol:chloroform and one chloroform extraction. The mixture was vortexed and centrifuged at 14,000xg at 4°C for 10 min. The RNA was precipitated by adding 2 volumes of cold absolute ethanol and incubated for 20 min at -20°C. The solution was centrifuged at 4°C for 10 min

at 14,000xg. The pellet was dried at 37°C. The pellet was resuspended in 250 µl DEPC-treated water followed by the addition of 62.5 µl of 10 M LiCl and left on ice overnight. The solution was centrifuged for 15 min at 4°C. The pellet was rinsed in 0.5 ml of 2 M LiCl and centrifuged for 15 min at 4°C and dissolved in 400 µl DEPC-treated water. The solution was precipitated with 2 volumes of ethanol and 1/10 volume of sodium acetate (pH 6.0) and frozen at –20°C for 20 min. The sample was left at room temperature and centrifuged for 10 min at 14,000xg. The RNA pellet was dried and resuspended in 50 µl DEPC-treated water and stored at -80°C.

RNA samples were analysed in 1.5% MOPS agarose gel containing 2.0% formaldehyde. The agarose gels were run in 1x MOPS buffer. One µg of RNA was resuspended in 2 volumes of RNA loading buffer. The sample was denatured at 65°C for 10 min and loaded immediately. A Geldoc® enabled RNA visualisation under UV light.

2.3.2.5 First-strand cDNA synthesis

The RNA sample was treated with DNase prior to RT-PCR. One unit of DNase I, 1 µl of 10x DNase I reaction buffer were added to 1 µg of RNA sample. The reaction was incubated for 15 min at room temperature. DNase I was inactivated by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture and heated for 10 min at 65°C. After this step the RNA sample was ready for use in reverse transcription. One µl of 500 µg/ml reverse primer, 1 µl of 10 mM dNTP Mix and sterile water to make a total volume of 12 µl were added to the DNase I treated RNA sample. The mixture was heated

to 65°C for 5 min and quickly chilled on ice. Four microlitres of 5x First-Strand Buffer and 2 μl of 0.1 M DTT were added to the mixture and then incubated at 42°C for 2 min. Then 1 μl of 200 units of SUPERSCRIPT II was added and incubated for 50 min at 42°C. The reaction was inactivated by heating at 70°C for 15 min. The primer was removed from the reaction product by a PCR product purification column. The cDNA was then ready for use as the template for amplification in PCR.

2.3.2.6 Rapid Amplification of 5' cDNA Ends (5'-RACE)

The sll0337 cDNA had a 3' poly(dA) tail added using terminal transferase. The reaction mixture contained 10 μl of 5x reaction buffer, 5 μl of 25 mM $CoCl_2$, 1 μl of 10 mM dATP, 15 μl of purified cDNA, 18 μl of sterile water and 1 μl of terminal transferase. The mixture was incubated at 37°C for 30 min then heated at 70°C for 10 min for inactivation. The poly(dA) tail cDNA was used for first round PCR by using the polyT forward primer and sll0337 reverse primer 1. The first PCR product was then amplified using anchor forward primer and sll0337 reverse primer 2. The PCR fragment was cloned into pGEMT-Easy. The universal primers were used for sequencing.

2.3.2.7 Reverse transcription(RT)-PCR

The sll1299 and slr2132 cDNA were amplified by using forward and reverse primers. In addition, *psbB* cDNA was also amplified to show constitutive gene expression.

2.3.3 Partial purification of acetate kinase and its properties

2.3.3.1 Acetate kinase assay

The reaction mixture 0.20 ml contained 50 mM Tris-HCl (pH 8.3), 50 mM sodium succinate, 5 mM ATP, 5 mM $MgCl_2$, 50 mM sodium acetate and 0.7 M neutralized hydroxylamine. The reaction was started by the addition of enzyme solution, allowed to proceed at 37°C for 20 min. The solution was stopped by addition of 0.3 ml of $FeCl_3$-trichloroacetic acid solution (0.37 M $FeCl_3$, 0.2 M trichloroacetic acid, 0.68 HCl). The $OD_{540\ nm}$ of ferric acetyl hydroxamate complex was measured. For reagent calibration, aqueous solution containing various known amounts of acetyl phosphate were added to the reaction mixtures instead of enzyme solution. (Yu, et al., 2001)

2.3.3.2 Cell extraction

Approximately 3 g of *Synechocystis* sp. PCC 6803 cell pellet grown in BG-11 was resuspended in 10 ml of 50 mM HEPES (pH7.5) containing 5 mM DTT, 1 mM benzamidine, 5 mM $MgCl_2$ and 10% Glycerol. The cells were broken by French pressure cell at 12,000 Psi. The suspension was then centrifuged at 40,000xg for 20 min at 4°C to collect crude enzyme in the supernatant, protein content was determined according to method of Bradford (1976). The 30% ammonium sulfate was added to the crude extract and centrifuged at 10,000xg for 15 min. The supernatant was then subjected to the phenyl-sepharose column chromatography.

2.3.3.3 Phenyl-sepharose Column Chromatography

Approximately 20 ml of commercial phenyl-sepharose was packed into a column. The column was equilibrated with 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT and 10% glycerol containing 1 M ammonium sulfate. The crude enzyme was loaded and allowed to be absorbed. Elution was carried out by linear gradient of 1-0 M ammonium sulfate in 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM DTT and 10% glycerol. The protein content of fractions was monitored by measuring the $OD_{280\ nm}$. The acetate kinase activity was detected as described in section 2.3.3.1. The fraction showing high activity of acetate kinase from this step was collected and analyzed by SDS-PAGE and also for the study of acetate kinase properties.

2.3.3.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular mass of acetate kinase in the denatured state was determined by SDS-PAGE according to Laemmli (1970). For this, the sample was treated with the sample buffer for SDS-PAGE and boiled for 5 min prior to application to the gel and then loaded to a slab gel (10% of separating gel and 5% of stacking gel). The electrophoresis was performed at the constant current of 10 mA per slab. The gel was stained with coomassie blue. The protein molecular weight markers were phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactabumin (14.4 kDa)

2.3.3.5 Staining of acetate kinase activity

After electrophoretic separation in 12% native polyacrylamide gel was placed on a glass plate and covered with two sheets of Whatman paper soaked with staining solution. The paper sheets were placed in close contact to the surface of the gel without any air bubbles and covered with a second glass plate. Colour was developed during incubation at 37°C for 30 min. The staining solution contained the following components (final concentration in 10 ml solution): 40 mM imidazole/HCl (pH 7.3), 20 mM glucose, 4 mM ADP, 10 mM $MgCl_2$, 1.3 mM NADP, 10 mM acetyl phosphate, 0.3 mg hexokinase, 0.15 mg glucose-6-phosphate dehydrogenase, 0.5 mg phenazine methosulfate and 1.35 mg nitro blue tetrazolium were added immediately before use.

2.3.3.6 The properties of acetate kinase

The partially purified acetate kinase was characterized with respect to temperature, pH, substrate specificity, effect of Mg^{2+} concentration, K_m and V_{max}.

2.3.3.6.1 Effect of pH and temperature on acetate kinase activity

The partailly purified acetate kinase was assayed in the reaction mixture with appropriate amounts of enzyme. In each assay, the pH was adjusted with appropriate buffer, i.e. HEPES-KOH buffer for pH 6.5-7.5 and Tris-HCl buffer for pH 8.0-8.5. For the temperature effect, the enzyme was incubated for 20 min at 25, 30, 35, 37, 40, 45, 50°C.

2.3.3.6.2 The kinetic of acetate kinase

The kinetics of the acetate kinase were studied by the addition of varying concentrations of either acetate or ATP. The Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated from the intercepts of x-axis and y-axis of the double-reciprocal plot, i.e., 1/[S] vs 1/[V], respectively.

2.3.3.6.3 The substrate specificity

The substrate specificity of acetate kinase was studied by adding propionate and butyrate replaced acetate, other triphosphorylated nucleotides replaced ATP.

2.3.3.6.4 Effect of the $MgCl_2$ concentration on acetate kinase activity

The partailly purified acetate kinase was assayed in the reaction mixture with appropriate amounts of enzyme by adding various $MgCl_2$ concentrations of 0-10 mM to the reaction mixture.