Functional characterization of a cAMP binding transcriptional regulator, Sll1371 in *Synechocystis* sp. PCC 6803

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

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November, 2019

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(A Central University established in 1974 by act of parliament)

HYDERABAD – 500 046, INDIA

DECLARATION

I **Lingaswamy Bantu** hereby declare that this thesis entitled "Functional characterization of a cAMP binding transcriptional regulator, Sll1371 in *Synechocystis* sp. PCC 6803 submitted by me under the guidance and supervision of **Prof. Jogadhenu S. S. Prakash** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date ;
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CERTIFICATE

This is to certify that this thesis entitled "Functional characterization of a cAMP binding transcriptional regulator, Sll1371 in *Synechocystis* sp.PCC 6803 submitted to the University of Hyderabad by **Mr. Lingaswamy Bantu** bearing registration number **11LTPH17** for the degree of doctor of philosophy in Department of Biotechnology&Bioinformatics, School of Life Sciences, University of Hyderabad. is based on the studies carried out by him under my supervision.

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Details of research paper published in referred journals with the list of authors:

- Singh, D. K., **Lingaswamy, B.**, Koduru, T. N., Nagu, P. P., & Jogadhenu, P. S. S. (2019). A putative merR family transcription factor Slr0701 regulates mercury inducible expression of MerA in the cyanobacterium *Synechocystis* sp.PCC6803. ***MicrobiologyOpen*, e838**.

- Cyclic AMP dependent SyCrp1meadiates inorganic carbon response in *synechocystis* sp.pcc 6803 **(Lingaswamy B. *et.al*, .2019. Manuscript under preparation).**

Presented in the following conferences:

- L.S.Bantu, Subhashini.M, J.S.S.Prakash (2015),"cAMP binding transcriptional regulator,Sll1371 regulates the low Ci inducible transporters in *Synechocystis* sp.PCC 6803 Poster presentation at International conference **"All India Microbiologists Association" 2015** at JNU Delhi, India.

- L.S.Bantu, Subhashini.M. J.S.S.Prakash (2016), "Functional characterization of a cAMP binding Transcriptional regulator, Sll1371 in *Synechocystis* sp.PCC 6803" Poster presentation at **"Frontiers in Life Sciences International Conference" 2016** at University of Hyderabad India.

Further, the student has passed the following courses towards fulfillment of coursework requirements for the award of Ph.D.

Sr.No	**Course code**	**Subject name**	**Credits**	**Pass/Fail**
1	BT 801	Seminar	1	Pass
2	BT 802	Research Ethics & Management	2	Pass
3	BT 803	Biostatistics	2	Pass
4	BT 804	Analytical Techniques	3	Pass
5	BT 805	Lab Work	4	Pass

Supervisor
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Acknowledgements

This Report would not have been possible without the essential and gracious support of many individuals.

I take immense pleasure in thanking ***Prof. Jogadhenu S. S. Prakash****,* ***Laboratory of microbial genes to functions*** *group, who had been a source of inspiration and role model in any given way, for his constant moral and personal support and guidance throughout my doctoral research. The leadership and critical assessment capabilities of* ***Prof. Jogadhenu S. S. Prakash****, allowed me to mobilize my collective knowledge and capacity in the best way possible and created an enabling environment to complete my research work. I thank the present and former Deans, school of Life Sciences,* ***Prof. Dayananda, Prof. Reddanna, Prof. M. Ramanadham*** *and* ***Prof. A. S. Raghavendra****. The present and former Heads, Department of Biotechnology & Bioinformatics,* ***Prof. Padmasree, Prof. Anand Kumar Kondapi Prof. Prakash Babu,and prof Niyazahmed*** *for allowing me to use the facilities of the School and the Department. I sincerely acknowledge the infrastructural support provided by UGC-SAP, DST-FIST, DBT-CREBB to the Dept. of Biotechnology & Bioinformatics and the DBT, Govt. of India for the research fellowship.*

I wish to express my deep sense of gratitude to ***Dr. Iwane Suzuki*** *and* ***Prof. Hagemann. M,*** *who graciously extended their laboratory facilities for Immunogold labeling and photosynthetic measurements.*

This thesis is not only the result of my scientific works but the outcome of a long road and I am glad to acknowledge everyone who has contributed to the build-up of my scientific personality and therefore, to the completion of this thesis.

My heartfelt thanks are also due to my colleague scholars of Microbial genes to functions group ***Dr. P. Bhavani Devi, Dr. P. Sankara Krishna, Dr. M. Subhashini, Dr. P. V. P. Sai Arun, B. Radha Rani, Mr.Deepak Kumar Singh, Afshan Srikumar, Dr. Reetika Chaurasia, D. Somasekar, Jahnabi Ramchiary,***

Pankaj Leonard Tirkey, Ranjana, Shubhangi Padhi, Ramana Murthy for their timely help and constant encouragement.
I would like to thank all my dearest friends ***Dr. Rambabu Undi, Afshan srikumar, ch.Swathi,Deepak kumar singh, M.sudhindrakumar, Srinivaschary, Dr. Damuka Naresh, Dr. Santosh, Swroop Vanka, Durgesh, Elsin Raju, Dr.Chaitanya,Ravi*** *and* ***Uha akola*** *who made my stay always cheerful in HCU.*

Eight long years in the same place is for sure a long time and with time come friends. I thank all my friends at University of Hyderabad for making my stay a delightful one.

This dissertation work at Laboratory of Microbial genes to functions (MGF) group could never run smoothly without all the help I got from lab helpers. I wish to thank, ***Ch. Sridhar Yadav*** *and* ***Manohar*** *for their assistance in the lab.*

I am thankful to all my batch mates and other research scholars of the School of Life Sciences for their timely help.

I highly acknowledge the efforts of ***Rajashekar, Murali, Rahul, Shekar, Mohan Rao***, ***Sudarshanam*** *and other non-teaching staff.*

I would like to express my special thanks of gratitude to my parents ***B Ramulu*** *and* ***B.lakshmi Devamma*** *as well as My sister* ***Shobarani*** *and my brother* ***B Krishna*** *who gave constant sapport during my phd.*

I always thankfull to my inspiring personality the great leader ***Dr B.R ambedkar*** *who provided the opportunity to complete my studies upto phD.*

Saving the most important for the last, I would like to express my heartfelt thanks to my guide, Prof. J. S. S. Prakash and his family for their blessings, wishes, support and constant encouragement throughout my Ph.D. tenure.

Thank you
Lingaswamy Bantu

ABBREVIATIONS

µg	:	microgram
µM	:	micromolar
°C	:	degree centigrade/degree Celsius
Abs	:	absorption
AC	:	Adenylate Cyclase
ATP	:	adenosine tri phosphate
cAMP	:	cyclic adenosine mono phosphate
BiC	:	Bicarbonate Transporter A
BCT-1	:	Bicarbonate transporter CmpA-D
Bp	:	base pair (bp)
BSA	:	bovine serum albumin
CA	:	Carbonic anhydrase
CCM	:	Carbon Concentrating Mechanism
CO_2	:	Carbon dioxide
CRP	:	cyclicAMP receptor protein
DEPC	:	diethylpyrocarbonate
DMSO	:	dimethyl sulfoxide
DNA	:	deoxy ribonucleic acid
dNTPs	:	deoxy nucleotide triphosphates
DTT	:	dithiothreitol
EDTA	:	ethylene diamine tetra acetic acid
gm	:	gram
h	:	hour(s)
HEPES	:	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt, N-(2- Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) sodium salt
IgG	:	immunoglobulin G
IPTG	:	isopropyl β-D-thiogalactoside
kb	:	kilo base pair
kDa	:	kilo dalton
2α-KG	:	2α-ketoglutaric acid
L	:	litre
LB	:	Luria-Bertani
M	:	molar
Mb	:	Mega base pair
mg	:	milligram
min	:	minute

ml	:	milliliter
mM	:	millimolar
Ni-NTA	:	nickel-nitroacetic acid agarose
N-terminal	:	amino terminal
OD	:	optical density
ORF	:	open reading frame
PAGE	:	polyacrylamide gel electrophoresis
PCR	:	Polymerase chain Reaction
2PGA	:	2-phosphoglyceric acid
PMSF	:	Phenylmethylsulfonylfluoride
ppm	:	Parts per million
qRT-PCR	:	quantitative real time PCR
RNA	:	ribonucleic acid
rpm	:	revolutions per minute
RT-PCR	:	reverse transcriptase-polymerase chain reaction
RuBiSCo	:	Ribulose bisphospho Corboxylase
SbtA/B	:	Sodium dependent bicarbonate transporters A/B
SDS	:	sodium dodecyl sulphate
Sec(s)	:	seconds
SEM	:	scanning electron microscopy
TBS	:	Tris Buffer saline
TE	:	Tris-EDTA
TEM	:	transmission electron microscopy
Tris	:	Tris-(Hydroxymethyl) aminoethane
V	:	volts

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1. INTRODUCTION

1. INTRODUCTION

Oxygenic photosynthesis – Cyanobacteria

Photosynthesis is the essential photochemical reaction on earth which had led to the oringin of an advanced life form. In the primitive environment the photosynthetic reaction was carried out by photosynthetic microscopic organisms, utilising H_2O, H_2S or ferrous iron [1]. Later after evolution of cyanobacteria on earth the oxygenic photosynthesis was performed by light driven splitting of water, electron transport to reduce NADP and simultaneous production of ATP by photophosphorylation. The reduced NADPHs are used for the reduction of CO_2 in Calvin cycle[2]. Cyanobacteria, important members of global ecosystem are prokaryotic organisms which perform oxygenic photosynthesis like higher plants. Every year they are responsible for the production of up to 30% atmospheric oxygen by actively performing photosynthesis [3].

Photosynthesis is the process of synthesis of organic compound in presence of light (as energy source) by utilizing CO_2 and water, mainly it is performed by green plants and photosynthetic bacteria like cyanobacteria. Photosynthesis consists of two phases; one is light reaction and other one is dark reaction. Light reaction occurs in the thylakoid membranes, which are present in the plant chloroplasts and in cyanobacterial cells. In the light reaction, NADPH and ATP are generated through photolysis of water which occur at PSII complex. The NADPH and ATP are utilized for CO_2 fixation in the dark reaction[4]. Cyanobacteria form different self-maintaining groups, indicating the flexible physiology and resistance towards environmental stress. It is well-known fact globally that cyanobacteria contribute greatly to primary productivity on earth [5]. Active performance of the photosynthesis mainly depends on the inorganic carbon availability. Cyanobacteria obtain inorganic carbon through the air in natural environment or through the liquid medium in the form of inorganic bicarbonate in laboratory conditions. In higher plans it is difficult to resolve many biological question thus cyanobacteria serving as a model organism to reseach many biological aspects. Many cyanobacterial strains are naturally transformable and they take in DNA during incubation of cells with linear or circular DNA fragments. The cyanobacterium *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*) used as one of the best model cyanobacterium to study abiotic stress responses, because it is not only naturally transformable, also various genetic tools for targeted inactivation of genes, overexpression of proteins and reporter studies, have been developed. *Synechocystis* is a unicellular, (~1.5µm diameter) fresh-water organism. It is not a nitrogen fixing cyanobacterium. It is a mesophilic microorganism can grow both autotrophically as well as heterotrophically in the presence of glucose [6]. *Synechocystis* contains 7 plasmids and 8-10 copies of circular chromosomes. It is the first photosynthetic bacteria to have its genome fully sequenced [7].

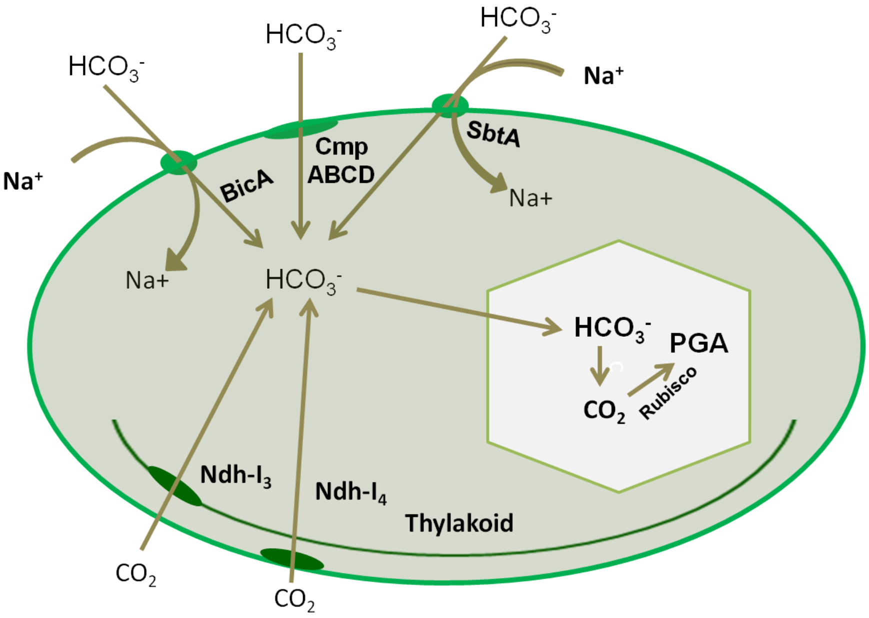
Cyanobacteria and unicellular green algae have acquired a mechanism to actively concentrate available inorganic carbon into the cells when they experience the low CO_2. The molecular mechanism by which cyanobacteria respond to changes in the CO_2 availability is known as Carbon Concentrating Mechanism (CCM). The CCM enhances the photosynthesis and helps to acclimatise to limiting CO_2 condition. The CCM transports and increases the inorganic carbon inside the cell thereby it will be utilised to increase the CO_2 concentration in the carboxysome around the vicinity of the ribulose bisphosphate carboxylase-oxygenase (RuBisCO) [8].

Carbon Concentrating Mechanism

In the ancient time the atmospheric CO_2 level was probably much higher compared to today's suggesting that the CCM would not have formed in the primitive cyanobacteria to perform effective photosynthesis [9] [10]. The present environment on earth has low levels of CO_2 and high levels of O_2 compared to ancient days. This limited CO_2 resources for photosynthesis might have lead to the development of a special mechanism known as Carbon Concentrating Mechanism (CCM) to increase the inorganic carbon levels inside the photosynthetic cyanobacterial cells [11][12]. The CCM helps cyanobacteria to survive under Ci limitation by acquiring both CO_2 and HCO_3^- into the cell even at very low concentration. This specialised mechanism for carbon concentration is known to exist in most of the cyanobacteria, algae, and embryophyta [13]. The performance of photosynthesis in cyanobacteria depends on the inorganic carbon availability and the activity of the main carbon establishing enzyme, Ribulose-1,5 Bisphosphate Carboxylase / Oxygenase (RuBisCO). The CCM fixes the carbon dioxide in a micro-compartment called carboxysome where the RuBisCO is localised, thus not allowing RuBisCO enzyme from oxygenation, thereby enhancing the efficiency of the photosynthesis [14]. The CCM of cyanobacteria involves in active transport of CO_2 and HCO_3^- which results in the accumulation of inorganic carbon source inside the cell, due to which the carbon concentration increases towards the cytosol in the form of HCO_3^- compared to external environment. The HCO_3^- will be utilised as the carbon source in the photosynthesis with the help of the carboxysomes. Carboxysomal membrane being freely permeable for the HCO_3^-, converts it to CO_2 by the catalytic activity of carbonic anhydrase thus the CO_2 levels increases in the carboxysome near RuBisCO.

In cyanobacteria the CCM is involved in various crucial functions namely (1) uptake of dissolved inorganic carbon as CO_2 and HCO_3^-, (2) accumulation of HCO_3^- in cytosol, (3) HCO_3^- passage into carboxysome (4) Conversion of HCO_3^- to CO_2 in vicinity of RuBisCO (5) fixation of CO_2 (6) prevention of leakage of CO_2 from the carboxysome and the cell [15]. The cyanobacteria contain five different Ci uptake systems, among these BicA, SbtA/B, BCT1(CmpA-D) are present on the plasma membrane which have main role in the transport of HCO_3^- and the other two are, $NdhI_3$ localized on the thylakoid membrane, $NdhI_4$ localized on the plasma membrane are involved in the uptake of CO_2 (Figure.1.1).

Figure1.1: Diagramatic representation of the cyanobacterial CO_2 concentrating mechanism



(adapted from James V. Moroney *et.al* 2007)

Figure.1.1. Arrows indicate a variety of active CO_2 and HCO_3^- uptake systems. A hexagonal structure is an internal micro-compartment, called carboxysome. The bicarbonate transporters BicA, CmpA-D, SbtA/B are located on plasma membrane, CO_2 transporters $NdhI_3$ and $NdhI_4$ located on thylakoid and plasma membranes respectively. RuBisCo is localised in the carboxysome a hexagonal structure present in the cyanobacteria.

Bicarbonate Transporters

In cyanobacteria, the protein complexes involved in bicarbonate transportation are well characterised [16]. The BicA, BCT1(CmpA-D) and SbtA/B are the known bicarbonate transporters listed in the Table 1.1. Among the bicarbonate transporters, BCT1 (CmpA-D) is a high-affinity HCO_3^- transporter [17] encoded by a operon called *cmpABCD*, it belongs to the ATP binding cassette (ABC) transporter family [18]. It is highly active under Ci limitation [19][20][21]. It is a uniporter for HCO_3^- having four different protein subunits CmpA, CmpB, CmpC and CmpD. CmpA is a plasma membrane-bound lipoprotein has affinity to bind HCO_3^-. CmpB is a membrane protein, forms a path for transportation of the bicarbonate across the membrane. CmpC and CmpD are the rest two subunits contain ATP binding site and are present towards the cytoplasmic side [22][23]. SbtA is a sodium-dependent bicarbonate transporter. It was identified in *Synechocystis* as a single subunit membrane attached cytoplasm protein [24]. The expression levels of SbtA increase under Ci limiting conditions [25]. In *synechocystis*, SbtA is in operonic structure with SbtB, which is located to the downstream of SbtA and has been shown to have enhanced levels along with SbtA under Ci limitation thus suggesting SbtB might have similar function to that of HCO_3^- transport [21]. SbtA is well conserved, and homologs are known to exist in many cyanobacterial species [16][10][26]. SbtA protein from *Synechocystis* [27] and *Synechococcus* PCC 7002 (hereafter *Synechococcus*) [28] are the only proteins which are well studied at genome level in cyanobacteria. SbtA, a Na^+/HCO_3^- symporter is a homotetrameric protein complex having ten membrane-spanning domains which are hydrophobic in nature and two hydrophilic domains facing towards the cytosol with molecular mass of approximately 160 kDa. [5]. It transports both sodium and bicarbonate depending on the electrochemical sodium gradient [23]. Rapid activation of HCO_3^- transportation is linked with sodium ion levels [29]. It is also suggested by Ogawa and Kaplan that the link of HCO_3^- transports with the sodium ions are may be important for the balance of internal pH [30]. Another transporter, a Na^+ dependent bicarbonate transporter, BicA was first noticed in the *Synechococcus* [28]. Homologues of BicA transporter protein are found in many cyanobacterial species and it is well characterized in *Synechococcus* WH 8102 and *Synechocystis* [31]. In *Synechococcus* the activity of BicA transporter is high under low Ci condition but in *Synechocystis*, the BicA gene is constitutively expressed [21]. BicA is also a main element of Ci uptake in most of the oceanic/marine cyanobacterial species. The growth of the cyanobacteria at low CO_2 conditions leads to the activation of HCO_3^- uptake systems. The uptake of Ci into the cells is an energy reuired mechanism thus the uptake of Ci is tightly regulated [32]. It is well known that phosphorylation of serine-threonine kinase signalling is involved in the HCO_3^- transport, mainly in *Synechococcus* PCC 7942 and *Synechococcus* PCC 7002 under Ci limitation.

Table.1.1. Low CO_2 responsive genes and their transcriptional regulators involved in carbon sensing and balancing in *Synechocystis*

Operons / regulators	***ORF No.***	***Gene symbol***	**Function**
cmpA-D / CmpR(Sll0030)	*slr0040*	*cmpA*	Bicarbonate transport system substrate-binding protein
	slr0041	*cmpB*	Bicarbonate transport system permease protein
	slr0042	*proB*	Probable porin; Major outer membrane protein
	slr0043	*cmpC*	Bicarbonate transport system ATP-binding protein
	slr0044	*cmpD*	Bicarbonate transport system ATP-binding protein
***sbtA-B* /** NdhR(Slr1594)	*slr1512*	*sbtA*	Sodium – dependent bicarbonate transporter
	slr1513	*sbtB*	Periplasmic protein function unknown
NDH-1_3 / NdhR(Slr1594)	*sll1732*	*ndhF3*	NADH dehydrogenase subunit 5
	sll1733	*ndhD3*	NADH dehydrogenase subunit 4
	sll1734	*cupA*	Protein involved in low CO_2 – inducible, high light affinity CO_2 uptake
NDH-1_4 (Constitutive)	*sll0026*	*ndhF4*	NADH dehydrogenase subunit 5
	sll0027	*ndhD4*	NADH dehydrogenase subunit 4
	Sll1302	*cupB*	Protein involved in constitutive low affinity CO_2 uptake

CO_2 uptake transporter

The main inorganic carbon source for cyanobacteria to perform active photosynthesis is CO_2. It enters into the cell by passive diffusion. It is also well known that water channels that are present in the plasma membrane, such as aquaporins support the entry of CO_2 [33]. Earlier studies reported that NDH-1 complex has role in transport of CO_2 through the thylakoid membrane by converting it to bicarbonate, but the actual mechanism is still unclear [27]. In cyanobacteria, there are two forms of NDH-1 multi subunit structures listed in the Table 1.1. NDH-1 is a proton-pumping multi-subunit structure in most of the cyanobacteria that work similar to that of mitochondrial respiratory complex I [34]. Several forms of NDH-1 exist in cyanobacterial species. It has fourteen different subunits (Ndh-ABCEGHIJKLMNOS). The different forms of NDH-1 contain different groups of NdhD and NdhF families [35][36]. But the complex differs in the pattern of NdhF and NdhD family sub units in cyanobacteria [37]. For example, *Synechocystis* genome codes for six NdhD (D1-D6) together with three NdhF (F_1, F_3 and F_4) proteins [38]. The NDH-1 complexes differ in specific hydrophobic members of NdhD and NdhF and also the presence of specific hydrophilic proteins CupA and CupS (for NDH-1MS) or CupB (for NDH-1MS′). The NDH-1L complex contains $NdhD_1$, $NdhF_1$ and recently two more subunits NdhP and NdhQ were found. An another complex, NDH-1L' has $NdhD_2$ instead of NdhD1. NDH-1MS consists of CupA, CupS, NdhD3 and $NdhF_3$ subunits, whereas NDH-1MS' has the subunits of $NdhD_4$, CupB and $NdhF_4$. The NDH-1 variants are known to perform multiple functions. The mutation survey in NdhF and NdhD revealed that NDH-1MS' and NDH-1MS are the two NDH-1 forms and play roles in CO_2 uptake [24]. In *Synechocystis,* the subunits CupA (Sll1734) and CupS (Sll1735) are associated with NDH-1MS and CupB (Slr1302) of NDH-1MS' reported to have role in the conversion of CO_2 to HCO_3^- [25]. Although they are involved in conversion of CO_2 to HCO_3, they do not show any similarity with known carbonic anhydrases. The CupA, CupS and NdhD3(Sll1733) are reported to be up regulated when cyanobacterial cells experience CO_2 limitation, thus from the literature survey it is clear that under limited CO_2 condition NDH-1MS is inducible whereas NDH-1MS' is essentially expressed [25]. However, the functional role of these complexes is still unclear.

Carboxysomes

In cyanobacteria, carboxysome is a proteinaceous polyhedral micro-compartment (organelle) located in the cytoplasm, which harbors Carbonic Anhydrase (CA) and RuBisCO. The RuBisCO is located inside the carboxysomes minimises its exposure to oxygen and thereby reduces the photorespiration. Photorespiration is a phenomenon in which the high oxygen concentration will turn RuBisCO towards the oxygenation reaction and forms glyoxalate compound instead of 3-PGA with CO_2. Bicarbonate, as it can be freely diffusible into the carboxysomes, creating a carbon-rich environment inside the carboxysomes for efficient carbon fixation by RuBisCO [39]. Bicarbonate which enters and gets accumulated in the cytosol is converted to CO_2 within carboxysomes to increase the CO_2 levels near the RuBisCO for enhanced photosynthesis efficiency of cyanobacteria [40]. Based on the similarity of RuBisCO as well as the protein-shell composition of carboxysome, carboxysomes can be divided into two categories such as α-carboxysomes and β- carboxysomes [41]. The α-Carboxysomes are typical part of marine cyanobacteria and chemo-autotrophs. The proteins of shell in α type carboxysomes are products of genes from *cso* operon. The β-carboxysomes found in freshwater cyanobacteria. The CCM genes code for the shell-proteins are dispersed in entire genome [16][39][42][43]. The structure and composition of the β- carboxysomes are well characterised and most extensively studied [44][16][15]. Mainly five proteins CcmK, CcmL, CcmO, CcmM and CcmN are observed in the β – carboxysomes, where as CcmK, CcmL , CcmO or CsoS1 , CsoS2 and CsoS3 are observed in the α-carboxysomes. But in *Synechocystis* additional proteins such as CcmK and CcmO are observed to occur [16][45]. The HCO_3^- freely permeable through the shell protein concealing of carboxysome. The CO_2 convereted from HCO_3^- by carbonic anhydrase inside the carboxysome resulting in CO_2 elevated levels in and around of the RuBisCO to perform carboxylation reaction [46]. When cyanobacteria experience low CO_2 (20-50 ppm), carboxysome-number is increased together with an enhanced RuBisCO activity [47] and such a decrease in carboxysome number leads to an in increase in the uptake of CO_2 and HCO_3^- through the transport systems [48].

Carbonic anhydrase

Carbonic anhydrase is an enzyme localised in the carboxysome catalyses the conversion of HCO_3^- to CO_2, thus increasing the CO_2 levels around the RuBisCO for the carbon fixation in cyanobacteria. The conversion of the HCO_3^- to CO_2 takes place inside the carboxysome. To provide CO_2 continuously to the RuBiSCO, the HCO_3^- molecules enter into the carboxysome from the cytosolic pool of cyanobacteria, where the carbonic anhydrase is catalytically active. Different forms of carbonic anhydrases discovered in different species of prokaryotes, *algae*, *Archaea*, *eubacteria*, plants and animals[49]. There are atleast four different types of CA present and named as α-, β-, ϒ- and δ-type carbonic anhydrases, among these CA, only α -CA and β-CA are predominant in several cyanobacterial species [50]. These carbonic anhydrases are metalloenzymes contain Zinc as cofactor at active site. Zinc is required for the enzymatic activity of the carbonic anhydrase [51]. *Synechocystis* has a gene called *icfA* in its genome, encodes for the functional β-CA. Cys101, His98, Asp41, and Cys39 are consensus amono acids in β-CA. These residues are crucial which binds by covalent co-ordination with cofactor Zn^{2+} at the active site[52]. Mutation studies of *icfA* results in accumulation of the Ci inside the cytosol and carboxysome have shown the impaired cell growth at air levels of CO_2. These reports suggest the important role of the carboxysomal CA in carbon sequestration mechanism of cyanobacteria. β-CA is well characterized in the cyanobacterial species such as *Synechococcus* PCC 7942 and *Synechocystis*. It has been reported, α-CA homolog also exists in the *Synechocystis* genome, which is probably present in the periplasm, but the functional role of the α-CA is not yet known [53]. It is predicted that α-CA takes CO_2 and transports it into the cytosol as HCO_3^- [10]. However, NDH-1 complex is also known to perform functions similar to that of α-CA in cyanobacterial species. NDH-1 complex is known to consists of CupA and CupB or ChpX and ChpY subunits [27][54]. As mentioned above, these proteins are involved in CO_2 transportation and exhibit CA like activity in the cytosol of the cyanobacteria. The available carbon source in the cytosol of cyanobacteria is in the form of HCO_3^- [55].

RuBisCO

Ribulose 1, 5-bisphosphate carboxylase oxygenase catalyses primary course of action of CO_2 fixation in CBB cycle. RuBisCO enzyme is a dimer consists of two monomeric subunits. These two monomeric subunits combine in a specific manner such that C terminal of one monomer binds with the N terminal of the second monomer to form two active sites in the dimeric form of RuBisCO [56]. The active site has different amino acid residues from both the subunits where the carbon fixation, carboxylation reaction and the oxygenation in the absence of the CO_2 occurs. Oxygen and CO_2 are the two substrates which compete for the active site of the RuBisCO. The rate of the reaction weather it is carboxylation or oxygenation with RuBisCO depends on its catalytic activity and also the availability of the inorganic carbon source. They are classified into four groups based on the organisation of the genes in the genomic setup and the subunit-nature.

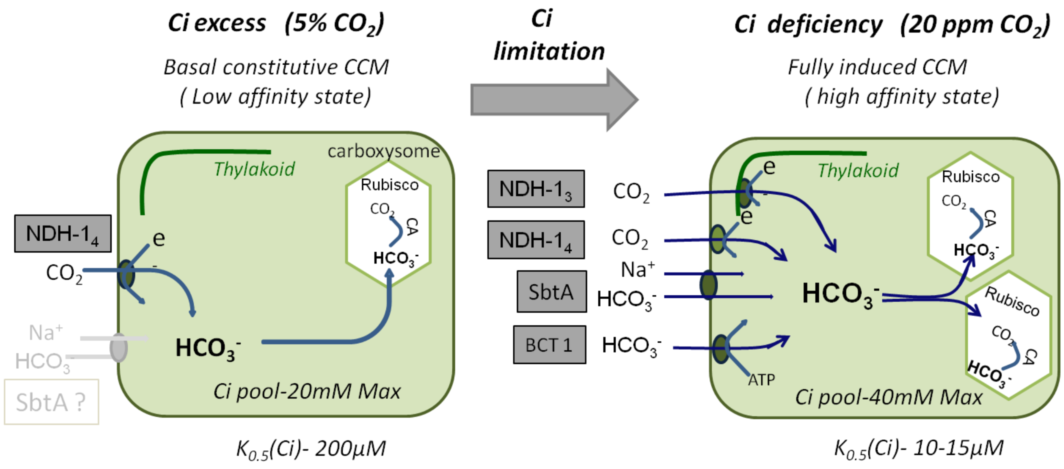
From of the 4 groups, form-I is the most predominant RuBisCO, which is present in bacteria and eukarya [41]. RuBisCO-I exists in L8S8 form (L-large subunit, S-small subunit). Phylogenic studies of the RuBisCO of different autotrophic bacteria indicate that the cyanobacteria contains two forms 1A or 1B RuBisCO[57]. On the basis of RuBisCO form caynobacteria are categorized in two, α-cyanobacteria (e.g. *Prochlorococcus marinus* MEO4) contain form 1A and the one with 1B form RuBisCO is β- cyanobacteria (e.g. *Synechocystis*) [10]. The 1B is further sub-classified into IB and IBc. IBc is the form of RuBisCO present in the cyanobacteria and is associated with carboxysome [58][59]

Regulation of the carbon concentrating mechanism in cyanobacteria

An important feature of the cyanobacterial carbon concentrating mechanism (CCM) is the presence of its functional form of CCM proteins even when cells are grown at the optimum concentration of Ci. The constitutive expression of CCM genes and the activity of associated proteins can be observed when cyanobacterial cells were grown at the optimal level of CO_2 (e.g. 1% CO_2). It clearly suggests that those cells can uptake and utilise both CO_2 and HCO_3^- as carbon source with the moderate affinity transporters. This type of basal expression of CCM can present in the cultures which were bubbled with air [14]. *Synechococcus* PCC7942, which is a freshwater habitat strain, acquires the CO_2 through the constitutive CO_2-uptake system (NDH-I_4). It is well studied and demonstrated that mutation in carboxysome forming genes (e.g. Δ*ccmM)* produces high carbon require phenotype which can grow at high carbon condition and are unable to grow at ambient air levels of CO_2 (0.04% CO_2). The cyanobacterial cells grown at low carbon concentration show actively functional carboxysome system [55][60][61].

Cyanobacteria, when exposed to Ci limitation, exhibits enhanced level of CCM activity and associated increase in the activity of carboxysome [62][47] (Figure 1.2). The changes in the CCM activity at Ci limitation is due to an increase in the RubisCO activity [44]. Due to the changes in the CCM and RubisCO activity, the affinity of CO_2 and HCO_3^- uptake also gets increased [10][15][16][48]. It is experimentally demonstrated that the maximum activity of CCM will attain within 4 hours of transfer of cyanobacterial cells from high carbon condition to low carbon condition (up to 20 ppm CO_2) or air [14] [63]. Sometimes the maximum activity of CCM can reach very fast within 2 hours when cells were grown at high carbon concentration are washed with low carbon media and shifted to low carbon [20].

Figure 1.2. Schematic presentation of the CCM at high and low carbon conditions.



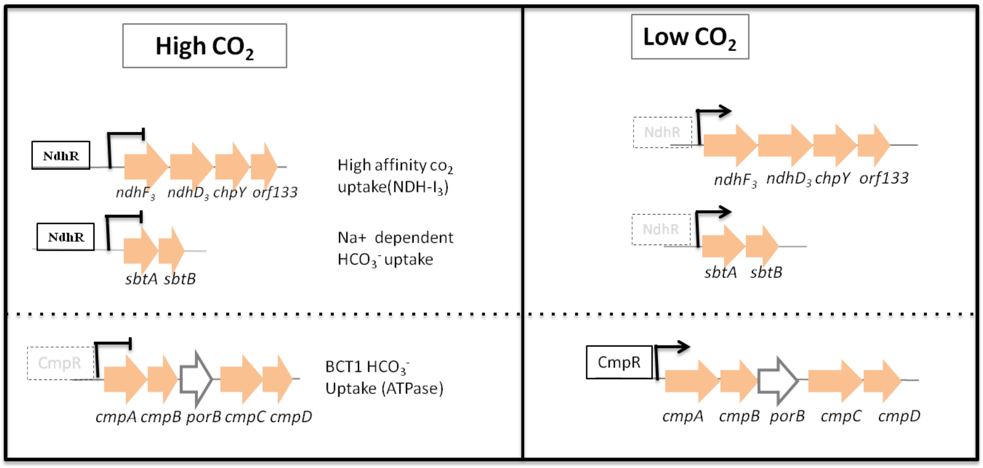
(Adapted from G.D.Price *et.al*,2003)

Figure 1.2. The genes associated with CCM exhibit basal level of expression when cyanobacterial cells experience high CO_2. At high CO_2 only low-affinity carbon transporters are active. At limiting CO_2 conditions, CCM is in fully induced sate, all the bicarbonate transporters are active to uptake inorganic carbon in Ci deficiency state.

Transcriptional Regulation of CCM

Cyanobacteria acclimatise to Ci limitation by inducing the expression of genes coding for high affinity Ci transporters such as NDH-I_3, NDH-I_4 SbtA,(SbtA/B) BCT1(*cmpA-D*), and BicA (high affinity CO_2 and HCO_3^- uptake systems) [26]. Shifting the cultures from high carbon to low carbon condition results in enhanced activity and increased transcription of both CO_2 and HCO_3^- uptake systems [19][20][64][65][66][67]. The high-affinity transporters are the structural components of the CCM and are encoded by genes, which are typically organized as operons. Some of the genes coding for these transporters are constitutively expressed and some are further induced immediately after shift of cultures to Ci limited condtion [21][65]. As mentioned in previous sections, among the Ci transporters the protein components of the high-affinity HCO_3^- transport system, BCT1 are encoded by an operon called *cmpA-D*. It was observed that in *Synechocystis* this operon has been linked with a divergently transcribed gene, called as *cmpR* [68]. The CmpR is an activator of *cmpA-D* operon, belongs to LysR family transcriptional regulators. In general, LysR family contains the transcriptional regulators, which act as both repressors as well as activators [69][70]. Normally, a LysR transcriptional regulator is activated by small effector molecule which binds to it and brings the conformational changes in the DNA binding site[69][70]. CmpR specifically binds to the upstream of the *cmpA-D* operon. In low carbon condition the binding of the CmpR increases to the upstream DNA sequence of *cmpA-D* operon and thereby activating the low-affinity *cmpA-D* operon (Figure 1.3) [71]. The NdhR (also called CcmR) is another transcriptional regulator also belongs to the LysR family and functions as a repressor [21][72]. Deletion mutation of *ndhR* gene has shown alteration in the expression of approximately 20 genes including Ndh-I_3 subunit genes *ndhF3/ndhD3/cupA/sll1735* that code for the high-affinity CO_2 uptake system and *sbtA/B* that codes for the Na^+/HCO_3^- transport systems [21]. Inactivation of *ndhR* leads to de-repression of the above operons in *Synechocystis* [73]. Thus, NdhR functions as a regulator of the high-affinity CCM by negatively regulating the *Ndh-I_3*, and *sb*tA/B operons under high carbon condition (Figure1.3) [21]. It is known that an AbrB family transcriptional regulatory protein called cyAbrB2 also functions as a repressor of NDH-I_3 and SbtA/B [74]. Another LysR family regulatory protein called CbbR is known to regulate the Calvin Benson cycle metabolic pathway. CbbR and its homologs are mainly observed in the proteobacteria has a DNA-binding domain and a sensory domain involved in sensing the adverse conditions [75]. Inactivation of CbbR-like protein, Sll0998 (RbcR), indicates its involvement in the regulation of *rbcLs* operon and is also essential for the survival of the cell [68][76]. Many of the regulatory genes pertain in the modulation of expression of low Ci inducible genes along with carbon transporter genes. Slr1214 (Rre15), a two-component response regulator, sigma factor Sll0184 (SigC), Sll0359 and Sll0822 (CyAbrB- like proteins), IcfG and PII are some of the regulatory genes involved in the regulation of low Ci inducible genes [77][78][79][74]. However, it is not clear as to how the Ci levels are sensed initially to regulate the genes involved in carbon concentrating mechanism.

Figure 1.3. Transcriptional regulation of inorganic carbon transporters in response to CO_2



(Adapted from G.D. Price *et.al*,2008)

Figure 1.3. In high CO_2 condition, NdhR binds to the upstream binding element and represses the transcription of NDH-I_3 and SbtA/B operons. CmpR is a transcriptional activator does not get activated to induce the expression of *cmpA-D* operon. At low CO_2 conditions, NdhR releases from the target binding sites leading to derepression of corresponding genes. CmpR activates the *cmpA-D* operon.

Metabolite signalling of CCM regulation

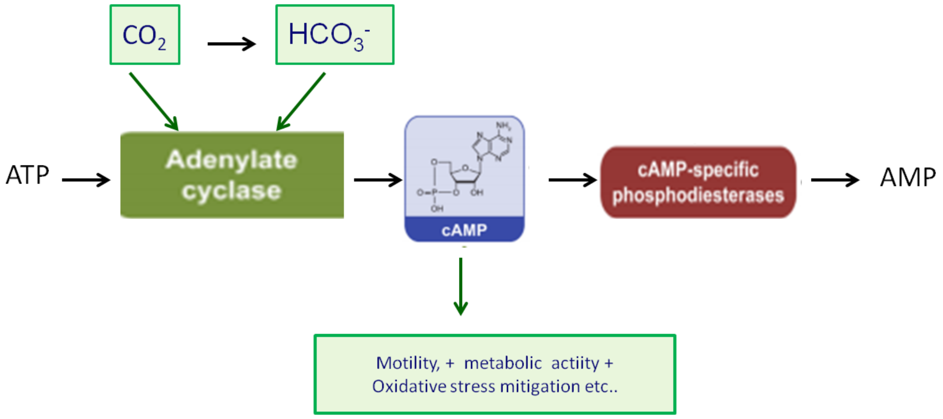
The main role of the CCM is to transport the Ci to the vicinity of the RuBisCO enzyme in the form of CO_2 inside the carboxysome. Under high Ci, most of the carbon is utilised in the carbon fixing metabolic process called CBB cycle to synthesise carbohydrates. When cyanobacterial cells experience low CO_2 condition the carbon available inside the cytosol is consumed in glycolysis and tricarboxylic acid (TCA) cycle to synthesise amino acid. When the cyanobacterial cells are shifted from high CO_2 to low CO_2 conditions the flow of carbon to photorespiration process gets increased [80]. Due to the photorespiration the cellular carbon levels are altered.

These alterations result in a change in the cellular metabolic activities due to which the cellular levels of some metabolites get altered [81]. These changes in the metabolite levels are the signals in response to the Ci limitation enhance the activity of CCM. Two such metabolites RuBP and 2-PG are observed to get accumulated during Ci limitation [82][83]. It was reported that the accumulation of the RuBP and 2-PG enhances the CmpR binding to the target DNA binding site [84]. Thus these small molecules act as a cofactors or activators and upon their binding to CmpR brings conformational change in the protein, consequently leading to activation of CmpR. Similarly, the NdhR another regulator of CCM genes also affected by the metabolite levels in the cell. The intracellular levels of αKG and $NADP^+$ are known to decrease when the cells are shifted from high Ci to low Ci. The Surface Plasmon Resonance (SPR) study revealed that in the presence of the $NADP^+$ and α-Ketoglutarate (α-KG) the binding efficiency of NdhR to its target DNA binding site increases under excess Ci concentrations in the cells, thus indicating that these molecules act as co-repressors which bind to the ndhR through their allosteric interactions [64]. These studies suggest that metabolites are the main signaling molecules for the activation of a CCM system.

The role of HCO_3^- and cAMP levels in cyanobacteria

Signalling mechanism(s) through which cyanobacteria sense low levels (fluctuations) in CO_2 / HCO_3^- concentrations is not yet known. Various research groups have made suggestions on possible sensory mechanisms of Ci fluctuations in cyanobacteria; (i) fluctuations in the redox state of electron transport chain could be a signal [85] (ii) changes in metabolite concentration due to the photorespiratory mechanism as mentioned above [86] and (iii) directly sensing the internal low Ci levels [28]. It is also suggested that cells may sense the low Ci through HCO_3^- levels in the cells. Recent studies have shown that HCO_3^- activates the adenylate cyclase(ACs) which is involved in the formation of cAMP from ATP. The cAMP is a global signalling molecule involved in cAMP-mediated signal transduction [87][88]. In the mammalian systems, the HCO_3^- has been demonstrated to be involved in the stimulation of soluble adenylate cyclase (sAC) [89]. Cyanobacteria also has sequence similarity in AC domain of sAC [89]. In cyanobacteria, the activation of AC by HCO_3^- is conserved, it has been also reported that HCO_3^- regulates the AC in *Spirulina platensis* [90]. The ACs are classified into six types, Class I, II, III, IV, V, VI. The cyanobacterial ACs share homology with the catalytic domain of eukaryotic sACs and are comes under class III type. The CyaC of *Anabaena* belongs to the class III adenylate cyclase. In this type of adenylate cyclases an active-site (Asp→Thr) polymorphism has been proposed as a site for HCO_3^- responsive stimulation [91]. The *Synechocystis* genome contains two genes, *sll1161* and *slr1991* which encode for two adenylate cyclases. Like CyaC from Anabaena, the Slr1991(Cya1) also belongs to Class III and gets activated by inorganic carbon sources [7][91]. Recent studies also shows that adenylate cyclases also gets stimulated or activated in responds to CO_2 [90].

Figure 1.4. External factors effects the adenylatecycalse activity



(adapted from Marco Agostoni *et al*.2014).

Figure 1.4. Inorganic carbon alters the intracellular cAMP levels through altering adenylate cyclase activity, there by altering the cAMP-dependent cellular processes in cyanobacteria. Adenylate cyclase (AC) converts ATP into cAMP. The cAMP is altered to AMP by cAMP-specific phosphodiesterases. The cellular levels of cAMP influence the motility and other other cellular processes.

Cyclic AMP is the most widely available molecule in prokaryotes including cyanobacteria and [92] the variation in the cAMP levels regulate the expression of certain genes [93]. The cAMP is produced from ATP in the presence of catalytic activity of adenylate cyclase (AC). The cAMP molecules can be converted to AMP by the hydrolysing activity of cAMP-specific phosphordiesterases (PDE). The cAMP levels inside the cyanobacterial cell are regulated by the light. Compared to the red and far-red light, blue light stimulates the formation of cAMP in *synechocystis*[94]. and *Microcoleus chthonoplastes* PCC 7420[95]. Whereas in case of *Anabaena cylindrica*, far-red light increases the intracellular cAMP levels and red light decreases the cAMP level inside the cell [96]. The cAMP usually functions as a secondary messenger in most of eukaryotic cells, mainly in the mammalian cells but in the case of prokaryotes like *E.coli* and cyanobacteria, it functions not only as second messenger but also acts as an activator or cofactor for some regulatory proteins named as cAMP receptor proteins (CRP) [90]. It is reported that some cyanobacterial genomes such as *Prochlorococcus* and *Synechococcus* lack cAMP receptor proteins to which cAMP can bind to and activate the transcriptional regulator CRP[97]. In *Synechocystis* three putative cAMP binding proteins were identified and they are Slr0842, Slr0593 and Sll1371 (Table1.2) [98]. The cAMP receptor protein of *E. coli* is a well characterised allosterically regulated protein. It is known to function as a transcriptional regulator of several genes [99]. Allosteric proteins get activated, which undergo conformational changes when bound to a small cofactor or activator. The activated CRP binds to its specific DNA binding site thereby regulates the transcriptional activity of the target genes[100] [101]. In *E.coli* all the cAMP related responses are regulated by the CRP proteins, these cAMP receptor proteins directly or indirectly are shown to be involved in the pH regulation, sugar metabolism and taxis [93]. The *synechocystis* CRP protein SyCrp1, a cAMP-receptor protein has a key role in the motility and is also involved in the regulation of pili formation [102]. Mutation in the *sycrp1* has shown changes in the pilli formation and phenotypic changes in pili appearance.

Table 1.2. Putative cAMP binding proteins in *Synechocystis*

S.No	***ORF No.***	***Gene symbol***	***Function***
1	*slr0842*	-	Unknown
2	*slr0593*	*samp*	cAMP binding membrane protein
3	***sll1371***	**SyCrp1**	**Regulation of transcription**

The inorganic carbon sources, HCO_3^- and CO_2 are reported to stimulate adenylate cyclase (AC) enhancing the intracellular cAMP levels in cyanobacteria. Therefore, it is likely that the carbon concentrating mechanism is regulated via intracellular cAMP whose levels are in turn depends on inorganic carbon availability. However, the cAMP binding protein SyCrp1 functional role in response to inorganic carbon limitation is not studied in *Synechocystis.* As the cellular levels of cAMP is affected by the CO_2 availability it is likely that the SyCrp1 might be involved in carbon concentrating mechanism. However the role of SyCrp1 in regulation of CCM has not been studied in detail. To get insights into the role of SyCrp1 in response to C_i conditions and CCM regulation we have chosen to unravel functional role of SyCrp1 in regulation of carbon concentrating mechanism in *Synechocystis* with the following objectives.

2. OBJECTIVES

Proposed objectives

1. Identification of genes regulated by SyCrp1 through reverse genetics in *Synechocystis*.

- Homology modelling and identification of cAMP binding site.
- Targeted inactivation of *SyCrp1* by *in vitro* transposon mutagenesis
- Identification of the genes regulated by SyCrp1 using DNA microarray.
- Quantitative PCR analysis of low Ci inducible genes in Δ*SyCrp1*.

2. Localization of SyCrp1, a transcriptional regulator in *Synechocystis*.

- Heterologous expression of SyCrp1 using *pET-28a*(+) expression vector.
- Localization of SyCrp1 by western blotting and immunogold labeling
- Localization of SyCrp1 at High and low CO_2 conditions
- Binding analysis of SyCrp1 to the upstream of CCM regulators using electrophoretic mobility shift assay.

3. Metabolite profiling of Δ*SyCrp1* under carbon limiting conditions

4. Effect of Δ*sll1371* on cellular physiology.

- Phenotype analysis of Δ*SyCrp1* in comparison with the wild type cells.
- Analysis of photosynthetic performance in the Δ*SyCrp1* in comparison with the wild type cells.
- Changes in the carboxysome number due to mutation in Δ*SyCrp1*

3. MATERIALS & METHODS

***Synechocystis* sp. PCC 6803 GT-1:**

A glucose tolerant *Synechocystis* PCC 6803 strain was initially obtained from Dr. J. G. K. Williams (Dupont de Nemours, Wilmington, DE) was used as wild-type (WT). The wild type cells were grown photo-autotrophically at 34°C in BG-11 medium buffered with 20 mM HEPES-NaOH (pH 7.5) under continuous illumination of 70 µmol photons m^{-2} s^{-1} and shaking at 110 rpm as described previously in (Wada and Murata 1989) [104]. The media to grow the Δ*sll1371* culture, generated by disrupting *sll1371* gene in the genome with kanamycin-resistance gene (Kan^R) cassette, was supplemented with the antibiotic kanamycin (25 µg mL^{-1}) in pre-cultures and the cultures were grown under the same conditions as described for WT. The growth of the culture was frequently monitored by measuring the optical density at 730nm using a spectrophotometer (Thermo Scientific™ NanoDrop 2000). For high CO_2 treatment The wild-type and Δ*sll1371 Synechocystis* cells were bubbled with ambient air until the OD of the cultures reached to ~0.6 OD units at 730nm, and then bubbled with air enriched with 5% CO_2 for high CO_2 treatment, initially the wild-type and Δ*sll1371 Synechocystis* cultures were bubbled with air until the OD of the culture at 730nm reached to 0.6 units considered as low CO_2, then collected the cells and resuspended into a fresh BG-11 medium. The cells were collected before and after the treatments (for high and low CO_2 treatment) were used for the isolation of the cellular proteins or total RNA.

BG-11 media composition:

Following stock solutions were used for preparing the BG-11 medium for maintaining and growing the *Synechocystis* wild-type as well as Δ*sll1371* mutant cells.

Stock 1: 0.3 g of Ferric ammonium citrate, 0.05 g of EDTA sodium salt and 0.3 g of Citric acid was dissolved in 100 ml of milliQ water filter sterilised and then stored in the dark at 4°C.

Stock 2: 30 g of Sodium Nitrate , 0.7 g of K_2HPO_4 and 1.5 g of $MgSO_4.7H_2O$ were dissolved in 1 L milliQ water.

Stock 3: 1.9 g of $CaCl_2.2H_2O$ was dissolved in 100ml of milliQ water and autoclaved.

Stock 4: 2 g of $Na_2CO_3$was dissolved in 100ml of milliQ water and autoclaved.

Stock 5: 32.86g of H_3BO_3- 1.81g of $Mncl_2.4H_2O$- 0.222g of $ZnSO_4.7H_2O$, 0.391g of $Na_2MO_4.2H_2O$, 0.079g of $CuSO_4.5H_2O$ and 0.049 of $Co(NO_3)_2.6H_2O$-, was dissolved in 1 litre of milliQ water.

Stock 6: HEPES-119.15g weighed and dissolved in 750 ml of milliQ water, the pH was adjusted to 7.5 with 2M NaOH, and the volume was made up to 1 litre with milliQ water. All stocks were prepared and stored at 4°C.

Table 3.1: Preparation of BG-11 from stocks

Stock solution	1X	2X
Stock 2	50ml	50ml
Stock 3	2ml	2ml
Stock 4	1ml	1ml
Stock 5	1ml	1ml
Stock 6	40ml	40ml
MilliQ Water	Up to 1000ml	Up to 500ml

Preparation of BG-11 Agar plates

For the preparation of BG-11 agar plates, 2X BG-11 solution and 2X agar agar (3% agar agar Himedia RM301) were prepared and sterilized separately. After the sterilization, the 2X BG-11 solution and the 2X agar agar solutions were mixed together in 1:1 ratio before pouring the plates. Stock-1 (double to the totalvolume of 2X BG-11 in µl) and 100mM $Na_2S_2O_3$ (10ml for 1 lit) , mixed gently and poured up to 25 ml in to a petriplate .

BG-11 antibiotic medium Preparation

All the antibiotic stock solutions prepared were filter sterilized. Whenever required, these antibiotics were added to the BG-11 medium. The final concentrations of kanamycin and chloramphenicol used were 25µg / ml and 20µg / ml respectively.

Escherichia coli

Escherichia coli strain,DH5α was used for molecular cloning and expression and purification various plasmid DNA constructs. For the induction and expression of the recombinant Sll1371 protein *E. coli* strain BL-21 (DE3) was used. These bacterial cultures were grown with appropriate antibiotic(s) in Luria Bertani (LB) broth or LB plates maintained at 37 °C.

Luria and Bertani medium (L.B. MEDIUM)

LB medium was prepared by dissolving yeast extract (5gm), NaCl (10gm) and tryptone (10gm) in 950ml of double distilled water (DDW) and the volume was made upto to 1L with double distilled water(DDW). For preparation of LB-agar plates, agar powder (w/v)(G Biosciences Cat.No.RC1011) was added to the LB solution at a final concentration of 1.5%, followed by sterilization (15 psi for 20 min). For casting the LB agar plates, the media was allowed to cool down to ~50°C before adding the appropriate antibiotics. About 25 ml of LB agar solution was poured into a 90 mm petri-plate and allowed to solidify, after which the plates were stored at 4°C till use.

Preparation of Antibiotics

Stocks of the antibiotics kanamycin, spectinomycin and chloramphenicol were prepared, and filter sterilised using 0.45 µm syringe filter. These antibiotics, when required were added to the LB medium to a final concentration of 25 µg / ml, 20 µg / ml and 20 µg / ml respectively.

Kits, enzymes, chemicals and reagents

The enzymes and molecular biology kits were obtained from MBI Fermentas (Germany) Takara bio (Japan) and Sigma-Aldrich (USA) and the materials were handled as per the manufacturers' instructions. Analytical grade chemicals and reagents were purchased from SRL (India), Himedia (India), Sigma-Aldrich (USA), Agilent Technologies (USA), GE HealthCare (USA), Fermentas (Germany).

Molecular biology protocols

To standardise protocols for genomic DNA isolation, plasmid DNA isolation, digestion, agarose gel electrophoresis, ligation, transformation and competent cell preparation, [103] was referred and used for guidelines.

Plasmid DNA vectors

For TA cloning and blue-white screening experiments, the vector pTZ57R/T also commonly known as TA cloning vector from Fermentas / Bangalore Genei was used. The cloning and the recombinant protein expression studies of Sll1371 was performed by using a prokaryotic expression vector *pET28a(+)*,

Quantification of DNA and RNA:

The concentration of RNA and DNA was checked using a spectrophotometer (Thermo Scientific™ NanoDrop 2000). The OD values were noted by measuring the absorbance at 260 nm for DNA / RNA and 280 nm for protein. A value of $O.D_{260}$ of 1 corresponds to 50 µg/ml for DNA, while $O.D_{260}$ of 1 corresponds to 40 µg / ml for RNA. A value at the ratio of OD_{260}/OD_{280} between 1.8 and 2.0 was considered to be DNA sample without contamination and that of RNA the ratio was considered to be between 1.9 and 2.0. The quality of the DNA and RNA was also checked by running it on the ethidium bromide containing agarose gel electrophoresis.

Oligonucleotides and DNA sequencing analysis

All the DNA oligonucleotides (primers) were synthesised by either Sigma Aldrich or MWG Biotech (Eurofins genomics India Pvt.Ltd Bangalore). DNA fragments cloned into suitable vectors for gene inactivation and/or overexpression were sequence confirmed by di-deoxy termination sequencing reaction (Eurofins genomics India Pvt.Ltd Bangalore)Please write details of the company).

DNA microarray chip

Genome-wide analysis of transcript levels was performed with custom made DNA microarray chips (*Synechocystis* microarray chip with 8 x 15 K format, Agilent, La Jolla, CA) that covered 3611 genes including the genes of all native plasmids of *Synechocystis*. The chips used in these studies were custom made using Agilent's noncontact inkjet technology. The arrays were printed with probes of 60-mer oligomers selected from the 3' end of the genes. On an average, 3 to 5 probes per transcript were present on each array. cDNA was prepared using the fair playIII microarray labelling kit (Cat.No.252009, Agilent, La Jolla, CA). Cy3 and Cy5 dyes (Cat.No. PA23001 and PA25001, GE healthcare) were coupled to the prepared cDNA as per the manufacturer's instructions. Dye coupled cDNA was purified using microspin columns and hybridised to the DNA microarray chip as per the manufacturer's recommendations (Gene expression hybridization kit, Cat No. 5188-5281).

Extraction of total RNA

RNA was isolated from the *Synechocystis* using the method described in Los et al., 1997[104]. The *Synechocystis* wild-type or Δ*sll1371* mutant cells were allowed to grow actively (OD_{730} of 0.4 to 0.6) by constantly bubbling with sterile air. Fifty ml of cultures were killed immediately by addition of equal volume of chilled 5% (w/v) phenol-ethanol solution. The cells were then harvested by centrifugation at 3500 rpm for 5 min at 4^{o}C and the cell pellets thus obtained were flash-frozen in liquid nitrogen and stored at -80^{o}C until used. Cell lysis was performed using hot phenol method by treating the cells with acid phenol (Cat. No. P4682, Sigma-Aldrich) in the presence of a solution containing 50mM Tris-HCl (pH 8.0) , 5mM EDTA and 0.5 % SDS at 70ºC. The obtained nucleic acids were then treated with DNaseI and ProteinaseK to remove any contaminating DNA and proteins in the solution and purified using phenol/chloroform/isoamylalcohol. The purified RNA was then precipitated with 3M NaOAc (pH 5.2) and chilled absolute ethanol and the obtained pellet was dissolved in 20 µl of DEPC.

Generation of *sll1371* mutant

We have generated Δ*sll1371* by insertional inactivation of the *sll1371* gene. A DNA fragment containing the *sll1371* ORF with downstream, upstream flanking region was amplified using PCR with the *sll1371*-TF-F and *sll1371-TF*-R primers (F-5' ATG TCT ATC AAT GCT TAC AAA CTA GCT ACG 3' and R-5'GCG AAG CTT ACC GAG TTT AAA AAC ATG GG G 3'). The resulting 842 bp including upstream and downstream fragment was ligated to a linear TA vector (GeNei™ INSTANT Cloning kit, Cat No: 107416, Bangalore Genie Pvt. Ltd., Bangalore, India) to generate pTsll1371. This construct was used to inactivate the *sll1371* gene by transposon mediated insertional inactivation with kanamycin cassette transposon kit provided by the company(EZ*::*Tn5™<KAN-2> kit,Cat. No. EZ1982K, Epicentre). The plasmid DNA construct in which the *sll1371* ORF was disrupted with the Kan^R cassette was designated as pT*sll1371::kan*^{R}. This construct was used to transform wild type *Synechocystis* cells. Mutant colonies that had developed were subsequently re-plated on the BG11 medium, which contained increasing concentrations of kanamycin and finally maintained on BG-11 plates with 25 µg / ml final concentration of kanamycin. Genomic DNA of the *Δsll1371* mutant cells were isolated and the extent of the replacement of wild-type copies of *sll1371* with the *sll1371::kan*^{R} was checked using primers *sll1371*-TF-F **(**5'-gtac GCTAGCGTGAGCATTATGTTAACCGTCAGC-3'**)** and *sll1371*-TF-R 5'gcat GAATTCCTAAGTCAACTGCTCATTTAACAAAC-3') The mutant thus generated was named Δ*sll1371*.

Preparation of cDNA for DNA microarray analysis

cDNA was prepared using the fair playIII microarray labeling kit (Cat.No.252009, Agilent, La Jolla, CA). Cy3 and Cy5 dyes (Cat.No. PA23001 and PA25001, GE healthcare) were coupled to the prepared cDNA as per the manufacturer's instructions. Dye coupled cDNA was purified using microspin columns and hybridized to the DNA microarray chip as per the manufacturer's recommendations (Gene expression hybridization kit, Cat No. 5188-5281).

DNA microarray analysis

The genome-wide analysis of Δ*sll1371*/WT transcript levels was performed with DNA microarrays as described previously[105]. In short, to perform DNA microarray we used *Synechocystis* DNA microarray chip The labelled cDNAs was used for hybridisation with the DNA microarray, was carried out at 65°C for 16 h. Total 45µl of the hybridisation mix with Cy3 and Cy5 labelled DNA, were allowed to hybridise in a hybridisation chamber. After completion of the hybridisation, the microarrays were washed with 2x SSC (1x SSC is 150 mM NaCl and 15 mM sodium citrate) at room temperature. They were rinsed with 2x SSC at 60°C for 10 min and with 0.2x SSC, 0.1% SDS at 60°C for 10 min and then washed with distilled water at room temperature for 2 min. Prior to scanning, moisture was removed with the air spray. The chip was scanned using Agilent microarray chip scanner (G2505B, Microarray scanner, Agilent technologies).Scanning was performed using green and red PMT at 100% (XDR Hi 100%) as well as at 70%. Feature extraction was done using Agilent feature extraction (FE) software version 9.5.1 as per protocol mentioned in the web site (www.agilent.com/chem/feprotocols). Each gene signal on the microarray was normalised by reference to the total intensity of signals from all genes, except the genes for rRNAs. Then we measured the change in the level of each gene transcript level to the total amount of mRNA.

Quantitative PCR

RNA isolated from wild type and Δ*sll1371* cells were used for cDNA synthesis with the Affinity Script cDNA synthesis kit, following the manufacturer's protocol (Cat No: 600559, Agilent Technologies Inc.). qRT-PCR was carried out using the Power SYBR Green Master Mix kit (Cat No: 4368708, Applied Biosystems, Cat No.F16L, Thermofischer). Each reaction was performed in a 15 µl volume containing 7.5 µl of SYBR green master mix, 0.3 µM of each of the forward and reverse primers and 3µl of diluted cDNA (3 ng/ µl). All reactions were run in triplicate, using a qRT-PCR instrument (Mx3005P, Agilent Technologies Inc.,). The instrument was programmed for 95°C for 10 min, 40 cycles of 30 s at 95°C, 60 s at 60°C and 60 s at 72°C. To check the amplification of the target gene by the corresponding primers, the melting curve was analysed for each reaction. Expression levels of target genes were normalised by using the *16s rRNA* gene as an internal reference. Primers used for qRT-PCR are listed in chapter Primer sequences.

Metabolic profiling, Sample collection and Extraction of polar metabolites:

Metabolite profiling was carried out by GC-MS analysis. A 5 to 10 ml culture containing 1 X10^9 *Synechocystis* cell were harvested at 3000 rpm for 5 minutes. The supernatant was discarded, pellets were frozen in liquid nitrogen and stored at -80^{o}C. A 300 ul of 80 % methanol was added to the freeze-dried pellet followed by the addition 30µl of the ribitol (0.02 % in milliQ water). The sample was mixed thoroughly and agitated for 5 minutes at 70^{o}C. Allowed the samples to reach room temperature and added 200 µl of chloroform followed by agitation for 5 min at 37^{o}C. A 400 µl of water was added to induce phase separation, thoroughly mixed and centrifuged at 14000 rpm for 10 min at 4^{o}C. The upper polar phase (approx 320µl) and lower non-polar phase were collected into different tubes and dried by vacuum centrifugation. The lyophilized polar phase was mixed with 50µl (20mg/ml) of alkoxyamine hydrochloride in pyridine and incubated at 300c for 90 minutes(Carbonyl moieties were protected either by methoximation or by ethoximation) (polar phase was methoximated and trimethylsilylated for the analysis of hydroxy- and amino acids, sugars, sugar alcohols, organic monophosphates, (poly)amines, and aromatic acids).Acidic protons were derivatized with 50µl of N-methyl N-tert-butyldimethylsilyltrifluoroacetamide (MTBSTFA) at 70°C or N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) at 37°C. One-microliter aliquots of these solutions were injected at a split ratio of 1:25 into a GC/MS system consisting of an AS 2000 auto sampler, a GC 8000 gas chromatograph, and a Voyager quadruple mass spectrometer including a dynode/phosphor/ photomultiplier detector (all ThermoQuest, Manchester, U.K.), manufactured in 1998. Tuning was done according to the instrument manual using tris (perfluorobutyl) amine (CF43) as reference gas. Special attention was paid to the high mass resolution which was manually improved to gain resolution up to Rfwhm) 2800 at m/z614. Installation requirements of Thermo Quest Voyager GC/ MS instruments are at leastRfwhm) 1500. Mass spectra were recorded from m/z50 to 600 at 0.5 s scan for trimethylsilylatedsamples (TMS) and from m/z 50 to 800 at 0.7 s scan for tertbutyldimethylsilylated samples (TBS). Accurate mass measurements were made using a Finnegan MAT magnetic sector field instrument (Finnegan, Bremen, Germany). Chromatography was performed using a 30m 250ím SPB 50 column (Supelco, Bellefonte, PA) or a 30m 250ím DB 5-MS column (J&W Scientific, Folsom, CA). Injection temperature was 230°C, the interface was set to 250°C, and the ion source was adjusted to 200°C. Helium flow was 1 mL min^{-1}. After a 5-min solvent delay time at 70°C, the oven temperature was increased at 5°C min-1 to 310 °C, 1 min isocratic, cool-down to 70°C, followed by an additional 5 min delay.

Expression and purification of sll1371 protein (His-tag)

DNA fragment covering the *sl1371* open reading frame was amplified from *Synechocystis* genomic DNA by PCR using the primer set, sll1371-exp-F:5' GATGTTTCAACCATTGTTTGCCAG-3' and sll1371-exp-R 5'-R:CTAGTGTGACATACTATGTTTCG A- 3'. The amplified fragment was digested with *NdeI* and *E.coRI*, and then ligated to pET28a (+) vector (Cat No: 69864-3, Novagen), which had also been digested with the same enzymes to generate the His-Sll1371-pET28a construct. The N-terminal 6x-histidine tagged Sll1371 recombinant plasmids were transformed into BL21(DE3) *E. coli* competent cells and was purified with Ni-NTA column. To overexpress the recombinant protein in *E. coli*, 400 μM IPTG was added to the cells then the cells were collected by centrifugation after 3 h of induction, the pellet was re-suspended in a buffer solution with 100 mM Tris-Cl pH 8.0, 300 mM NaCl and 1mM PMSF[106]. The resuspended cells were subjected to the lysis by French press, thrice at a pressure of 1000 psi and then centrifuged at 10,000 rpm for 10 min at 4°C to remove unlysed cells, the supernatant was filtered through a 0.45 µm filter to remove remaining cell debris. The expressed protein was purified using the Nickel affinity column (His60 Ni superflow resin, Cat No: 635660, Clonetech laboratories Inc) column after that washed twice with 20 mM Tris-HCl, pH 7.5/500 mM NaCl /40 mM imidazole sequentially filtered supernatant of Sll1371 protein was loaded onto the Nickel affinity column, were eluted with 20 mM Tris-HCl, pH 7.5/500 mM NaCl /100mM, 200mM,300mM and 400mM imidazole respectively. The purity of each fraction was examined by SDS-gel electrophoresis. The fractions which gave a single band at the expected region on the gel were pooled and dialyzed against 20 mM Tris-HCl, pH 7.5/ 150mM NaCl. The purity of both the proteins was examined by SDS-gel electrophoresis and these pure protein fractions were pooled and used for the generation of the antibody and also used in the western blot analysis to show no expression of Sll1371 in mutant strain.

Preparation of antibodies against Sll1371

Antibodies against Sll1371 were raised in Rabbit with purified His-tagged Sll1371 protein. The purified protein was used to generate anti-Sll1371 antibody in New Zealand white rabbits with Freund’s complete adjuvant (primary) and Freund’s incomplete adjuvant (booster). After the second booster, serum was collected and diluted 3000 times in 3% BSA and used for detecting Sll1371 protein on the membrane blots.

Isolation of total membranes and soluble proteins

Wild-type and Δ*sll1371 Synechocystis* cells were lysed by mechanical disruption using glass beads (106-µm diameter, Cat. No G4649, Sigma). Isolation of total membrane and soluble fractions was carried out as described previously [107]. To check if Sll1371 protein is membrane localized or a soluble protein, Wild type and Δ*sll1371* *Synechocystis* cells were grown at 34^{o}C under continuous illumination of 70 µmol photons m^{2} s^{-1} light and constant bubbling of air until an OD_{730nm} of around 1.0 was reached. Fifty ml of cells were harvested by centrifugation and resuspended in 500 µL of TBS buffer containing 50 mM Tris-HCl (pH 8.0), 150mM NaCl and 1mM PMSF. To test the localization of the Sll1371 protein up on cAMP treatment and shift to high carbon condition, The wild type and Δ*sll1371 Synechocystis* cells were grown by continuously bubbling ambient air until an OD_{730nm} of around1.0 was reached.. Fifty ml of the cells were collected and resuspended in 500µl of TBS buffer containing 50 mM Tris pH 7.5,150 mM NaCl and 1mM PMSF. For cAMP treatment, untreated cells were washed with BG- 11 followed by addition of 2 mM cAMP to the cells suspended in 500 µl of TBS, for high carbon treatments, the cells were grown at ambient air (0.04% CO_2) were shifted to high CO_2 (5%CO_2). Fifty ml of the treated cells were harvested by centrifugation and resuspended in the 500µl of TBS buffer without cAMP. An equal volume of glass beads was added to the cell suspensions and lysed by vigorous vortex for 30 seconds on a vortex mixer followed by 1 min cooling on ice. The mixing on vortex and cooling on ice was repeated 14 times to get maximum lysis of the cells. The unlysed cells were removed by the centrifugation at 2000 rpm for 2 minutes. The mechanically lysed cells were centrifuged at 25,000 g for 30 min to separate the soluble and the insoluble fractions. The resultant supernatant is the soluble protein fraction and the pellet is the membrane fraction. The membrane fraction was dissolved in 80µl of TBS buffer. Both the membrane and the soluble protein samples were loaded on to SDS-PAGE gel on equal protein basis (30 µg soluble and membrane protein in each well). After electrophoresis, the separated proteins from the SDS-PAGE were transferred onto polyvinylidene fluoride membrane (Immobilon-P; Millipore) by semidry transfer apparatus (TE77-PWR semi dry transfer unit, GE health care). The levels of Sll1371 protein were determined with NBT-BCIP chromogenic detection system according to the manufacturer's instructions (Sigmafast BCIP/NBT, Cat No: B5655, Sigma-Aldrich). The primary antibody (polyclonal, 1:2000) used for Sll1371 protein to develop the western blot was raised in a rabbit against His-Sll1371 protein and the secondary antibody used(1:5000) is an alkaline phosphatase-linked antibody raised in goat against rabbit IgG (Cat. No. NA934V, Amersham Biosciences)).

Immunogold detection of Sll1371

Wild type *Synechocystis* cells were harvested by centrifugation at 3500 rpm for 5 min and the cell pellets were fixed for 2 hr in 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). After fixation, the cells were rinsed twice with sodium phosphate buffer, the moisture was removed by passage through a graded ethanol series (30- 95%). After the dehydration, the cells were subjected to sectioning. Thin sections were placed on to nickel grids in such a way that section-side down on a drop of blocking solution [20 mM Tris/20 mM NaN3/150 mM NaCl, pH 8.2/0.2% (vol/vol) BSA] for 20 min. It was then incubated with the specific antibodies against the Sll1371 for 18 hrs. After the incubation, the thin sections were washed to remove the unbound primary antibodies. After washing, the sections were next incubated with secondary antibody which was coupled to 10-nm colloidal gold particles (GAR10; Biocell Laboratories). It was followed by washing twice with sodium phosphate buffer. After the washes, the sections were stained with uranyl acetate and examined under the transmission electron microscope (model 100CX; JEOL). The number of gold particles per unit area was calculated with known magnification. The areas of cell periphery and the cytosol were calculated for the Sll1371 protein localisation.

Gel mobility shift assays

To perform the gel mobility shift assay, the upstream region from the translation start site of selected genes *sll0030, sll1594* and *slr1838* were amplified by PCR using primers designed for the purpose. The PCR amplified product of the upstream region of the selected genes were purified and was incubated with varying concentrations of His–Sll1371 in 2 µl of binding buffer (750 mM KCl 50 mM Tris pH 7.4, 0.5 mM EDTA, 0.5 mM dithiothreitol). To check the binding of Sll1371 to DNA in the presence of cAMP, 2µl of the 20µM cAMP for 20 mins at room temperature. The reaction mixtures prepared were subjected to electrophoresis on a native 6% polyacrylamide gel at 150-200 V in cold room. The gel was stained by using SYPRO Ruby EMSA stain and Gel image analyses was carried out using epiluminiscence and a 490nm long-pass filter such as SYPRO filter (S6656) in an image analyser.

Table 3.2.Primers designed to amplify the upstream region of the selected genes for EMSA:

S.No	Gene	Forward primer	Reverse Primer
1	Sll0030 (276bp)	5′-TTAGTCATTGTCCTCGGGCAGG-3′	5′-AAGGGTTAGTACCGGTGCTGGC-3′
2	Sll1594 (235 bp)	5′-GATAACCAATGATAGAATAAAGCC-3′	5′-CGGTGTAGGCTCCTTTCTG-3′
3	Sll1838 (327bp)	5′-CCTTGTTGGTCCGGTGTAGGC-3′	5′-GGGCATGGAAAATTCCTCTAGGG-3′

Photosynthetic activity measurements:

To assess the photosynthetic performance of the wild type and Δ*sll1371* in response to external Ci, the photosynthetic O_2 evolution was measured to know the function of the mutant in the Ci concentration. It was performed using a Clark type O_2 electrode (PS2108, Passport dissolved O_2 sensor) as described by Kaplan et al. [60]. Wild type and Δ*sll1371* cells were grown simultaneously in ambient air and in high carbon (5% CO_2). The cells were harvested by centrifugation at 3,500 rpm for 5 min and then resuspended in a CO_2-free medium containing 10 mM NaCl and 20 mM HEPES, pH 7.5. The cells were then placed in the O_2 electrode chamber at 30 °C, under illumination at 70 µmol photons m^{-2} s^{-1}, and allowed to use the leftover Ci in the medium until the CO_2 compensation point was reached. Following this, 500 to 2000 µM of $NaHCO_3$ was injected into the chamber to raise the Ci concentration which was measured through the increased rate of O_2 concentration in the chamber.

Electron Microscopy for Carboxysome count

Wild type and Δ*sll1371* were grown in high carbon (5% CO_2) and in ambient air, the cells were harvested by centrifugation, fixed with glutaraldehyde (2.5 %, v/v) in 0.1 M phosphate buffer. The fixed cells were dipped in low melting agarose, later fixed with 2 % osmium tetroxide, followed by the dehydration in ethanol and finally the cells were embedded in EPON resin. Ultrathin sections were observed under the transmission electron microscope arranged with a digital (CCD) camera (161k). In the sections of WT and Δ*sll1371* the numbers of carboxysomes were counted approximately 50 cells (sectioned approximately in the middle of the cell) from each culture were considered.

Motility Assay

Synechocysits cultures containing approximately 100-500 cells were spotted onto the surface of BG11 agar (0.8 %). The cells were incubated under unidirectional light of 20 µmol m^{-2} s^{-1} for 5-8 days to check colony movement.

4. RESULTS

Results

Functional characterization of Sll1371

In *Synechocystis* genome three genes encode for putative cAMP receptor proteins. One of these cAMP binding proteins, Sll1371 also called as SyCrp1 has been reported to have a functional role in the motility of the *Synechocystis*[102]. The SyCrp1 has been reported to be a transcriptional activator for *slr1667, slr1668, slr2015, slr2016, slr2017* and *slr2018* [108]. However, the cyanobacterial cAMP binding proteins have not been functionally well characterized. Therefore, we inactivated the *sll1371* gene to investigate the functional role of this transcriptional regulator by reverse genetics approach. As we also got a clue from the literature that the cAMP levels are altered by inorganic carbon availability we investigated its role in the regulation of carbon concentrating mechanism.

Sll1371 is a conserved protein in cyanobacteria

When we used Sll1371 protein sequence as a query and searched against the protein database using NCBI BlastP resulted in catabolite gene activator proteins (CRP) as top hits for SyCrp1. Therefore, to determine the conserved regions in the protein sequence, multiple sequence alignment was performed with top ten best hits of SyCrp1 and the alignment result is shown in Figure 4.1. The protein has two domains, a N-terminal cAMP binding domain and a C-terminal DNA binding domain. *In silico* studies were performed to determine the cAMP binding site in SyCrp1 by building homology model using crystal structure of CRP from *E. coli* (PDB Id: 2GZW) as a template and docking studies were carried out with cAMP through Swissdoc software (Figure 4.2). The studies revealed that the protein has six β-sheets and a helix turn helix located at N-terminal and C- terminal respectively. In *E. coli* the helix turn helix motif of CRP was reported to bind to the DNA sequence and was observed to interact with arginine (R-180), glutamine (N-181) and arginine (R-185) (RNTVXR) [109]. The same amino acids (RNTVXR) were conserved in HTH motif of SyCrp1 in *Synechocystis*. Further, we docked the homology modelled protein with cAMP molecule and observed that it forms hydrogen bond interactions with Glu-90 and Ser-100 at N-terminal region and Arg-140 of C-terminal region of SyCrp1 protein (Figure 4.2). It is known that the *E.coli* cAMP-CRP forms hydrogen bonds with Ser-83, Glu-72, Arg-82 and gly-71 suggesting that similar pattern of interaction is followed by the cAMP -SyCrp1 complex in *Synechocystis* [110]**.**

Figure 4.1. Multiple sequence alignment of Sll1371 and its top 10 best cyanobacterial orthologs identified by NCBI-Blast search

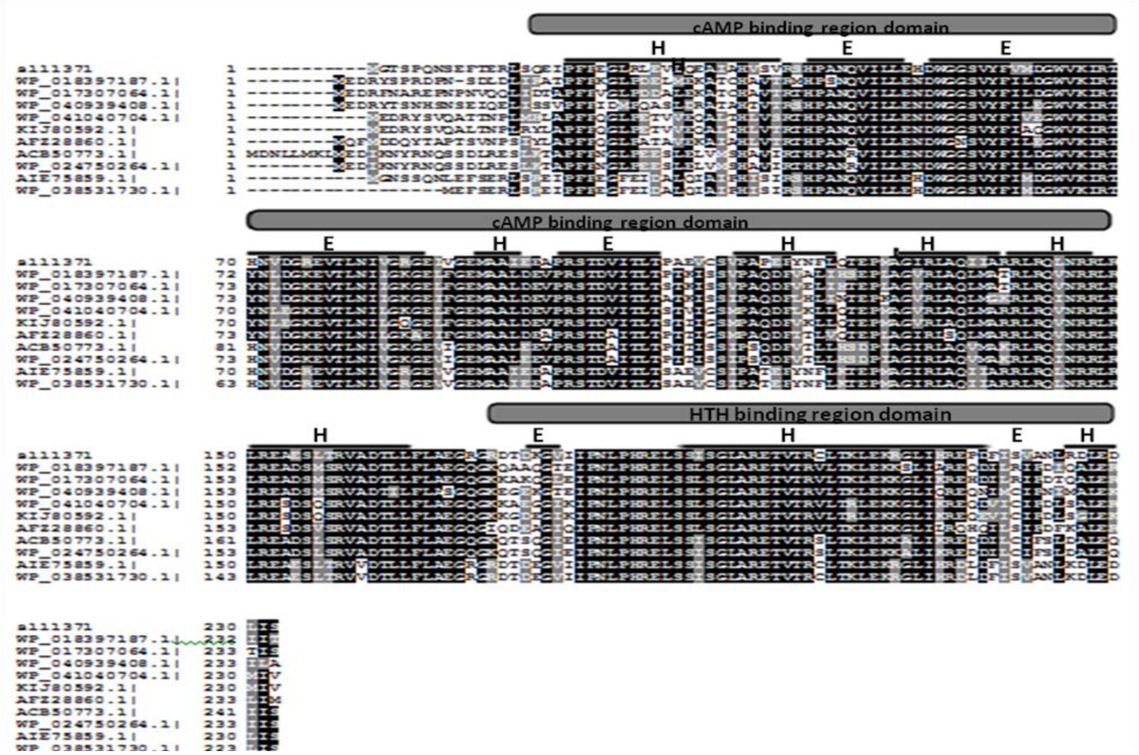


Figure 4.1: The protein sequences were used for multiple sequence alignment by using ClustalW algorithm. Similar amino acids are shown in black and grey colour indicates the conserved amino acid substitutions. Intial Six sheets (E) are conserved among all orthologues of Sll1371.Secondary structure regions were found using the Prosite secondary structure finding tool. By using Pfam, SMART databases search The cAMP binding region on SyCrp1 protein was detected . Sll1371, *Synechocystis* sp. PCC 6803; AIE75859.1, *Synechocystis* sp. PCC 6714; WP_038531730.1, *Synechocystis* sp. PCC 6714; ACB50773.1, *Cyanothece* sp. ATCC 51142; WP_024750264.1, *Cyanothece*; WP_041040704.1, *Tolypothrix campylonemoides*; WP_018397187.1, cyanobacterium ESFC-1; WP_040939408.1, *Prochloron didemni* ; KIJ80592.1, *Scytonema tolypothrichoides* VB-61278; AFZ28860.1, *Gloeocapsa* sp. PCC 7428; WP_040934598,

Figure 4.2. Homology model of SyCrp1 and its interacting region with cAMP

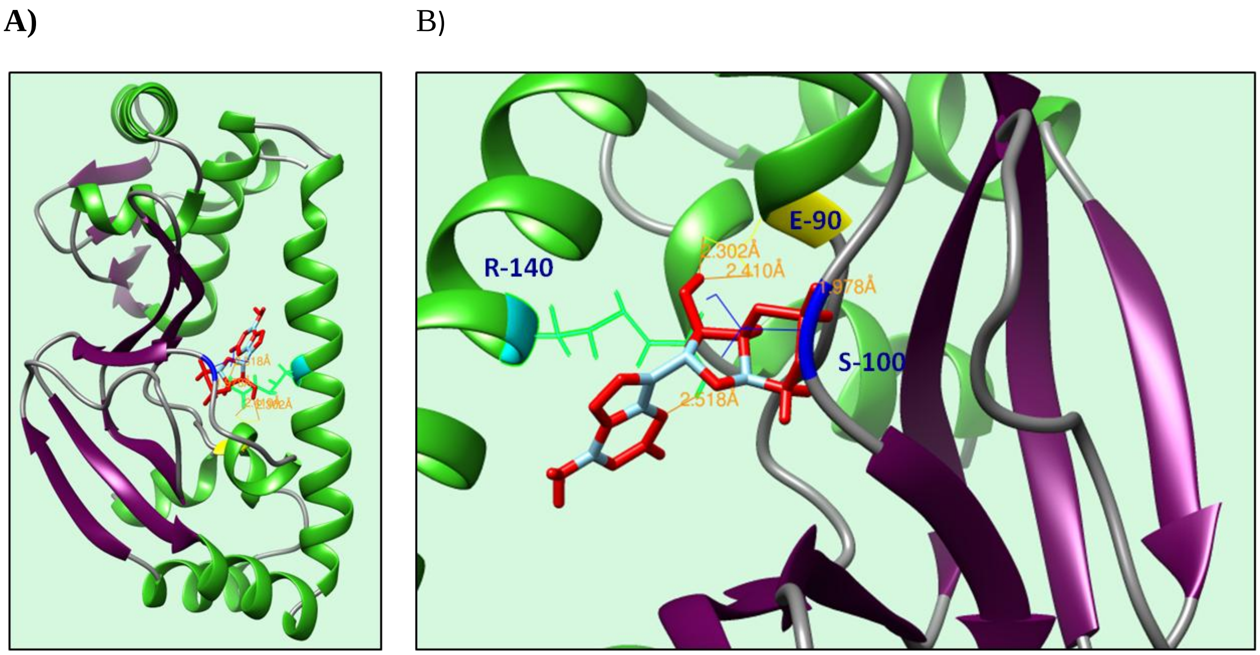


Figure 4.2. (A) SyCrp1 homology model prepared through swissdoc software.The studies depicted that the protein has six β-sheets and an helix turn helix located at n-terminal and c-terminal respectively. (B). Interacting amino acids on SyCrp1 with cAMP. cAMP binds to SyCrp1 by forming hydrogen bonds with Glu-90 and Ser-100 at N-terminal region and Arg-140 of C-terminal H-T-H domain

Segregation of disrupted copy of the *sll1371* gene in the multiple genomes of *Synechocystis:*

To elucidate the functional role of SyCrp1, the *sll1371* gene was disrupted by insertional inactivation as shown in the schematic presentation (Figure 4.3A). As *Synechocystis* contains multiple copies of identical genomes, to obtain a mutant, the mutated copy of the gene should replace the wild type copy in all the identical genomes. This can be achieved by repeated culturing of partially segregated mutant under the increasing concentration of antibiotic used for disruption of the gene. Segregation analysis of Δ*sll1371* mutant showed that it was complete i.e, all the *sll1371* gene copies in the genomes of Δ*sll1371* cells were modified by the inactivated version after many generations under antibiotic selection pressure (kanamycin, 25μg / ml). Complete replacement of the *sll1371* gene with that of modified copies of *sll1371::km*^{R}, was verifyied by comparing the size of amplified PCR products. The amplification of the *sll1371* with gene specific primers (*sll1371*TF-F and *sll1371*-TF-R) by using the wild type genomic DNA showed a PCR product of 842 bp equivalent to the undisrupted *sll1371*. Whereas, the amplified product of the *sll1371* from the genomic DNA of Δ*sll1371* cells with the same set of primers, has shown the approximately 2 kb size DNA sequence eqvivalent to the wild type fragment (842 bp) including the kanamycin resistant gene which is inserted (1200 bp) (Figure 4.3B), which indicates that complete replacement of wild type copy of *sll1371* with mutant copy of *sll1371*.

Figure.4.3. Strategic disruption of *sll1371* gene by insertional inactivation

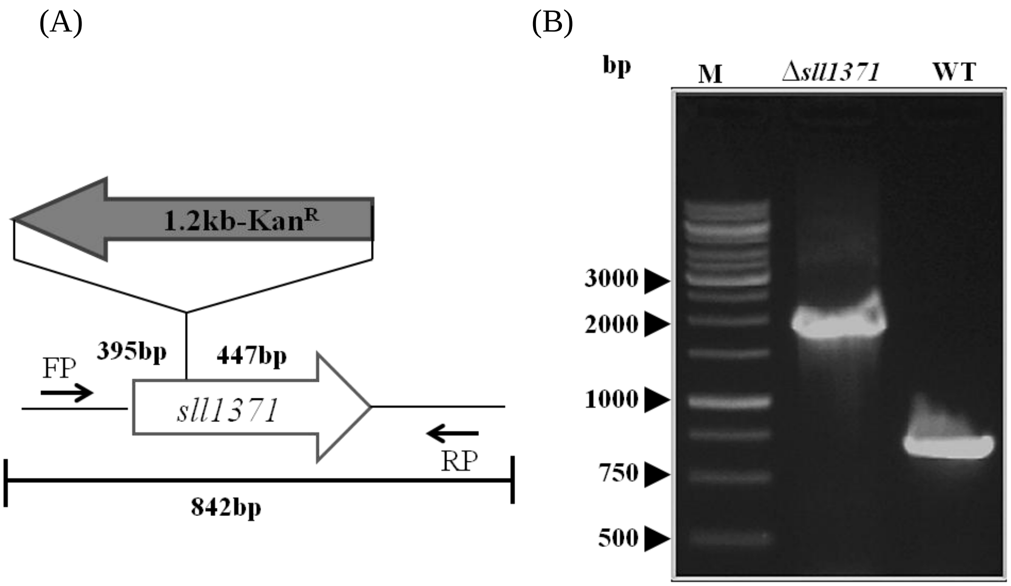


Figure 4.3. The *sll1371* gene from wild-type was completely altered by the mutated version of the gene in *sll1371* mutant cells. 842 bp length of DNA from the *sll1371* gene was disrupted with a Kan^R (1200 bp) cassette. (A) diagrammatic presentation of the genotype of the *sll1371* mutant. Open arrow represents the *sll1371* gene, the Kan^R cassette is shown with filled arrow. sll1371-F (FP) and sll1371-R (RP) are indicated by thick arrows, these two primers were used for PCR amplification of the *sll1371* gene from wild-type and that of the Kan^R cassette. (B) segregation analysis with the primers indicated in (A). M, DNA ladder; *Δsll1371* PCR product with mutant genomic DNA ; WT PCR product with wild-type genomic DNA as a template.

Δ*sll1371* mutant has exhibited a slow growth phenotype:

The Δ*sll1371* mutant has shown slower growth compared to wild type at the optimum growth temperature of 34°C due to complete inactivation of the *sll1371* gene in the Δ*sll1371* mutant. The growth pattern of Δ*sll1371* and wild type at different pH were shown in Figure 4.4.

Although, the Δ*sll1371* showed a slow growth phenotype when compared to the wild type *Synechocystis* culture at all the pH conditions tested, as the pH is close to near-neutral or neutral the difference in the growth was prominent. This suggests that at limiting CO_2 conditions Δ*sll1371* exhibited a clear slow growth phenotype than that of the wild type cells.

Figure 4.4 Phenotypic characterisation of the Δ*sll1371* mutant

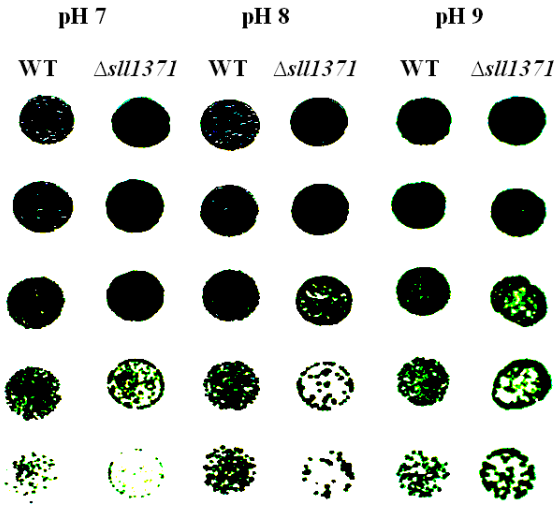


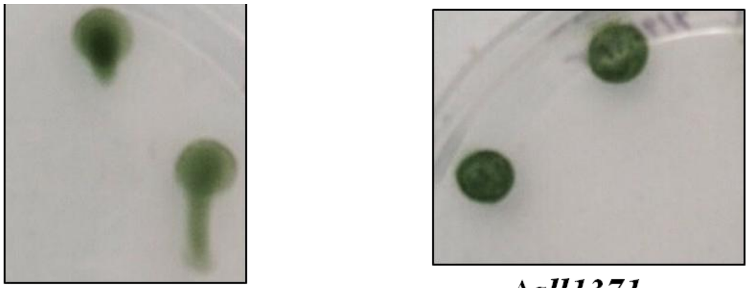
Figure 4.4. Growth of wild type (WT) and *Δsll1371* mutant cells on solid BG11 medium at different pH values under ambient air conditions. Wild type and Δ*sll1371* mutant cells were grown at low CO_2 until attain 0.6 $O.D_{(730nm)}$ and then serially diluted. Five µl of each of the dilution series of cultures were spotted onto BG11 agar plates of pH 7, 8 and 9 respectively. The plates were incubated for 5 days.

The Δ*sll1371* is a non-motile strain:

In order to check the effect of mutation on motility of *Synechocystis* we have analysed the cell phototactic movement of Δ*sll1371* and comapared with the wild type *Synechocystis* (Figure 4.5).

The phototactic movemnt of *Synechocystis* purely depends on the thick pilli [111] [102]. It was observed that the wild type has moved towards the unidirectional light, but the *Δsll1371* has shown impaired in its movement (Figure 4.5).

Figure.4.5. Directional motility assays



WT

Δsll1371

Figure 4.5: Phototactic movement of the motile wild type *Synechocystis* and the Δ*sll1371*. The cells were grown on a sold BG-11-agar plate for 5 days under continuous illumination of unidirectional light 40 μE m^2 s^1. *Synechocystis* WT strain showed a movement towards the light source**.** The Δ*sll1371* has not showed phototactic movement.

Changes in gene expression pattern of *Synechocystis* due to mutation in *sll1371*

In silico studies indicated that Sll1371 has a cAMP binding domain and a DNA binding domain, this suggests that it might function as a transcription factor for regulating expression of a set of genes by sensing the levels of cAMP. For further investigation to elucidate its function as a transcriptional regulator we performed DNA microarray analysis of the mutant Δ*sll1371*. Gene expression profile of Δ*sll1371* cells was compared with that of wild-type cells. Both the starins

Δ*sll1371* and wild-type were maintained under favourable growth conditions [70 µmol photons m^{-2} S^{-1}; 34^{o}C, continuously supply of sterile atmospheric air (0.04 %CO_2)] were used for comparing changes in the gene expression pattern. In brief, the information about the effect of mutation in *sll1371* on genome expression profile is presented in Table 4.1. The Table 4. 1 shows the list of genes whose expression levels were sufficiently up-regulated due to inactivation of *sll1371* in *Δsll1371*. According to our previous studies, we consider the genes with induction ratios falling between 2.0 and 0.5 as genes with unaltered expression due to mutation or treatment [112]. The data points which appears higher than the induction factor 2.0 or lower than induction factor 0.5 shows those genes whose expression was increased or decreased respectively, due to inactivation of gene or by stress conditions. The listed genes in Table 4.1 have shown the mean induction greater than 3.0 *(*Δ*sll1371* / wild type). Notably, the expression of genes coding for bicarbonate transporter (CmpA-D, SbtA/B, BicA) and CO_2 transport system ($NdhI_3$ and $NdhI_4$) and NADH dehydrogenase complex genes (*slr1279* operon) along with several other genes coding for hypothetical proteins were strongly up-regulated due to mutation in *sll1371*. It has been well documented that these genes were upregulated strongly when cyanobacterial cells experience limiting CO_2 condition [17][19] [20][21[27] [28][68][113]. However, it is interesting to note that upregulation of genes coding for bicarbonate and CO_2 transporters without bringing any changes in the CO_2 supply to the Δ*sll1371* mutant cells suggests a possible regulatory role of SyCrp1 in carbon concentrating mechanism.

Table.4.1. **List of the genes up regulated with effect of mutation in *sll1371***

ORF No	Function	Δ*sll1371* / WT (Signal ratio)
slr0040	bicarbonate transport system substrate-binding protein	114.2 ± 13
slr0041	bicarbonate transport system permease protein	108.9 ± 37.2
slr0042	probable porin; major outer membrane protein	56.1 ± 6.4
slr0043	bicarbonate transport system ATP-binding protein	53.1 ± 9.1
slr1512	sodium-dependent bicarbonate transporter	37.9 ± 3.4
slr1513	periplasmic protein, function unknown	32.8 ± 2.3
slr0044	bicarbonate transport system ATP-binding protein	28.7 ± 1.8
sll1734	protein involved in low CO2-inducible, high affinity CO2 Uptake	13.1 ± 0.4
sll1732	NADH dehydrogenase subunit 5 (involved in low CO2-inducible, high affinity CO2 uptake)	13.1 ± 0.6
sll1733	NADH dehydrogenase subunit 4 (involved in low CO2-inducible, high affinity CO2 uptake)	11.9 ± 0.5
slr0616	unknown protein	9.4 ± 0.8
ssr7036	unknown protein	8.9 ± 0.5
sll0199	Plastocyanin	7.9 ± 0.2
slr1279	NADH dehydrogenase subunit 3	6.6 ± 0.2
slr1281	NADH dehydrogenase subunit I	6.3 ± 0.5
slr1291	NADH dehydrogenase subunit 4	5.9 ± 0.4
sll1735	hypothetical protein	5.9 ± 0.3
sll1594	$NdhF_3$ operon transcriptional regulator, LysR family protein	5.7 ± 1.9
ssr1528	hypothetical protein	5 ± 2.4
sll0519	NADH dehydrogenase subunit 1	4.9 ± 0.2
slr1280	NADH dehydrogenase subunit NdhK	4.8 ± 0.3
slr0006	unknown protein	4.4 ± 0.2
slr1350	acyl-lipid desaturase (delta 12)	4.2 ± 2.7
sll0811	unknown protein	3.8 ± 0.0
sll0016	probable transglycosylase A	3.8 ± 4.6
slr0789	hypothetical protein	3.7 ± 0.2
sll0529	hypothetical protein	3.7 ± 0.2
slr0009	ribulosebisphosphate carboxylase large subunit	3.4 ± 0.3
sll0550	Flavoprotein	3.3 ± 0.2
sll0043	positive phototaxis protein, homologous to chemotaxis protein CheA	3.3 ± 5.1
sll0520	NADH dehydrogenase subunit NdhI	3.3 ± 0.6
sll0018	fructose-bisphosphatealdolase, class II	3.2 ± 1.6
sll0788	hypothetical protein	3.1 ± 0.0

Confirmation of DNA microarray data by qRT-PCR analysis

DNA microarray analysis indicated a strong up-regulation of the inorganic carbon transporter genes namely *cmp*A-D operon, *sbt*A/B operon and *sll1732* operon in Δ*sll1371*. The gene expression changes observed using DNA microarray were further confirmed by real time PCR. The real-time PCR data of the Δ*sll1371* / wild type confirmed the up-regulation of inorganic carbon transporter genes (Figure 4.6). As described in the previous sections, genes coding for CO_2 transporters $NdhI_3$ and $NdhI_4$, and the HCO_3^- transporters CmpA-D and SbtA/B are usually expressed only when there is a limitation in the supply of inorganic carbon source (CO_2) [5][17][114] [115]. However, the up-regulation of these genes in Δ*sll1371* mutant, when both wild-type and Δ*sll1371* cells were grown at constant supply of air indicates that SyCrp1 might be directly or indirectly involved in the carbon concentrating mechanism in *Synechocystis*. These findings evidence that SyCrp1 might function like a transcriptional regulator for the inorganic carbon transporters.

Figure.4.6. qRT-PCR confirmation of gene expression changes, analysed by DNA microarray in the Δ*sll1371*

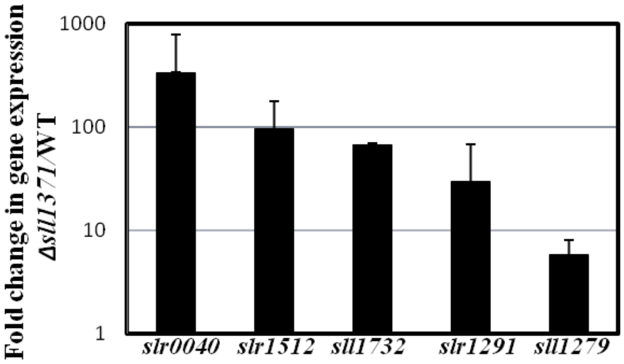


Figure.4.6. Eexpression changes of the genes involved in carbon concentrating mechanism due to inactivation of *sll1371*. qRT-PCR expression levels of *slr0040* (*cmp*A, first ORF of *cmp*A- D operon), *slr1512* (*sbt*A, first ORF of *sbt*AB operon), *sll1732* (*ndh*F3, first gene of *sll1732*-*sll1734* operon), *slr1279* (*ndhC*), and *slr1291*(*ndh*D2). All these genes were strongly upregulated in the Δ*sll1371* mutant.

Changes in the metabolite levels due to inactivation of *sll1371*

Polar metabolites of wild-type and Δ*sll1371* cells were compared by using GC-MS profiling. The disruption of *sll1371* resulted in an increased concentration of 2- phosphoglycerate of C_2 cycle intermediate metabolite (Figure 4.7), which is a common phenomenon in the low carbon condition. As 2-PG is an allosteric modulator of CmpR, it is meaningful to observe an enhanced expression of Ci transporters (*cmpA-D* genes). Glyoxalate levels of the C_2 cycle was also increased in the Δ*sll1371* mutant. Several metabolic intermediates of Calvin Benson Cycle (CBB), Oxidative pentose Phosphate pathway (OPP), and glycolysis were also exhibited marked differences in their levels. The primary product of CBB cycle, 3-phosphoglycerate (3-PG) level was increased about 64 fold in the Δ*sll1371* (Figure 4.7), Increase in the 3-PG levels has been reported earlier when the photosynthetic cyanobacterial cells, *Synechococcu*s sp. PCC 79421, experiences low carbon condition [80]. Schwarz et al., suggested that such an increase in 3-PG levels indicates an increased rate of glycolysis to that of CBB under low carbon conditions. In the Δ*sll1371* mutant, 6-phospogluconate (6-PG), an OPP pathway intermediate also increased about 5 fold, indicating carbon utilisation shifted from CBB towards OPP (Figure 4.7). Besides, decreased levels of TCA cycle intermediates also explain the shift of intermediates to glycolysis [83]. All these changes were previously reported to occur when cyanobacterial cells experience low CO_2 condition and while they attempt to acclimatise to the low carbon environment

[116][86]. However, in the present study, the disruption of *sll1371*, without inducing any change in the CO_2 levels, exhibited the above metabolic changes when compared to wild-type cells, indicates that the SyCrp1 might be involved in signalling carbon levels and subsequent regulation of the target genes involved in carbon transporters.

Figure.4.7. Metabolite profiling of wild type and Δ*sll1371* cells under optimal growth

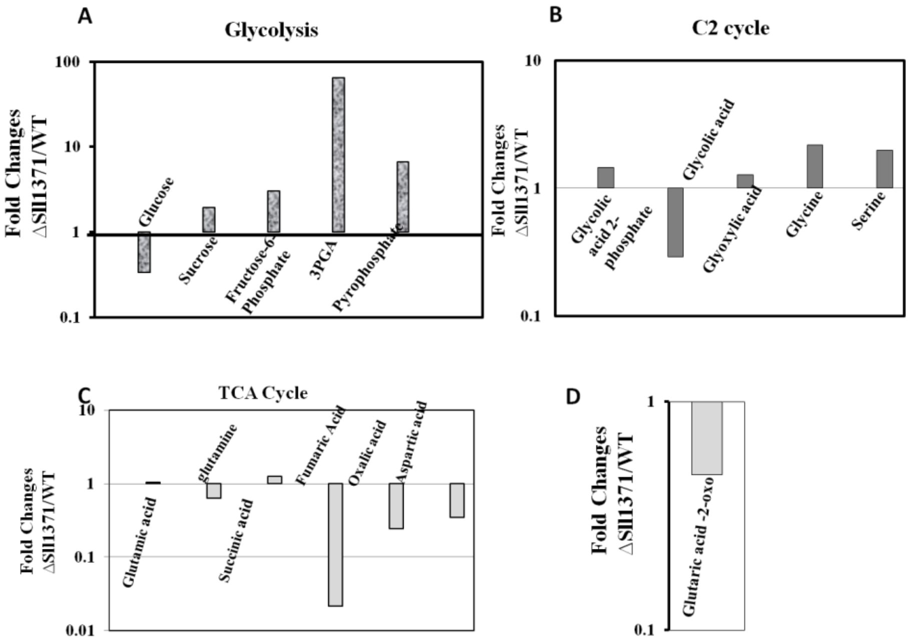


Figure 4.7. Changes in the metabolite levels in Δ*sll1371* as compared to wild type (WT). The metabolic intermediates of C_2 Cycle. OPP, Glycolysis and TCA cycle are shown marked changes in Δ*sll1371* when compared to WT. (A), Glycolysis. (B) C2 cycle.(C), TCA cycle. and (D). GS/ GOGAT).

6x-Histidine tagged Sll1371 has been purified and antibody is generated:

In *E. coli* BL21 the Sll1371 protein was expressed as N-terminal His-Sll1371 by adding the 1mM final concentration of IPTG. The actual size of the Sll1371 protein is 27 kDa. We detected the Sll1371 protein at approximately 30 kDa region in the soluble fraction of the *E. coli* lysate, but not in the pellet containing membranes. As expected the protein was not detected in the un-induced protein samples from cell lysates. The amount of the protein expressed gradually increased during IPTG induction and reasonably good amount of protein was accumulated after induction for 3 hours. (Figure. 4.8). The protein bands appeared close to the expected molecular mass. The purified protein was confirmed to be Sll1371 a protein by MS-MS analysis. The purified Sll1371 protein from elutes was pooled and dialysed against 150 mM NaCl and 20 mM Tris-HCl to take away the imidazole left in the samples. Then the dialysed samples were used for the generation of polyclonal antibody.

Figure 4.8. Expression and purification of Sll1371 protein

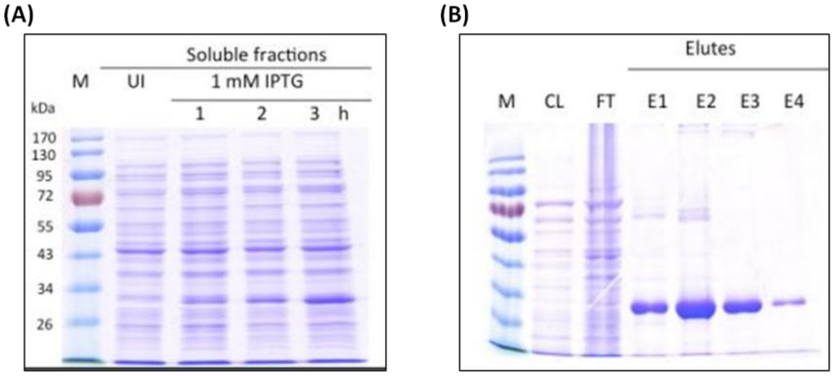


Figure 4.8: (A) In *E. coli* BL21 the Sll1371 protein was expressed as a N-terminal His-Sll1371 by adding the 1mM final concentration of IPTG. Sll1371 protein band can be seen at approximately 30 kDa region in the soluble fractions. UI, un-induced soluble protein fraction; M, protein marker (B) Affinity column purified protein appeared in the Elutes 1 to 4 (imidazole concentrations 100 mM, 200 mM, 300 mM and 400 mM respectively).

SyCrp1 is a membrane anchored protein:

Assuming SyCrp1 functionally is involved in regulating the carbon concentrating mechanism in *Synechocystis* and might function as a transcriptional regulator for carbon transporter genes, we further proceeded to investigate its localisation by performing western blotting using anti-Sll1371 polyclonal antibody raised against purified His-SyCrp1 in Rabbit. The isolated membrane and soluble fractions from the wild type and Δ*sll1371* cells of *Synechocystis* were used for the detection of SyCrp1. Interestingly, the anti-SyCrp1 antibody detected SyCrp1 protein in the membrane fraction of the wild type *Synechocystis* cells (Figure 4.9). As expected in the Δ*sll1371* mutant cells SyCrp1 protein was not identified in both insoluble as well as in the soluble fractions. Since we have not observed any membrane spanning domains in the protein using bioinformatics tools; we assumed that the protein is probably associated with the membrane by electrostatic interactions, but not spanning across the membrane. Thus, it clearly indicates that SyCrp1 is a membrane anchored protein and was not detected in the soluble fraction (Figure 4.9).

Figure.4.9. Immunodetection of SyCrp1 in wild type and Δ*sll1371*

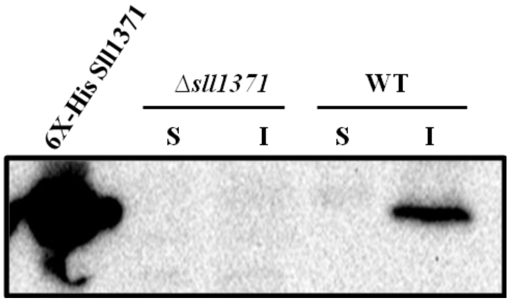


Figure 4.9 : Westrn blot analysis of SyCrp1 protein in the cytoslic (S) and membrane (I) fractions separated from Δ*sll1371* and wild type *Synechocystis* cells; anti-SyCrp1 antibody also detected the purified His-Sll1371. I – Insoluble membrane, S – Soluble cytosolic fraction, 6X-His- Sll1371 – Pure SyCrp1 protein.

SyCrp1 (Sll1371) is confirmed to be associated with the membrane:

We carried out the immunogold detection for further confirmation of the localisation of SyCrp1 protein in wild type cells. Thin sections of wild-type *Synechocystis* cells were used for immunolabeling with antibodies against the SyCrp1 protein. It is clearly seen that 3-4 layers of thylakoid membranes separated from one another and a well-separated cytoplasmic region in the thin section of wild-type cell (Figure 4.10). More number of the gold particles were localised at the membrane part of the wild type cell. Compared to membrane region a very few gold particles were observed towards the cytoplasmic part of the cell. The number of gold particles observed per unit area; the same was calculated for different regions of the thin section of wild-type *Synechocystis* cells. The number of gold particles were more in the membrane region than that in the cytosolic region of the wild type *Synechocystis* cells. It suggests that when cells grown at optimal growth conditions the SyCrp1 is mostly abundantly localised in the membrane.

Figure 4.10: **Localization of SyCrp1 in the *Synechocystis* with purified gold labeled anti-Sll1371 antibody.**

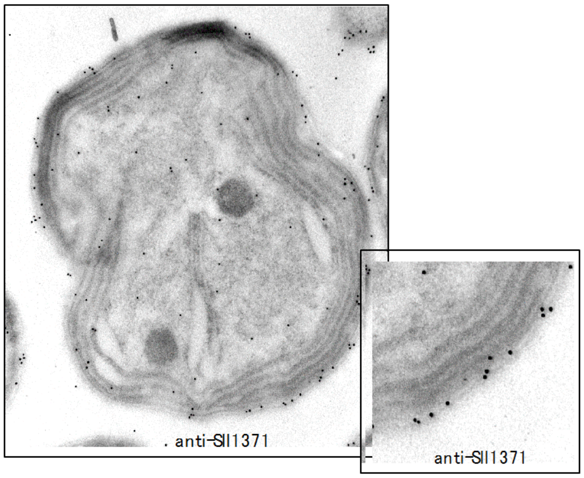


Figure 4.10: Immunogold labeling of wild type cells with purified antibodies against SyCrp1.The black spots indicate the gold particles. The number of gold particles localizaed in membrane was higher than th number of gold particles observed in cytoplasmic region.

The cAMP induced the detachment of SyCrp1 protein from the membrane to soluble region

It has been well established that the cAMP receptor proteins function as transcriptional regulators after binding to cAMP and binding of cAMP leads to confirmational change in the protein structure allowing the cAMP-CRP complex to bind to target DNA region [100][101]. The SyCrp1 being a transcriptional regulator its localisation in the cytoplasm is reasonable, but it is predominantly localized on the cytoplasmic membrane. Therefore it is likely that binding of cAMP would release the protein from the membrane to cytoplasm. To check whether cAMP binding releases the SyCrp1 protein into cytoplasm or not, we immuno-detected the SyCrp1 protein in the *Synechocystis* cells before and after addition of cAMP. To detect SyCrp1 protein, the wild type and Δ*sll1371 Synechocystis* cells were resuspended in TBS buffer, followed by the addition 2mM cAMP then separated the membrane (insoluble) and cytosolic protein fractions. The isolated total membrane and cytosolic protein fractions of wild type and Δ*sll1371* cells of *Synechocystis* in the presence of cAMP were used for the detection of SyCrp1. SyCrp1 protein was identified in the membrane fraction of the wild type *Synechocystis* cells in the absence of cAMP (Figure 4.11A). In the presence of cAMP most of the SyCrp1 protein was detected in the soluble fraction, while a little in the insoluble membrane fraction indicating that the cAMP binding to SyCrp1 leads to change in the localisaiton of the protein from the membrane to cytoplasm. As expected, in the Δ*sll1371* the SyCrp1 protein was completely (Figure 4.11B).

Figure 4.11. Localization of SyCrp1 protein the wild type and Δ*sll1371* *Synechocystis*

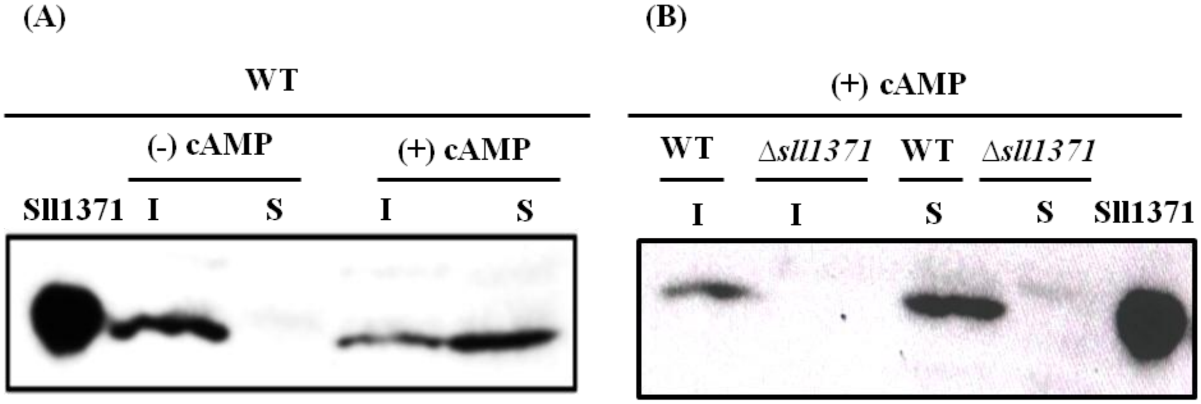


Figure 4.11: (A) In the absence of cAMP, the protein was identified in the insoluble fraction of wild-type *Synechocystis* cells. In the presence, major part of protein is localised in the membrane fraction. (B) In the Δ*sll1371* mutant, the Sll1371(SyCrp1) was not detected. I, insoluble membrane fraction; S, soluble protein; WT, wild-type; Δ*sll1371*, *Synechocystis* mutant strain in which *sll1371* gene was inactivated by inserting kanamycin resistance gene cassette within the coding region.

Carbon dioxide induced the transfer of membrane attached SyCrp1 to cytosol

Collectively, the data clearly indicated that the inactivation of *sll1371* leads to alteration in the transcript levels of genes encoding for inorganic carbon transporters, changes in the metabolites responsible for allosteric regulators of the same genes. In addition, we also observed that the membrane associated SyCrp1 has been released into the cytoplasm in the presence of increased levels of cAMP. These results indicated that SyCrp1 could be a possible regulator of the carbon concentrating mechanism and such a regulation might be mediated via cAMP levels. It is known that increased levels of CO_2 activate the adenylate cyclase thereby accumulation of cAMP in the *Synechocystis* cells [90]. This finding together with our observations on cAMP dependent release of SyCrp1 and changes in the expression of CCM genes, indicated that the CO_2 levels might influence the intracellular cAMP levels, which in turn regulate the release of SyCrp1 from the membrane into the cytosol for regulation of genes involved in bicarbonate / CO_2 transporters. To investigate the role of CO_2 on SyCrp1 localization, we have checked the levels of cytoplasmic and membrane-bound SyCrp1 in the wild type *Synechocystis* cells due to limiting and sufficient CO_2 supply (Figure 4.12). When *Synechocystis* cells were allowed to grow in the atmospheric air (0.04% CO_2) for 16 hours, SyCrp1 was observed to be present in the insoluble membrane fraction, but not in the soluble cytoplasmic protein fraction as expected (Figure 4.12A). When the cells which were grown by continuous supply of ambient air for 16 hours were supplied with 5% CO_2 in air, the SyCrp1 protein appeared in both membranes as well as soluble fractions. A gradual increase in the SyCrp1 levels was detected in the soluble fraction with a concomitant decrease in the membrane fraction during onset of bubbling the cultures with 5% CO_2 in air (Figure 4.12B). The isolated membrane and cytosolic fractions of wild type *Synechocystis* grown at either ambient air and or high CO_2 levels (5% CO_2 in air) were used for the detection of SyCrp1. The anti-Sll1371 antibody detected SyCrp1 protein in both the cytosolic and insoluble fractions of the wild- type *Synechocystis* cells, as the time course of high CO_2 supply increased (shift from ambient air to 5% CO_2). These observations suggest that in the presence of high CO_2

the membrane-bound SyCrp1 protein releases into the cytosol, whereas at ambient air level grown wild type cells, SyCrp1 was present only in the membrane. It implies that in the presence of high CO_2 the membrane-bound SyCrp1 protein gets released from the membrane into the cytosol of the cell.

Figure.4.12. Localization of SyCrp1 in *Synechocystis* cells during the course of high-CO_2 bubbling

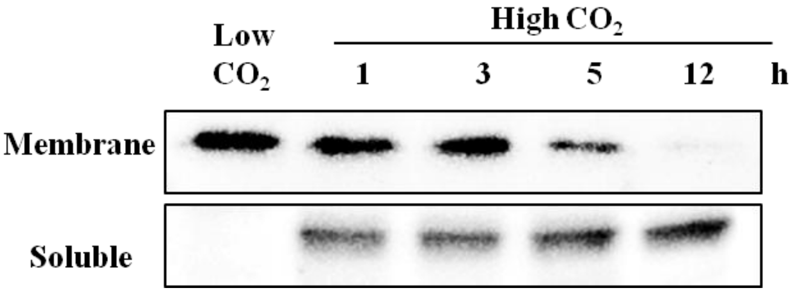


Figure 4.12: SyCrp1 was identified using an anti-SyCrp1 antibody in the membrane and cytosolic soluble protein fractions isolated from the wild type *Synechocystis* cells. Gradual release of SyCrp1 protein from membrane to soluble fraction was observed when wild type cells were shifted to high CO_2 (5% CO_2 in air) from low CO_2 conditions (Only air).

Further, in order to examine the effect of limiting CO_2 supply to *Synechocystis* cells on the localisation of SyCrp1 protein, the wild type cells were grown at high CO_2 (5% CO_2 in air) continuously for 24 hours were washed and resuspended in a fresh BG-11 medium with ambient air (0.04% CO_2 in air). When *Synechocystis* cells were continuously maintained by supplying high-CO_2, SyCrp1 was observed to be abundantly present in the soluble fraction, and a little was also detected in the membrane fraction (Figure 4.13). When the high-CO_2 grown cells were washed and resuspended in BG-11, followed by bubbling with ambient air, the SyCrp1 was found still in the cytosolic frction, but not in the insoluble membrane fraction (Figure 4.13). Thus a gradual decrease in the SyCrp1 protein from the membrane to soluble fraction after a shift from low to high CO_2 condition suggests that the CO_2 induced activation of adenylate cyclase and subsequent increase in the cAMP levels could release the SyCrp1 into the cytoplasm. The SyCrp1 is a probable repressor of the genes that encode for the key transcriptional regulators of carbon transporters.

Figure 4.13. Localisation of SyCrp1 in high CO_2 acclimatised wild type *Synechocystis*

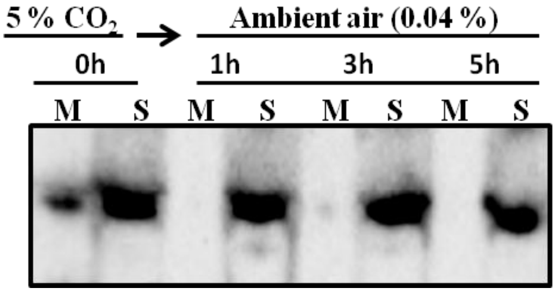


Figure.4.13. Localization of SyCrp1 in *Synechocystis* cells during the shift of high CO_2 to bubbling the cells with ambient air (0.04% CO_2 bubbling). *Synechocystis* cells were initially grown by bubbling with the air mixed with 5% CO_2 for 24 h followed by bubbling with ambient air (0.04% CO_2). SyCrp1 was identified using an anti-SyCrp1 antibody in the cytosolic protein fractions isolated from the wild type *Synechocystis* cells but not in the membrane . Due to high carbon in the cell SyCrp1 protein in membrane was not observed when wild type cells were shifted from high CO_2 (5% CO_2 in air) to low CO_2 conditions (Only air).

SyCrp1 binds to the upstream regions of genes encoding CCM transcriptional regulators

As mentioned before, when cyanobacterial cells experience low carbon condition the CmpR gets activated and binds to the upstream DNA binding region in the *cmpA-D* operon and there by triggers the expression of low-affinity *cmpA-D* operon [71]. As described in the earlier sections, NdhR is another transcriptional regulator functions as a repressor for the CO_2 transporters $NdhI_3$, $NdhI_4$ and bicarbonate transporter, SbtA/B. In order to check whether SyCrp1 is an additional alternate regulator of CCM working together with the known transcriptional regulators, CmpR and NdhR, we searched for its target DNA binding sites. A putative DNA binging site, ‘’TCAnnnnnAAAA’’ and ‘’TGAnnnnnTCA’’ was predicted in the upstreams of *cmpR* (*sll0030) sbtA (slr1512*), other CCM related gene, *ccmK*(*slr1838*) earlier [117]. A possible mechanism of SyCrp1 binding to its target site and the mechanism of regulation of gene expression was also reported for SyCrp1 with an experimental validation of the predicted binding sites. It was also experimentally demonstrated the SyCrp1 bidning to the promoter region of a bicarbonate transporter gene *SbtA (slr1512*). It was also suggested that promoter region of *ccmK* gene has a similar binding site for SyCrp1 [97][117]. Since, we observed that the membrane associated SyCrp1 gets released into the cytoplasm when bubbled with sufficient CO_2 and also the targeted inactivation of corresponding gene *sll1371* led to the upregulation of genes involved in inorganic carbon transporters, we further went ahead to check if this SyCrp1 functions cooperatively with the CmpR and NdhR regulators as an additional regulatory mechanism. The binding of SyCrp1 protein for the upstream sequence of the CCM regulatory genes namely *cmp*R and *ndhR*, and *ccmK*, a component of carboxysome was analysed by gel mobility shift assay. The DNA upstream regions were chosen in such a way that the predicted motif ‘’TCAnnnnnAAA’’ is located with in the DNA fragment used for binding assays. It was observed that His-Sll1371 protein binds to the upstream DNA fragments of *cmpR(sll0030), ndhR(slr1594), ccmK (slr1838)* and *sbtA (slr1512) (*Figure 4.14). The SyCrp1 was observed to bind to the upstream DNA fragments in the absence and also in the presence of 20μM cAMP, SyCrp1 has a grater affinity for target DNA binding site in the presence of cAMP. It is highly likely that the intracellular inorganic carbon levels are sensed through the adenylate cyclase and cAMP functions as a messenger for release of membrane bound SyCrp1 into cytoplasm under sufficient CO_2 conditions.

Figure 4.14. Binding of SyCrp1 to the upstream sequence of *cmpR, ndhR, ccmK ,sbtA* genes

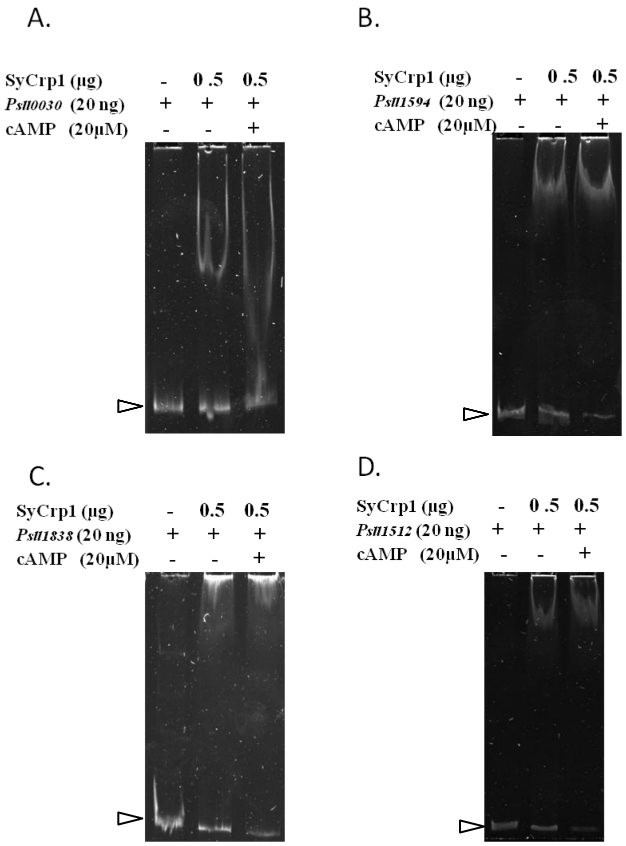


Figure 4.14. electrophoretic mobility shift assay was carried out with His–Sll1371 protein and a 276, 327 and 235 bp PCR amplified DNA upstream regions starting from the translation start site of *sll0030(A), sll1594(B) slr1838(C) and slr1512(D)* ORFs. The open arrowhead indicates free labelled DNA

Effect of inactivation of *sll1371* on the photosynthetic performance:

As the expression of the genes that code for inorganic carbon transporters was altered due to inactivation of *sll1371* and to get further experimental evidence that SyCrp1 is involved in control of inorganic carbon sensing and regulation, we analysed the inorganic carbon uptake affinity under excess CO_2 acclimatized and limited-CO_2 acclimatized Δ*sll1371* cells in comparison with the wild type cells. To determine the affinity of inorganic carbon uptake, bicarbonate dependent rate of photosynthesis was monitored in terms of oxygen evolved per µg chlorophyll per hour. We observed that the maximal photosynthetic rates of low carbon-acclimated Δ*sll1371* mutant cells were lower than that of the low carbon acclimated wild type cells (Figure 4.15). The lower V_{max} indicates that the carbon fixation ability in the carboxysomes is impaired in the Δ*sll1371* cells at low carbon acclimating conditions. This could be either due to reduced number of carboxysomes in which RuBisCO fixes the $CO_{2.}$ We have also observed that the affinity for bicarbonate is not affected at low CO_2 conditions in both the wild type and the Δ*sll1371* cells. At high carbon conditions, there was no significant difference in the V_{max} and affinity were noticed between the wild type and Δ*sll1371* mutant (Figure 4.15). This data indicates that SyCrp1 is functionally linked to low carbon acclimation in *Synechocystis*.

Figure 4.15. Photosynthetic Rate of wild type and Δ*sll1371 Synechocystis*

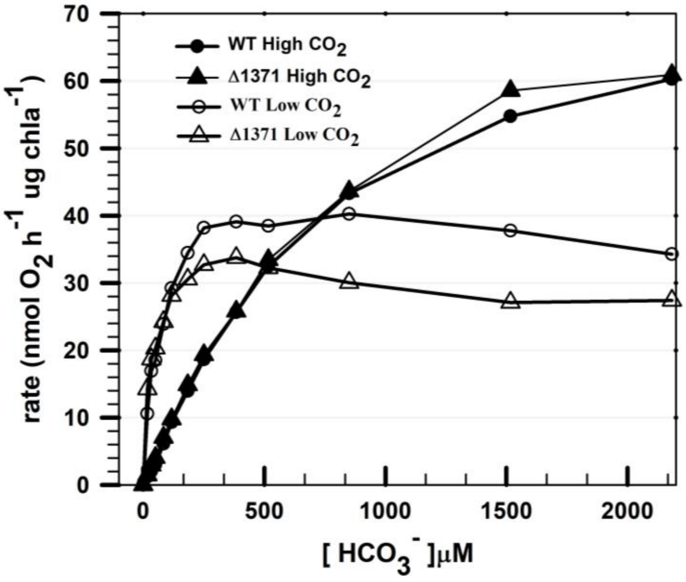


Figure 4.15. Cultures were grown till an OD $_{730nm}$ of 0.8, then the cells were collected to determine photosynthetic electron transport rates with increasing concentrations of Ci by adding $HCO_{3.}$

Low CO_2-acclimated Δ*sll1371* cells have relatively lower carboxysomes:

Carbonic anhydrase in the carboxysomes converts the dissolved bicarbonate into carbon dioxide for subsequent fixation into the calvin-benson cycle by RuBisCO. Since there was no significant difference in the affinity of bicarbonate uptake but significant decrease in the maximum rate of photosynthesis in the Δ*sll1371* under low inorganic carbon conditions, we expected to observe an overall decrease in the carboxysomes in which CO_2 is fixed by RuBisCO in the Δ*sll1371* mutant. The carboxysome number was counted in the transmission electron microscopic images of the Δ*sll1371* and wild type cells, which were grown in the presence of high carbon and low carbonacclimated cells (Figure 4.16A). There was no markable change in the carboxysome numbers were observed between the wild type and the Δ*sll1371* cells when grown by bubbling 5% CO_2 in air Strikingly, in the low CO_2 acclimated Δ*sll1371* cells, we observed relatively less number of carboxysomes were seen when compared to the wild type cells (Figure 4.16B). This observation is in consistent with the reduced rate of maximum photosynthesis when measured the photosynthetic rate as a function of increasing concentration of bicarbonate (Figure 4.15).

Figure.4.16. Changes in cell morphology at low CO_2 due to the mutation in *sll1371*

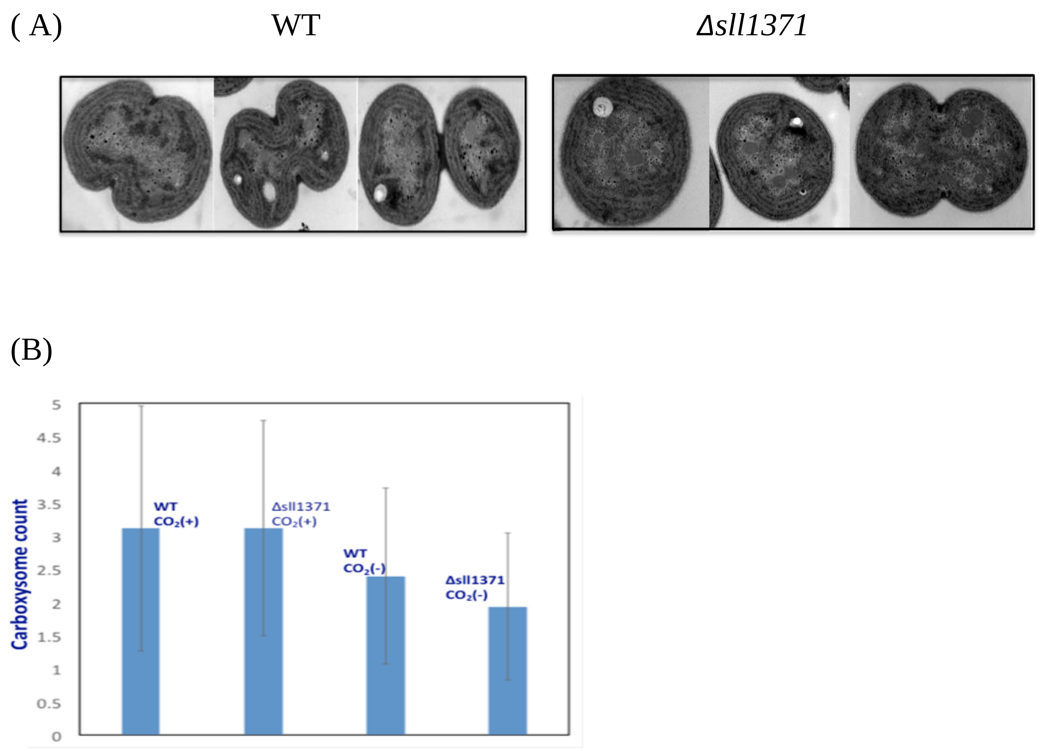


Figure.4.16. (A) Transmission electron micrographs of wild type and Δ*sll1371* cells. The Cells were grown in BG11 medium at low CO_2, samples were taken after 16h of the growth. (B). Carboxysome number counted (means and confidence intervals) per cell is shown. The carboxysome number was compared in the wild type and Δ*sll1371* grown at high carbon conditions and limited carbon conditions were indicated

5. DISCUSSION

5. DISCUSSION

1. Structural similarity of the SyCrp1 with cAMP receptor proteins(CRP)

Though *Synechocystis* is a well-studied model organism, only 50% of its genes were functionally characterised, and the remaining 50% are not functionally characterized [118]**.** The sequenced genome of the *Synechoystis* has shown cyclic nucleotide binding receptor protein annotated as cNMP whose functions are not studied. Three such kind of putative cNMP binding proteins were identified as Slr0842, Slr0593 and Sll1371 (SyCrp1) [98] (Table 1.2). SyCrp1 is a cAMP binding receptor protein also reported to be involved in the motility of the *Synechocystis*. The cAMP receptor protein was first reported in *E. coli* and is well characterised. *E. coli's* CRP is involved in the multiple cellular functions and is conserved among the bacterial species. SyCrp1 (Sll1371) is also conserved among the cyanobacterial species but has low sequence similarity with that of *E. coli* CRP. Homology modeling and docking studies have shown that SyCrp1 has cAMP binding site and DNA binding domain (Figure 4.1, Figure 4.2)

2. SyCrp1 regulates the genes involved in CCM.

The major inorganic carbon transporter proteins well studied at their functional level are CmpA-D, SbtA/B, $NdhI_3$, and $NdhI_4$. These transporters are involved in the carbon concentrating mechanism [22][28][24][114]. It is also known that the regulatory mechanism of these inorganic carbon transporter genes involve two regulators called CmpR and NdhR, depending on the availability of the inorganic carbon the carbon concentration mechanism (CCM) is regulated. There is a need to understand the detailed molecular mechanism of the inorganic carbon sensing and the process of signal perception for the uptake of inorganic carbon especially under limiting

CO_2 conditions. In *Synechocystis,* the regulation of the inorganic carbon transporters has been studied. However the detailed molecular mechanism involved in the sensing of CO_2 concentration and coordinated regulation of downstream processes of carbon sequestration was yet to be elucidated. For the first time in our study, we demonstrated that the SyCrp1 as a novel cAMP binding and membrane anchored protein involved in carbon concentrating mechanism. In DNA microarray and qRT-PCR of Δ*sll1371* mutant has shown the upregulation of the inorganic carbon transporter genes indicating that SyCrp1 has a role in the carbon concentrating mechanism (Table 4.1, Figure 4.6). We have confirmed that SyCrp1 binds to the target DNA sites located in the upstream of *cmpR*, *ndhR*, *ccmK* and *sbtA*, were observed in the retardation of the target DNA on polyacrylamide gel. Our data is in agreement with the previous reports on SyCrp1 target DNA binding elements and experimental demonstration of its binding to upstream of *sbtA* and *ccmK* [97][117]. These observations led us to report that the absence of the SyCrp1 in the Δ*sll1371* resulted in the up-regulation of the inorganic carbon transporters, suggesting that SyCrp1 is involved in the CCM regulation.

3. SyCrp1 is a membrane bound protein

Being a transcriptional regulator, SyCrp1 was expected to localised in the cytoplasm of *Synechocystis.* Interestingly, SyCrp1 was identified in the membrane fraction of the *Synechocystis* cells (Figure 4.9). As expected SyCrp1 was not detected and was completely absent in the *Δsll1371* as the disrupted copy of the *sll1371* completely altered the wild type copies of the of *sll1371* gene in all the multiple identical copies of the genomes in Δ*sll1371*(Figure 4.3). Immuno-gold labelling of the SyCrp1 confirmed its localisation in the membrane of *Synechocsysits* but some were also observed to be located in the cytosol (Figure4.10). This observation confirms that SyCrp1 is a constitutively expressed membrane protein but can be relocated to cytosol when required.

4. High CO_2 condition induces the release of the membrane bound SyCrp1 to the cytosol

In the direction to know the functional phenomenon of the SyCrp1, the SyCrp1 protein levels and its localisation was checked when cells were shifted from high to low CO_2 condition and vice versa. As the SyCrp1 is a cAMP receptor protein, the localisation of SyCrp1 was also checked in presence and absence of cAMP. The data clearly demonstrated that the increase in cAMP levels leads to release of SyCrp1 into the cytoplasm (Figure 4.11). It was reported that adenylate cyclase gets activated in presence of the CO_2 and generates the cAMP from ATP [88][94]. Based on this information, we studied whether the release of SyCrp1 from the membrane to cytosol is in turn regulated by CO_2 levels or not. Supporting our hypothesis, incubation of cells with high CO_2 releases the SyCrp1 into the cytosol from the membrane (Figure 4.12). Thus *Synechocystis* when experiences high CO_2, the SyCrp1 is translocated from the membrane to the cytosol.

5. Physiological role of SyCrp1 in *Synechocystis*

The complete mutation of *sll1371* has shown slow growth phenotype at low CO_2 condition. At high CO_2 both wild type and Δ*sll1371* had a similar rate of photosynthesis. However, at low CO_2 level the mutant showed decreased rate of photosynthesis as compared to the wild-type (Figure 4.15). The active performance of photosynthesis depends on the carboxysomes. It was observed that the number of carboxysomes in the *Δsll1371* and wild-type at high CO_2 condition was same but at ambient air level, *Δsll1371* has lesser number of carboxysome (Figure.4.16B). These observations suggest that *Δsll1371* has lower Vmax at low CO_2 due to the lesser number of carboxysomes.

Conclusions

SyCrp1 functions as a possible regulator of the CCM

The open reading frame *sll1371* encoding a SyCrp1 was disrupted by *in vitro* transposon mutagenesis. The Δ*sll1371* exhibited a slow growth phenotype when compared to wild type *Synechocystis* cells. We observed that bicarbonate dependent maximum photosynthesis rate was lower in the Δ*sll1371* mutant when compared to wild type cells, when grown by bubbling ambient air (Low CO_2). However, when these cultures were grown at 5% CO2 in air (High CO_2), the bicarbonate dependent photosynthesis rates were similar. The number of carboxysomes were significantly reduced in the Δ*sll1371* mutant could be the reason for its lower photosynthetic maximum . The DNA microarray analysis led to the upregulation of genes encoding inorganic carbon transporters in Δ*sll1371* cells when compared to the wild type cells at Low CO_2 and the same was confirmed by the qRT-PCR. In the wild type *Synechocystis* cells grown at Low CO_2, the SyCrp1 protein was observed to be localized in the membrane. However, when these cells were treated with cAMP, the SyCrp1 was released from the membrane to cytosol indicating cAMP triggers release of SyCrp1. Further upon shift of these cells from Low CO_2 to High CO_2 we observed the release of SyCrp1 into cytosol. High CO_2 enhances the adenylate cyclase activity, leading to enhanced levels of cAMP which in turn cause the SyCrp1 to release from membrane to cytosol. Thus high CO_2 is involved in the translocation of SyCrp1 protein from membrane to cytosol. The data suggested that the cAMP mediated release of SyCrp1 depends on inorganic carbon availability. In general, since CRP proteins are known to regulate the expression of genes by binding to their DNA upstreams, it is likely that the SyCrp1 is a membrane anchored protein releasing to cytosol when required. SyCrp1's target DNA binding element ($TTTCAn_9TCn_4AAA$) was reported in the DNA upstreams of various CCM related genes, where it's binding was also demonstrated. We also observed that 6xHis-SyCrp1 binds to the DNA upstreams of *ndhR, cmpR* and *ccmk.* Thus, collectively our data suggests a regulatory role for SyCrp1 in carbon concentrating mechanism in *Synechocystis.*

PRIMER SEQUENCES

Primer used for the amplification of *sll1371* to generate the sll1371 mutant

Name	Primer Seaquence
Sll1371 TF-F	5’ TCCAGAAATGCTCCCTAGCAAGTG 3’
Sll1371 TF-R	5’ ATTCTGTGGCAACAAGCCCAACCC 3’

Primers used for qRT-PCR

Name	Primer seaquence
Sll1594 F	5’CCCTCAAGGTGACTAACCATGAGC 3’
Sll1594 R	5’GACGGGCAATGACCACTAAAGG 3’
Slr0040 F	5’CGGAAGGGATCATTACCAATGGC3’
Slr0040 R	5’AGCCCTTGATATAGTCCGCCG3’
Sll1732 F	5’GGGGGTAGTTGCCCTCCTGCCTTTG 3’
Sll1732 R	5’CCAGAGATGGAGAGGAAATTGAGCGC-3’
Slr1291 F	5’GCGGCCCAGATTGGCGTGTTCGTGGC-3’
Slr1291 R	5’CGGCGGCGGTGTAGAGCAAAAACTTC-3’
Slr1279 F	5’GCCCATTGGCGGAGCTTGGATTCAG-3’
Slr1279 R	5’GCCCAATTGATTAAAGGCAACGGCCC-3’
Slr1512 F	5’CTGCCTAACGTCAGAACCGTGG-3’
Slr1512 R	5’ATCGCCGTTACCAGGGCAGG-3’
16s rRNA F	5’TCGCCTACCGTTGGAGCC3’
16s rRNA R	5’GGGCACAATGGCTTCAACAA3’

Primers used for the amplification of upstream of sll1371 binding genes

Name	Primer Seaquence
Sll1594-EMSA-F:	5’GATAACCAATGATAGAATAAAGCC 3’
Sll1594-EMSA-R:	5’CGGTGTAGGCTCCTTTCTG 3’
Sll0030-EMSA-F:	5’TTAGTCATTGTCCTCGGGCAGG 3’
Sll0030-EMSA-R	5’AAGGGTTAGTACCGGTGCTGGC 3’
Slr1838-EMSA-F	5’CCTTGTTGGTCCGGTGTAGGC 3’
Slr1838-EMSA-R	5’GGGCATGGAAAATTCCTCTAGGG 3’

Primers used for Expression of Sll1371

Name	Primer Seaquence 5’-3’	R.E site
Sll1371 Exp- F-	5’GACTCATATGGGCACTAGTCCCCAAAATTC-3’	NdeI
Sll1371 Exp-R-	5’GCTGAATTCAGGAAATTAGATCTTCTAAAT 3’	E.coRI

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Submission date: 29-Oct-2019 11:54AM (UTC+0530)

Submission ID: 1202638170

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