University of Alberta

**LexA-mediated repression of the
cyanobacterial RNA helicase, *crhR***

by

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Dedication

To my mom and dad

Abstract

Expression of the cyanobacterial DEAD-box RNA helicase, *crhR*, is regulated in response to conditions which elicit reduction of the photosynthetic electron transport chain. Transcriptional regulation of *crhR* expression was investigated in this thesis. DNA affinity chromatography and mass spectrometry identified that the *Synechocystis* sp. strain PCC 6803 LexA orthologue binds the *crhR* gene. Recombinant LexA (rLexA) interacts specifically with both the *crhR* and *lexA* genes identifying both as LexA targets. Transcript analysis was initially used to investigate the LexA/*crhR* regulatory relationship and indicated that that *lexA* and *crhR* are divergently expressed under the redox conditions examined suggesting LexA is a negative regulator of *crhR* expression. *In vivo* and *in vitro* analysis confirmed LexA repression of *crhR* expression. rLexA decreases expression in a linear manner in an *in vitro* transcriptional/translation while *in vivo* reduction of *lexA* levels in a *lexA* heteroploid mutant correlated with increased accumulation of the *crhR* transcript under oxidizing conditions. Transcript analysis also demonstrated that expression of the *crhR*, *recA* and *lexA* genes in *Synechocystis* is not inducible by DNA damage.

A combination of DNaseI footprinting, site directed mutagenesis and electrophoretic mobility shift assays identified that the LexA-orthologue binds to a CTA-N_9-CTA direct repeat conserved within the open reading frame and the 5' untranslated region of the *crhR* and *lexA* genes, respectively. Furthermore, gel exclusion chromatography and an electrophoretic mobility shift assay-based

method were used to demonstrate that rLexA exists as a monomer in solution and as a dimer when bound to DNA.

Insertional inactivation of *crhR* and *lexA* was used to further investigate the physiological roles of both proteins in the cell. *crhR* is a non-essential gene. CrhR is required at low temperature as demonstrated by the absence of growth at 20°C. In contrast, *lexA* is an essential gene, evidenced by the continual maintenance of wild type copies of the gene after several generations on selective medium.

The potential significance of a LexA-orthologue in the regulation of redox-responsive gene expression and consequently, the implications of this novel role performed by LexA in *Synechocystis* are discussed.

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List of Abbreviations

°C	degrees Celsius
A	absorbance
Ala	alanine
ATP	adenosine triphosphate
bp	basepair(s)
BSA	bovine serum albumin
CO_2	carbon dioxide
cpm	counts per minute
CTD	carboxyl terminal domain
CTP	cytidine triphosphate
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-ρ-benzoquinine
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP(s)	deoxyribonucleotide triphosphate(s)
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
EtOH	ethanol
Fd	ferredoxin
FTR	ferrodoxin-thioredoxin reductase
Gly	glycine
GSH	reduced glutathione
GSSG	oxidized glutathione
h	hour(s)
His	histidine
IPTG	isopropyl-beta-D-thiogalactopyranoside
J	joules
kb	kilobase(s)
kDa	kilodalton(s)
Km	kanamycin

KOH	potassium hydroxide
Kd	equilibrium dissociation constant
Kr	retardation coefficient
LB	Luria-Bertani
LC	liquid chromatography
LHCII	light harvesting complex II
μE	microeinstein(s)
μg	microgram(s)
μl	microliter(s)
μM	micromolar
M	molar
m	meter(s)
Mb	megabase(s)
M-C	mitomycin-C
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar
M-MuLV RT	Moloney Murine Leukemia virus reverse transcriptase
MS	mass spectrometry
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
ng	nanogram(s)
nm	nanometer(s)
nM	nanomolar
nt	nucleotide(s)
NTD	amino terminal domain
OD	optical density
ORF	open reading frame
PAS	Per-Arnt-Sim domain
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
PET	photosynthetic electron transport
pfu	phage forming unit(s)
pmol	picomole(s)
pM	picomolar
PNK	polynucleotide kinase
ppm	parts per million

PSI	photosystem I
PSII	photosystem II
Rf	retention factor
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase	ribonuclease A
rRNA	ribosomal RNA
rNTP(s)	ribonucleotide triphosphate(s)
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT-PCR reaction	reverse transcriptase polymerase chain
s	second(s)
SDS	sodium doedecyl sulfate
Sm	streptomycin
Sp	spectinomycin
ss	single stranded
SSC	saline sodium citrate
SSPE	saline sodium phosphate
STE	sodium chloride Tris EDTA
TAK	thylakoid associated kinase(s)
TBE	TRIS borate EDTA
TBS	TRIS buffered saline
TBST	TRIS buffered saline tween
Tris	Tris (hydroxylmethyl) aminomethane
tRNA	transfer RNA
U	units
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
w/v	weight per volume

Chapter 1: Introduction

Cyanobacteria frequently encounter changing light environments and must respond accordingly to ensure continued growth and photosynthesis. Light sensing occurs either via direct mechanisms involving photoreceptor proteins, or indirectly through light-driven changes in the redox status of the electron transport chain or soluble carriers (Mullineaux, 2001; Chen et al., 2004). Photoreceptor proteins, the phytochromes, cryptochromes, and phototropins sense different light qualities and direct the cell to respond accordingly (Chen et al., 2004). Photoreceptors respond and regulate processes to improve photosynthetic capabilities, for example, phototaxis in cyanobacteria (Choi et al., 1999), or shade avoidance (Franklin and Whitelam, 2005), chloroplast movements (Wada et al., 2003), and circadian clock functioning in plants (Yankovsky and Kay, 2003). In contrast, direct regulation of photosynthesis-related gene expression has been shown to involve changes in the redox potential of the electron transport chain and photosynthesis-associated redox active compounds; glutathione, thioredoxin and reactive oxygen species (Figure 1.1) (Maxwell et al., 1995; Pfannschmidt et al., 1999a; Pfannschmidt et al., 1999b; Pfannschmidt et al., 2001).

1.1 Redox-active compounds

The redox poise of plastoquinone and cytochrome b_6f within the electron transport system (Figure 1.2) provides a direct measure of PSII/PSI excitation and monitors the predominating environment for example, temperature and CO_2 availability (Myers, 1986; Gal et al., 1997; Pfannschmidt, 2003). Thioredoxin and
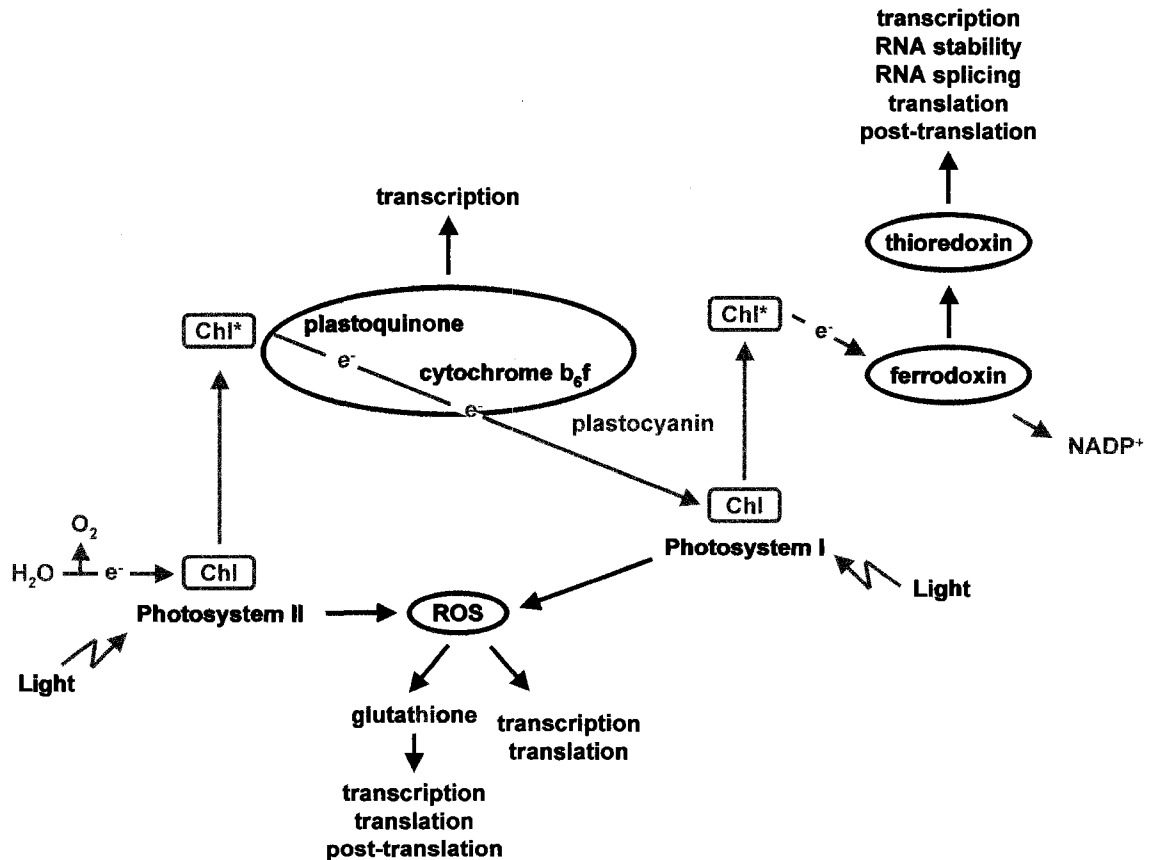
Figure 1.1 Photosynthetic redox carriers and their influences on gene expression. The electron transport chain of photosynthetic organisms is represented as a Hill-Bendall Z-scheme. The major photosynthetic redox carriers are represented by red ovals. The effects of the various redox carriers on short- and long-term responses are indicated in blue.

(Buchanan, 1991; Danon and Mayfield, 1994a; Escoubas *et al.*, 1995; Kim and Mayfield, 1997; Liere and Link, 1997; Pfannschmidt *et* al., 1999a; Pfannschmidt *et al.*, 1999b; Baena-González *et al.*, 2001; op den Camp *et al.*, 2003; Allen, 2004).
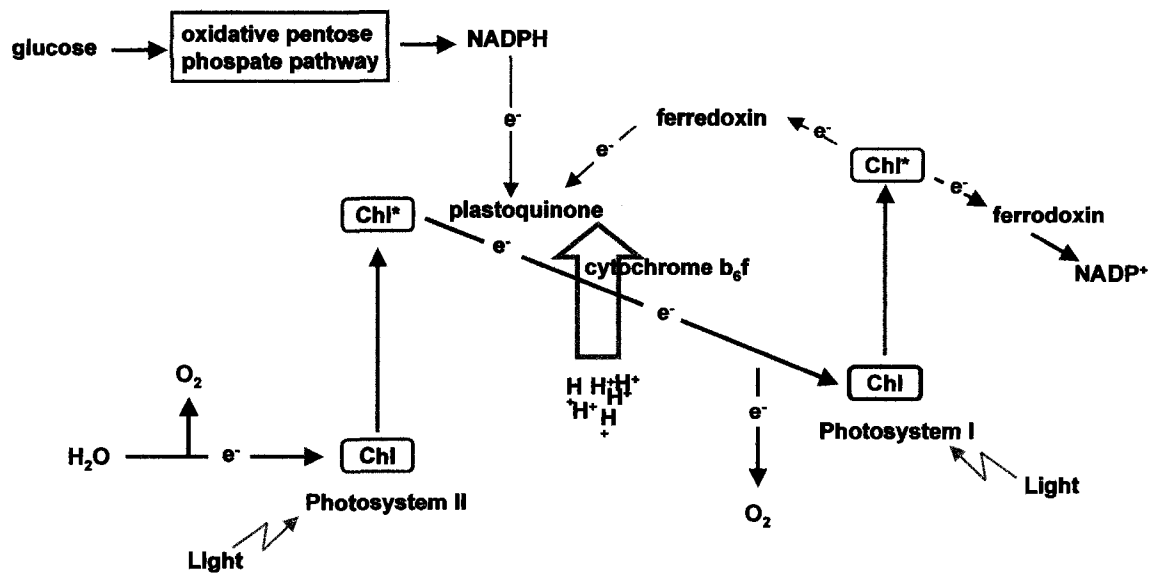
Figure 1.2 Photosynthethic and respiratory electron flow in cyanobacteria. The electron transport chain of cyanobacteria is represented as a Hill-Bendall Z-scheme. Electron flow common to both the photosynthetic and respiratory transport chains are depicted by the black arrows. Electron flow specific to the photosynthetic and respiratory transport chains are depicted by the red and blue arrows respectively. The ATP coupling site is represented by a green arrow (adapted from Allen, 2004)

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glutaredoxin are small, soluble redox active compounds responsible for maintaining the redox state of thiol groups (-SH) found within cellular proteins and enzymes (Lemaire, 2004; Michelet *et al.*, 2005; Lemaire *et al.*, 2007). Oxidation and reduction of thiol groups within a protein or enzyme may modify its cellular function. For example, in the chloroplast, light-induced thiol reduction performs well characterized roles in the activation and regulation of carbon fixation enzymes (Buchanan, 1991; Ruelland and Miginiac-Maslow, 1999) and the transcriptional and translational machinery (Danon and Mayfield, 1994a; Kim and Mayfield, 1997; Liere and Link, 1997; Fong *et al.*, 2000; Alergand *et al.*, 2006).

Thioredoxin activity is influenced by the electron environment of photosystem I (PSI) (Figure 1.2). The ferredoxin-thioredoxin system transfers electrons from PSI to thioredoxin via ferredoxin and ferredoxin-thioredoxin reducatase (FTR). Reduced thioredoxin is available to regulate downstream targets (Schürmann, 2003; Lemaire *et al.*, 2007). Glutaredoxins use glutathione as an electron donor to post-translationally modify target proteins through thiol reduction or the formation of a mixed disulfide between reduced glutathione and a redox-active cysteine residue on the target protein in a process known as glutathionylation (Lemaire, 2004; Michelet *et al.*, 2005; Michelet *et al.*, 2006). Glutathionylation is a common control mechanism in mammalian systems which modifies the activity of target proteins. (Michelet *et al.*, 2006). Recently, glutathionylated proteins have been identified in *Arabidopsis* including fructose-1,

6-bisphosphate aldolase and cytosolic triose phosphate isomrease (Ito *et al.*, 2003) extending this mode of enzyme regulation to other systems. In addition, the ratio of reduced to oxidized glutathione (GSH: GSSG) in the cell is an important indicator of redox status in animals, plants, and bacteria. In photosynthetic organisms, the GSH: GSSG ratio is influenced by the light environment, production of reactive oxygen species, and normal cellular metabolic activities (Baena-González *et al.*, 2001, Mullineaux and Karpinski, 2002; Meyer and Hell, 2005, Michelet *et al.*, 2006). Changes to the GSH: GSSG ratio induces modification of protein thiol groups influencing gene expression and/or protein activities (Baginsky *et al.*, 1997; Karpinski *et al.*, 1997; Baginsky *et al.*, 1999; Baena- González *et al.*, 2001).

Reactive oxygen species are produced as a result of excess electron flow from high light or environmental stress conditions (Mullineaux and Karpinski, 2002; Kimura *et al.*, 2003). Singlet oxygen is primarily produced by overexcitation of PSII while superoxide anion and H_2O_2 are formed at PSI through the Mehler reaction (Mullineaux and Karpinski, 2002; Beck, 2005). H_2O_2 and singlet oxygen species are important signaling molecules from the chloroplast to the nucleus in plants (Yabuta *et al.*, 2004; Beck, 2005) and may directly influence expression of both nuclear and chloroplast genes (op den Camp *et al.*, 2003).

1.2 Redox-regulated responses

Redox poise regulates short- and long-term responses in photosynthetic organisms. Initial data suggested independence among the various redox active compounds. However, emerging data has demonstrated the existence of

crosstalk and cooperation between the different redox carriers in regulating cellular responses (Rintamäki *et al.*, 2000; Trebitsh *et al.*, 2000). The redox regulation of state transitions and gene expression in higher plants and green algae described below demonstrates this interplay among the various redox carriers.

1.2.1 Short-term responses: the state transition

State transitions are short-term responses to excitation imbalances between the two photosystems. Efficient photosynthesis requires that both photosystems equally absorb light with imbalances resulting in diminished photosynthetic efficiency and increased light-induced damage. In higher plants and green algae, light is harvested by chlorophyll protein complexes (LHCII) and is subsequently transferred to the chlorophyll *a* dimer of the PSI and PSII reaction centers (Scheller *et al.*, 1997; Allen *et al.*, 1998). The light-harvesting machinery reversibly associates with the thylakoid membranes and is capable of lateral diffusion along the thylakoid membranes from PSI to PSII and vice-versa. This mobility and lateral diffusion of the light-harvesting machinery is the basis of the state transition. Reversible phosphorylation of the LHCII determines photosystem association. Under state 1 conditions, the proteins remain unphosphorylated and associate with PSII. Phosphorylation leads to a state 1 to state 2 transition involving protein dissociation from PSII and reassociation with PSI. This reversible association ensures balanced light-harvesting under the predominating light conditions. The reversible phosphorylation is catalyzed by a thylakoid membrane-associated kinase which senses and responds to the redox

poise of the plastoquinone and cytochrome b_6f electron carriers (Allen *et al.*, 1995; Gal *et al.*, 1997; Mullineaux *et al.*, 1997). Recently, the thioredoxin-ferredoxin system was also implicated in regulating activity of the LHCII kinase. The LHCII kinase contains a pair of redox-responsive cysteine residues susceptible to disulfide formation. Thioredoxin may provide the reducing power for disulfide formation and subsequent structural changes regulating kinase activity (Rintamäki *et al.*, 2000). The two mechanisms of regulation cooperate to regulate kinase activity ensuring activity when required by the cell (Rintamäki *et al.*, 2000).

Similarly, the redox poise between plastoquinone and cytochrome b_6f determines phycobilisome-photosystem association in cyanobacteria (Mao *et al.*, 2002). Unlike the situation in higher plants, the mechanisms controlling the state transition in cyanobacteria are not well-understood. Recently, a mutation within the *Synechocystis rpaC* gene was shown to block state transitions. The biochemical role of RpaC remains unknown and consequently its role in the state transition is still unclear (Emlyn-Jones *et al*,, 1999; Mullineaux and Emlyn-Jones, 2005).

1.2.2 Long-term responses: changes in gene expression

In higher plants and green algae, expression of both nuclear and chloroplast genes is regulated by redox (Table 1.1). Initially, regulated genes were believed to be directly involved in or connected to photosynthesis; however, recent transcriptome analyses have identified many genes unrelated to photosynthesis which respond to redox signals (Richly *et al.*, 2003; Fey *et al.*,

Table 1.1 Putative redox-regulated genes of green algae and higher plants. The gene, encoding genome, level of regulation and redox carrier (photosynthetic electron transport chain or soluble carrier) involved are indicated.

Gene	Genome	Organism	Level of Regulation	Redox carrier[a]	Reference
apx1, apx2	nuclear	Arabidopsis	transcription	PET	1, 2, 3
fed1	nuclear		post-transcription	thioredoxin	4, 5
cab/Lhcb	nuclear	Green algae	transcription	PET	6, 7
petE	nuclear	Pisium sativum	transcription	PET	8, 9
por		Marchantia paleacea var. diptera	post-transcription	?	10
psaD	nuclear	Sinapsis alba	transcription / post-transcription	?	8, 11
psaF	nuclear	S. alba	transcription	PET	8
psaAB	chloroplast	S. alba	transcription	?	12-15
psbA	chloroplast	S. alba	transcription	PET	12- 15
psbA	chloroplast	C. reinhardtii	translation / post-transcription	thioredoxin / PET	16-17
rbsC	nuclear	Arabidopsis (cell culture)	transcription	thioredoxin/ glutathione?	18
rbsL	chloroplast	C. reinhardtii		ROS/glutathione	19
p54		S. alba	post-translation	glutathione	20
PTK		S. alba	post-translation	glutathione	21-23

a. PET: photosynthetic electron transport, ROS: reactive oxygen species
1. Karpinski et al., 1997; 2. Karpinski et al., 1999; 3. Yabuta et al., 2004; 4. Petracek et al., 1997; 5. Petracek et al., 1998; 6. Escoubas et al., 1995; 7. Maxwell et al., 1995; 8. Pfannschmidt et al., 2001; 9. Sullivan and Gray, 2002; 10. Eguchi et al., 2002; 11. Sherameti et al., 2002; 12. Pfannschmidt et al., 1999a; 13. Pfannschmidt et al., 1999b; 14. Kovacs et al., 2000; 15. Tullberg et al., 2000; 16. Danon and Mayfield, 1994a; 17. Deshpande et al., 1997; 18. Oswald et al., 2001; 19. Irihimovitch and Shapira, 2000; 20. Liere and Link, 1997; 21. Baginsky et al., 1997; 22. Baginsky et al., 1999; 23. Baena-González et al., 2001

2005). Regulation of chloroplast gene expression was initially believed to occur predominately via post-transcriptional mechanisms: translation initiation, RNA processing, RNA stability, and protein stability (Gillham *et al.*, 1994; Rochaix, 1996). Basal transcription of all chloroplast genes including photosynthetic proteins in non-photosynthetic plastids supported post-transcription/translation as the main points of regulatory control (Deng and Gruissem, 1988). However, evidence from barley, mustard and *Arabidopsis* is emerging showing that transcriptional control in response to redox does play an important role in cellular responses for maintaining maximal photosynthetic efficiency (Klein and Mullet, 1990; Pfannschmidt *et al.*, 1999a; Pfannschmidt *et al.*, 1999b).

The *cab* and photosystem genes of higher plants are regulated at the transcriptional level by the redox poise of the photosynthetic electron transport chain (PET). For example, expression of the nuclear *cab* genes encoding the light harvesting LHCII proteins is regulated by the redox status of the plastoquinone pool. Specifically, *cab* gene transcripts accumulate when the plastoquinone pool is oxidized, while reduced plastoquinone is correlated with a 3-fold decrease in transcript levels (Escoubas *et al.,* 1995). In the chloroplast genome, expression of the *psbA* and *psaB* genes encoding the photosystem core proteins are differentially by the redox status of the electron transport chain. The *psbA* transcript accumulates when plastoquinone is oxidized while *psaB* transcripts accumulate under plastoquinone reducing conditions (Pfannschmidt *et al.*, 1999a; Pfannschmidt *et al.*, 1999b; Puthiyaveetil and Allen, 2008). Thus in many systems, the photosystem gene transcripts differentially accumulate in

response to the redox poise of PET, specifically accumulating under conditions where the light quality dictates their requirement.

In contrast, expression of the *psbA* gene in *Chlamydomonas reinhardtii* is regulated post-transcriptionally. Both *psbA* mRNA translation and splicing are responsive to the thioredoxin and PET redox poises (Danon and Mayfield, 1994a; Deshpande *et al.*, 1997; Lee and Herrin, 2003). Redox-regulated translation of the *psbA* transcript requires binding of a multi-protein complex to a short stem-loop containing sequence within the 5' UTR (Danon and Mayfield, 1991). The *psbA* translation complex is composed of 4 polypeptides, RB38, RB47, RB55 and RB60. RB47 is a chloroplast polyadenylate binding protein whose binding capabilities are modulated by the RB60 protein (Kim and Mayfield, 1997; Yohn *et al.*, 1998). Activity of the RB60 protein disulfide isomerase is responsive to the ferredoxin-thioredoxin system (Kim and Mayfield, 1997). The redox state of RB60 disulfide is coupled to RB47 binding capabilities. RB60 reduces the RB47 disulfide inducing a conformational change within the protein exposing RNA binding sites and consequently allowing interaction with the *psbA* mRNA and translation initiation (Fong *et al.*, 2000; Alergand *et al.*, 2006). Translation of the *psbA* message is additionally influenced by other cellular metabolic status indicators including ADP-dependent phosphorylation of the RB60 protein (Danon and Mayfield, 1994b; Kim and Mayfield, 1997) and the plastoquinone redox poise (Trebitsh *et al.*, 2001).

In cyanobacteria, both photosynthetic and non-photosynthetic genes have been shown to exhibit redox-dependent expression (Table 1.2). Glutamine

Table 1.2 Putative redox-regulated genes of *Synechocystis* sp. strain PCC 6803. The gene, level of regulation and redox carrier (photosynthetic electron transport or soluble) involved are indicated.

Gene	Level of Regulation	Redox carrier[a]	Reference
crhR	transcription	PET	1
glnA	transcription	PET	2
glnB	transcription	PET	3
ntcA	post-translational	thioredoxin	4
desABC	transcription	PET	5
secA	transcription	PET	6
trxA	transcription	PET	7
psbA	transcription	PET	8, 9
psaAB, psaC, psaD, psaL1	transcription	PET	10
psaE	transcription	PET	8, 9
apcABC, cpcBA	transcription	PET	8, 10
sigB	transcription	PET	11
sigD	transcription	PET	11
NADPH deydrogenase supercomplex	transcription/ post-translational	PET	12
pedR	post-translational	PET	13
PRK gene	post-translational	thioredoxin	14

a. PET: photosynthetic electron transport
1. Kujat and Owttrim, 2000; 2. Reyes and Florencio, 1995; 3. García-Dominguez and Florencio, 1997; 4. Alfonso et al., 2001; 5. Kis et al., 1998; 6. Mazouni et al., 1998; 7. Navarro et al., 2000; 8. Alfonso et al., 2000; 9. El Bissati and Kirilovksy, 2001; 10. Li and Sherman, 2000; 11. Imamura et al., 2003; 12. Ma et al., 2008; 13. Nakamura and Hihara, 2006; 14.Kobayashi et al., 2003

synthetase from *Synechocystis* sp. strain PCC 6803 was the first identified cyanobacterial gene regulated by a redox mechanism. *glnA* transcript accumulation is dependent on the redox poise of the electron transport chain as demonstrated by differential accumulation following incubation under different light environments or in the presence or absence of glucose and electron transport chain inhibitors (Reyes and Florencio, 1995). The role of thioredoxin and glutaredoxin in regulating cyanobacterial gene expression is only beginning to emerge with the identification of several targets of both redox carriers including proteins involved in for example light harvesting, the Calvin cycle and oxidative stress response (Sippola and Aro, 1999; Lindahl and Florencio, 2003; Li *et al.*, 2007).

1.2.3 Redox-regulatory mechanisms-sensing PET redox poise

Glutathione and thioredoxin directly influence gene expression and protein function through structural changes induced by disulfide reduction and oxidation (Buchanan, 1991; Ruelland and Miginiac-Maslow, 1999). Photosynthetic electron flow in chloroplasts is hypothesized to influence cellular activity and/or gene expression through the activity of chloroplast thylakoid membrane associated kinases. However, it is only in the last decade that these kinases, STN7/Stt7, STN8 and TAKs (thylakoid-associated kinases), have been identified (Snyders and Kohorn, 1999; Snyders and Kohorn, 2001; Bonardi *et al.*, 2005, Bellafiore *et al.*, 2005). The STN7/Stt7 kinases from *Arabidopsis* and *Chlamydomonas reinhardtii*, respectively are required for state transitions (Depège *et al.*, 2003;

Bonardi *et al.*, 2005). Mutants in the STN7 or Stt7 gene lacks the ability to undergo state transitions and exhibit reduced growth under conditions where light quality and quantity are frequently changing (Depège *et al.*, 2003; Bellafiore *et al.*, 2005). The identified TAKs are also proposed to be involved in phosphorylation of LHCII proteins during state transitions. Furthermore, the identification of multiple TAKs suggests the potential for a phosphorylation cascade in regulating protein phosphorylation which may or may not also involve the STN7 kinase (Snyders and Kohorn, 1999; Bonardi *et al.*, 2005). A phosphorylation cascade was previously suggested to link nuclear expression of the *cab* genes to redox signals within the chloroplast (Escoubas *et al.*, 1995). Chloroplast proteins including the thylakoid-associated kinases themselves exhibit reversible phosphorylation in response to redox (Snyder and Kohorn, 1999; Aro and Ohad, 2003; Depège *et al.*, 2003) and furthermore, inhibition of cytoplasmic phosphatase activity disrupts signaling between the chloroplast and the nucleus (Escoubas *et al.*, 1995; Durnford and Falkowski, 1997).

However, no conclusive evidence exists to link these thylakoid-associated kinases to either chloroplast or nuclear gene expression. Both STN7 and TAKs have been suggested as a potential link between redox poise and gene expression due to the impairment of long-term responses in kinase mutants (Snyders and Kohorn, 1999; Bonardi *et al.*, 2005). Alternative regulators identified as potential links between PET redox poise and gene expression include a 31 kDa spinach DNA binding protein and the plant-specific TSP9 protein (Cheng *et al.*, 1997; Carlberg *et al.*, 2003). Recently, a bacterial-like

sensor kinase and response regulator were also identified within the chloroplast as alternative mechanisms of sensing and responding to PET and modulating gene expression (Weber *et al.*, 2006; Allen *et al.*, 2007; Puthiyaveetil and Allen, 2008). Inactivation of the chloroplast sensor kinase led to loss of redox-regulated transcription of chloroplast genes (Allen *et al.*, 2008; Puthiyaveetil and Allen, 2008).

In cyanobacteria, modulators of the redox regulated expression of target genes at the transcriptional level have been proposed. Expression of photosynthetic and photopigment genes has been attributed to the RppAB two-component signal transduction system and the alternative sigma factors, *sigB* and *sigD* (Li and Sherman, 2000; Imamura *et al.*, 2003; Yoshimura *et al.*, 2007). The RppAB two-component system is composed of the sensor kinase, RppB, and the response regulator, RppA. RppB is proposed to sense the plastoquinone redox balance and regulate gene expression accordingly through phosphorylation-induced alteration of RppA DNA binding activity (Li and Sherman, 2000). The levels of sigma factors, SigB and SigD, are modified by the electron transport chain. Specifically, the SigB protein accumulates under oxidizing conditions while the SigD protein accumulates under reducing conditions (Imamura *et al.*, 2003). Transcriptome analysis of *sigB* and *sigD* mutants implicates them in redox- and light-dependent expression of both photosynthetic and non-photosynthetic genes (Imamura *et al.*, 2003; Summerfield and Sherman, 2007; Yoshimura *et al.*, 2007). Furthermore, it suggests that sigma factor availability under specific redox conditions is an

important regulator of gene expression (Imamura *et al.*, 2003; Summerfield and Sherman, 2007; Yoshimura *et al.*, 2007).

In addition, NblS is a membrane-associated sensor histidine kinase which responds and controls photosynthesis-related gene expression in response to high light and nutrient stress. The presence of a redox-sensing PAS domain and thylakoid membrane association of NblS are clues for its association with photosynthetic or cellular redox poise. However, no direct evidence of redox sensing has been demonstrated and to date, identities of transcriptional regulators responding to NblS remain to be identified (van Waasbergen *et al.*, 2002).

Redox potentials can also modulate protein activity at the post-translational level. For example, the transcriptional regulators, NtcA and PedR, regulate expression of target genes in response to redox-modulated changes in protein structure and activity (Jiang *et al.*, 1997; Alfonso *et al.*, 2001; Nakamura and Hihara, 2005). NtcA, controls expression of genes involved in nitrogen acquisition in response to both nitrogen levels and photosynthetic electron transport (Alfonso *et al.*, 2001). The binding activity of NtcA is regulated by the cellular thiol pool through one or more redox sensitive cysteine residues (Jiang *et al.*, 1997; Alfonso *et al.*, 2001). In contrast, PedR responds to PET, undergoing a conformational change in response to reducing conditions which alters its regulatory control of target genes including the pigment biosynthetic genes and a NAD(P)H dehydrogenase subunit (Nakamura and Hihara, 2006). The PedR protein is conserved among cyanobacteria and suggests the existence of a

common redox-sensing mechanism in cyanobacteria (Nakamura and Hihara, 2006).

While the number of known redox-sensitive regulators in cyanobacteria increases, the regulators of many known redox-regulated genes including the crhR RNA helicase remain undiscovered. The similarities between plant and cyanobacterial systems suggest that homologs of identified plant kinases and regulators may also be involved in cyanobacterial responses. However, most of the identified proteins in higher plants to date lack a cyanobacterial homolog (Weber et al., 2004; Carlberg et al, 2003). Therefore, our knowledge of signal transduction pathways responsible for regulating redox-responsive gene expression remains preliminary at best.

1.3 Cyanobacteria as a model organsism

The cyanobacteria are prokaryotic organisms distinguished by their ability to carry out oxygenic photosynthesis (Stanier and Cohen-Bazire, 1977). The largest, most diverse group of photosynthetic organisms and the accepted ancestor of the plant chloroplast, cyanobacteria exhibit tolerance to a wide range of environmental extremes permitting its widespread distribution in a variety of habitats (Stanier and Cohen-Bazire, 1977; McFadden, 2001). This diversity has led to a range of both harmful and beneficial roles including the production of toxic cyanobacterial blooms and a crucial position in the ocean food web and worldwide oxygen balance respectively (Stanier and Cohen-Bazire, 1977; de Figueiredo et al., 2004). The cyanobacteria are distinguished by their

developmental properties and structure resulting in six sub-sections of related

organisms (Stanier and Cohen-Bazire, 1977; Rippka *et al.*, 1979).

The organism of interest in this study is *Synechocystis* sp. strain PCC

6803 (referred to as *Synechocystis*), a member of subsection I or *Chroococcales*

(Stanier and Cohen-Bazire, 1977). Many features distinguish *Synechocystis* as a

model organism for the study of photosynthesis including its natural competency,

its ability to grow photoheterotrophically and the availability of its genome

sequence (Anderson and McIntosh, 1991; Grigorieva and Shestakov, 1992;

Kaneko *et al.*, 1995). The 3.6 Mb circular *Synechocystis* genome encodes 3186

putative proteins, 2 rRNA operons, and 42 tRNA genes (Kotani *et al.*, 1995

Kaneko *et al.*, 1996; Kaneko and Tabata, 1997). Sequence data is available from

the Cyanobase site (http://www.kazusa.or.jp/cyano/cyano.html).

1.3.1 Bioenergetics

Cyanobacteria preferentially grow photoautotrophically obtaining ATP and

reducing power for metabolism and growth from the photosynthetic conversion of

light energy into chemical energy (Chitnis, 1996). Photosystems and electron

carriers located within the thylakoid membranes of cyanobacteria are responsible

for the electron transfers and chemical reactions which produce ATP energy and

reducing power (Chitnis, 1996; Barber *et al.*, 1997).

Figure 1.2 represents the general electron flow from the primary electron

donor to the terminal electron acceptor, $NADP^+$, in cyanobacteria. At least two

patterns of electron flow, cyclic and non-cyclic, are possible. Non-cyclic flow

involves both PSI and PSII producing ATP, NADPH and O_2 while cyclic flow

involves only PSI and contributes solely to the ATP-generating proton motive

force (Allen *et al.*, 1995; Chitnis, 1996).

During non-cyclic electron flow, photons of light excite one electron from

the primary electron donors of PSI and PSII, initiating a series of electron transfer

reactions. Electron flow proceeds from PSII to PSI via an interphotosystem

electron system composed of membrane bound, cytoplasmic and lumenal

proteins (Scheller *et al.*, 1997), reaching the terminal electron acceptor $NADP^+$.

Reduction-oxidation reactions are coupled to proton translocation across the

thylakoid membrane and consequently to ATP generation via

photophosphorylation. Coupling of electron flow and proton translocation occurs

at the cytochrome b_6f complex. Electrons lost from the primary electron donors of

PSI and PSII are replaced by oxidation of plastocyanin and a PSII-associated

water splitting reaction, respectively (Chitnis, 1996; Barber *et al.*, 1997).

A secondary mechanism for ATP production; typical cyclic electron flow

involves only PSI. The electrons lost upon light excitation cycle back to PSI via

plastoquinone and the inter-photosystem electron transport chain. The reduction

in electron potential during transfer through the PET chain contributes to charge

separation across the membrane and ATP generation (Chitnis, 1996).

1.3.2 The photosynthetic machinery

The photosynthetic machinery is located on the thylakoid membrane and

consists of photosystems I and II, the interphotosystem electron transport chain

and the light-harvesting antennae. PSII and PSI catalyze the light-driven reactions of photosynthesis and are respectively, pheophytin-quinone type and iron-sulfur type systems (Allen and Williams, 1998). The photosystems are composed of a core reaction center containing the primary chlorophyll *a* electron donor and associated proteins and pigments required for the light harvesting and electron transfer reactions (Barber *et al.*, 1997; Scheller *et al.*, 1997; Xu *et al.*, 2001). PSII catalyzes the oxidation of water and the reduction of plastoquinone (Barber *et al.*, 1997). PSI catalyzes the oxidation of plastocyanin by transferring its electrons and donating them to ferredoxin. Reduced ferredoxin provides electrons to Fd-NADP$^+$ oxidoreductase for the production of NADPH (Chitnis, 1996; Scheller *et al.*, 1997; Kareptyan *et al.*, 1999).

The electron transport chain connecting PSII and PSI is responsible for both electron transfer and proton translocation (Figure 1.2). PSII-associated plastoquinone accepts electrons from PSII and transfers them to cytochrome b_6f. In its reduced state, plastoquinone is freely diffusible in the membrane, interacting with cytochrome b_6f to transfer electrons (Barber *et al.*, 1997). This electron transfer is coupled to proton translocation across the thylakoid membrane (Barber *et al.*, 1997; Allen, 2004). Plastocyanin is a lumenal copper protein responsible for providing electrons to oxidized PSI. Electron transfer within plastocyanin involves a redox-sensitive Cu^{2+} atom (Morand *et al.*, 1994). Ferredoxin is a soluble cytoplasmic carrier responsible for the transfer of a single electron from PSI to ferredoxin-NADP$^+$ oxidoreductase and ultimately the

terminal electron acceptor, $NADP^+$. NADPH production requires 2 electrons from ferredoxin (Morand *et al.*, 1994).

Light is harvested over a large surface area and focused to the reaction centers of PSI and PSII. A large surface area is necessary to ensure sufficient light is harvested for maintenance of photosynthesis (Scheller *et al.*, 1997; Allen *et al.*, 1998). The phycobilisomes are the light harvesting antennae in cyanobacteria. The phycobilisome is a macromolecular complex composed of the pigmented phycobiliproteins. Three major classes of phycobiliproteins exist; phycoerythrin, phycocyanin and allophycocyanin (Grossman *et al.*, 1993; Mullineaux *et al.*, 1997). The phycobiliproteins are composed of 2 subunits, α and β each carrying chromophores responsible for light absorption. Non-pigmented linker proteins help the phycobiliproteins assemble into the hexameric assemblages of the phycobilisomes. The phycobilisome has a "fan-like" arrangement composed of a cylindrical core and six rod-like structures radiating from the core (Grossman *et al.*, 1993; Mullineaux *et al.*, 1997). The phycobilisome antennas are anchored to the thylakoid membrane through a high MW protein. Harvested light is transferred along the phycobilisome antennas to the primary chlorophylls in both PSII and PSI (Grossman *et al.*, 1993; Mullineaux *et al.*, 1997).

1.3.3 Cyanobacterial respiration

Synechocystis is capable of photoheterotrophic growth in the absence of light. *Synechocystis* uses glucose to produce ATP energy allowing for survival

until the reintroduction of light (Scherer, 1990). Glucose is metabolized by the oxidative pentose phosphate pathway with NADPH produced being the primary electron donor to the respiratory electron chain (Schmetterer, 1994). The respiratory electron transport chain shares common electron carriers with photosynthetic electron transport including plastoquinone, cytochrome b_6f and plastocyanin. Plastoquinone is the established point of entry for electrons from both PSII and NAD (P) H-dehydrogenase (Hirano *et al.*, 1980; Scherer *et al.*, 1990). Electrons from respiratory metabolism enter at plastoquinone ultimately reaching O_2, the terminal electron acceptor, producing H_2O (Schmetterer, 1994).

1.4 Control of gene expression

As previously discussed, one way by which photosynthetic organisms respond to changes in light intensity and quality is through changes in gene expression. These responses to a new environment ensure only genes required under the predominating conditions are expressed. In bacteria, transcription initiation is a major point of expression control with multiple mechanisms existing. These mechanisms include promoter structure, sigma factor availability, bacterial chromosome structure and transcription factors.

The limiting levels of RNA polymerase (RNAP) in the cell results in a competition among sigma factors for available core polymerases and among promoters for the RNA polymerase holoenzyme. The limited availability of RNAP may control the level of gene expression from a specific promoter in two ways. Firstly, the strength of a promoter dictates its ability to compete for the available

pool of RNA polymerase. Promoter strength is influenced by the sequence and alignment of the -10 and -35 promoter elements. In *E. coli*, the -10 and -35 consensus sequences are 5'-TTGACA-3' and 5'-TATAAT-3' respectively. Sequences more similar to the consensus will be preferentially expressed over those genes containing divergent elements. Similarly, increasing or decreasing the spacing between the -10/-35 elements within the promoter will reduce promoter strength and consequently gene expression. Secondly, promoters recognized by an alternative sigma factor will be influenced by its availability. Expression of alternative sigma factors is environmentally or stress regulated. The differential expression of the sigma factor will determine when a regulated promoter is recognized and a gene expressed (Browning and Busby, 2004). For example, the *E. coli* σ^s sigma factor is only expressed during stationary phase and consequently, genes containing a σ^s promoter sequence are only transcribed during stationary phase growth (Loewen *et al.*, 1998).

The structure of the bacterial chromosome can also regulate transcription initiation events. DNA exists as a supercoiled structure interacting with protein and/or RNA. Therefore, the compact structure of the DNA will both expose and sequester different promoter elements. Non-specific binding by proteins such as Fis (factor for inversion stimulation), H-NS, and IHF (intergration host factor) can alter the DNA structure and consequently gene expression patterns through the differential exposure of specific promoter elements. Specific binding of the DNA by protein is the major mechanism by which the rate of transcription initiation is regulated. The actions of transcriptional regulators can increase or decrease the

RNA polymerase's affinity for the promoter (Figure 1.3) (Browning and Busby, 2004).

1.4.1 Transcriptional regulators in bacteria

Transcriptional regulators activate or repress gene expression. They are commonly differentiated by the location and sequence of their binding site. Activator binding sites are usually located from -30 to -100 (with respect to the transcriptional start site) and function to enhance DNA-RNAP contacts. Activators make pre-existing poor promoters more efficient by stabilizing open complex formation and/or altering DNA conformation. Conversely, repressor binding sites usually overlap important promoter elements restricting RNAP access and blocking RNAP-promoter complex formation (Choy and Adhya, 1996; Record *et al.*, 1996).

Commonly, steric hindrance is used by repressors to block RNAP binding (Figure 1.3 A). The LexA, LacR and λCI proteins are classical examples repressing transcription by binding DNA at sites overlapping promoter elements and blocking RNAP access (Majors, 1975; Little *et al.*, 1981; Little and Mount, 1982; Hawley *et al.*, 1985; Fernández de Henestrosa *et al.*, 2000). Alternative repressive mechanisms working in bacteria are DNA looping (Figure 1.3 B), promoter remodeling, and anti-activation. Proteins bound at distal sites can interact, looping the DNA to block RNAP access to the promoter. For example, maximal repression of the *lac* operon involves LacR binding at two distal sites and protein-protein interactions to loop the DNA (Oehler *et al.*, 1990). Repressor

A Steric hindrance
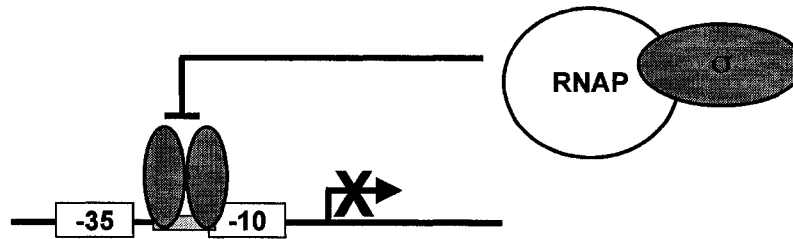
B DNA looping
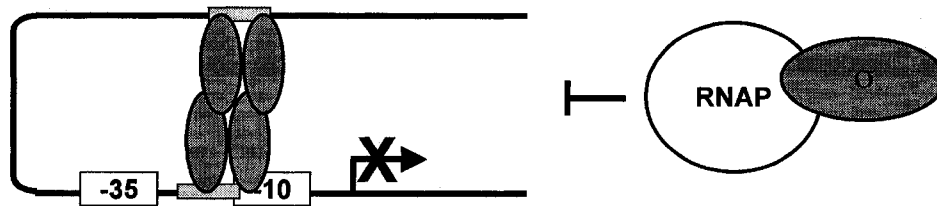
C Activation
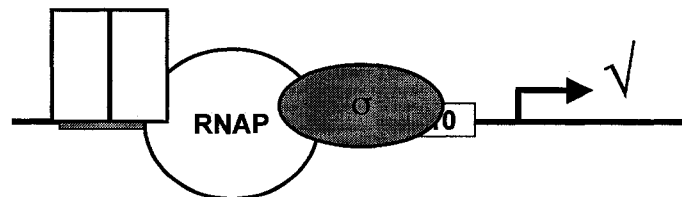
D Promoter remodelling
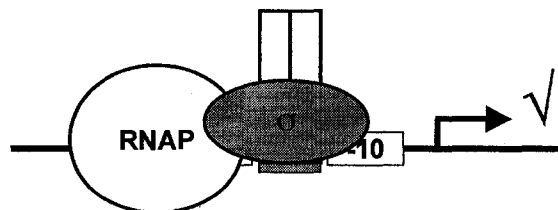
Figure 1.3 Transcriptional regulation of gene expression
A. Steric Hindrance. Protein binding at target sites within the promoter blocks RNA polymerase access and binding to the promoter
B. DNA looping. Repressor proteins bound within the promoter and at distal sites may interact, looping the DNA to block RNAP access to the promoter.

C. Activation. Protein-protein interactions between the RNA polymerase and the activator improves transcription from a promoter by increasing affinity of the RNAP for the promoter

D. Promoter remodeling. Protein binding within the promoter results in structural changes making the promoter more suitable for RNA polymerase binding and transcription initiation.

(Majors, 1975; Little *et al.*, 1981; Little and Mount, 1982; Hawley *et al.*, 1985; Heltzel *et al.*, 1990; Oehler *et al.*, 1990; Ansari *et al.*, 1992; Ebright, 1993; Kuldell and Hochschild, 1994; Li *et al.*, 1994; Valentin-Hansen *et al.*, 1996; Lonetto *et al.*, 1998; Fernández de Henestrosa *et al.*, 2000; Dove *et al.*, 2003; Browning and Busby, 2004).

proteins bound at distant sites are also hypothesized to influence gene expression through downstream structural rearrangements within the promoter making it less attractive to the RNAP (Pérez-Martín and Espinosa, 1991; Rojo and Salas, 1991; Sheridan *et al.*, 2001). The *E. coli* CytR protein represses expression of the *deo* operon by interfering with cyclic AMP receptor protein (CRP)-mediated activation of the operon. The CRP-CytR interaction may mask regions of CRP important for activation or reposition CRP in an inactive location on the DNA (Valentin-Hansen *et al.*, 1996).

Transcriptional activators increase the affinity of RNA polymerase for its promoter (Figure 1.3C). Protein-protein interactions between RNAP and the activator can improve transcription from a weak promoter by stabilizing complex formation. Two types of protein-protein interactions have been identified; λCI, PhoB and AraC proteins interact with domain 4 of σ^{70} while CRP contacts the α-carboxyl terminal domain of the core enzyme (Ebright, 1993; Kuldell and Hochschild, 1994; Li *et al.*, 1994; Lonetto *et al.*, 1998; Dove *et al.*, 2003). Promoter topology can also influence RNA polymerase binding. The binding of an activator protein can alter a promoter's conformation making it more attractive to RNA polymerase binding (Figure 1.3D). The -10 and -35 elements of the *E. coli mer* operon are separated by 19 nucleotides resulting in poor expression. MerR binds its recognition site within the promoter, locally unwinding and distorting the DNA to allow realignment of the -10/-35 elements and open complex formation (Heltzel *et al.*, 1990; Ansari *et al.*, 1992; Brown *et al.*, 2003).

Similar mechanisms are proposed to occur in cyanobacteria with the identification of differential gene regulation in response to alternative sigma factors and transcriptional regulators (Imamura *et al.*, 2003; Gutekunst *et al.*, 2005; Patterson-Fortin *et al.*, 2006 Yoshimura *et al.*, 2007). Differences in cyanobacterial RNA polymerase and promoter composition suggest that the mechanisms of gene regulation may differ slightly from the previously described paradigms in *E. coli*. The cyanobacterial RNA polymerase core enzyme is composed of four domains, α, β, β' and γ, unlike the *E. coli* core enzyme composed only of the α, β and β' domains. The structural differences have been proposed to influence protein-protein interactions and consequently the mechanisms of gene activation and/or repression. Additionally, promoter sequence alignments have revealed that while encoding an *E. coli*-like -10 element, the majority of cyanobacterial promoters analyzed lack a -35 element or encode an element exhibiting low sequence conservation with the *E. coli* consensus. Similarly, to the previously noted structural differences, promoter sequence variability may also influence the way by which transcription factors regulate gene expression (Curtis and Martin, 1994). For example, NtcA-activated gene promoters lack a -35 element which is replaced by a palindromic NtcA binding site, GTA-N_8-TAC (Luque *et al.*, 1994). Protein-protein interactions between bound NtcA and RNAP serve to activate transcription initiation (Luque *et al.*, 1994; Herrero *et al.*, 2001). The location of the NtcA binding site within the promoter also influences NtcA regulatory activity. The *Synechocystis gifA* and *gifB* genes are negatively regulated by NtcA bound at sites overlapping the -10

28

promoter element (García-Domínguez *et al.*, 2001) most likely through steric hindrance.

1.4.2 Protein recognition of the DNA and complex formation

Transcription factors recognize both the chemical "signature" of the DNA and sequence-dependent variations in the DNA, for example, bends and kinks (Pabo and Sauer, 1993; Garvie and Wolberger, 2001). The chemical "signature" is formed by the base pair functional groups exposed in the major and minor grooves of the DNA. Unique "signatures" form depending on the DNA sequence of the protein recognition site (Garvie and Wolberger, 2001). The majority of DNA binding proteins interact with the major grooves because it is larger and more accessible to proteins than the minor groove making it easier to distinguish functional groups (Pabo and Sauer, 1993). Notable exceptions exist including the PurR dimer and LacI family of repressors which interact with the DNA's minor groove (Schumacher *et al.*, 1994). Contacts are made between complementary surfaces of the protein and the nucleotide functional groups through Van der Waals forces, electrostatic interactions, salt bridges and hydrogen bonding with specific nucleotide-amino acid interactions favored. The interactions with the sugar-phosphate backbone of the DNA are non-specific and allow for stable protein positioning and indirect recognition of the binding site through structural features of the DNA backbone. (Pabo and Sauer, 1993; Garvie and Wolberger, 2001). DNA recognition involves a DNA binding domain composed of protruding α-helical or β-sheet structures. Families of DNA binding proteins share similar

protein folds responsible for sequence recognition. For example, *E. coli* TrpR

proteins use a helix-turn-helix motif to contact the DNA. The recognition helix

contacts the DNA through the major groove (Otwinowski *et al.*, 1988). Less

common is the use of β-sheet structures to interact with the DNA. The MetJ and

Arc repressors contact the DNA using an antiparallel β-sheet (Somers and

Philips, 1992; Raumann *et al.*, 1994).

1.5 *crhR* DEAD-box RNA helicase, a model

We are trying to elucidate the mechanisms of redox-regulated gene

expression in *Synechocystis* using expression of the DEAD-box RNA helicase,

CrhR (Cyanobacterial RNA helicase-Redox) as our model. Expression of *crhR* is

regulated by the redox poise of the electron transport chain between Q_a in

photosystem II and Q_0 of the cytochrome b_6f complex (Kujat and Owttrim, 2000).

crhR transcripts accumulate in the light or under photoheterotrophic growth when

the electron transport chain is reduced. In contrast, oxidation of the electron

transport chain during growth in the absence of light, decreases *crhR* transcript

accumulation. In addition, alterations in light quality or the addition of electron

transport inhibitors alter *crhR* induction confirming redox-regulated expression

(Kujat and Owttrim, 2000).

CrhR encodes a DEAD-box RNA helicase. RNA helicases are required for

virtually all biological properties involving RNA including splicing, ribosome

biogenesis, mRNA export, translation initiation, degradation of RNA molecules

and expression of yeast mitochondrial genomes (Rozen *et al.*, 1990; Tseng *et al.*,

1998; Daugeron *et al.*, 2001; Daugeron and Linder, 2001; Tanner and Linder, 2001). The DEAD-box family of RNA helicases are characterized by a conserved core region of about 400 amino acids containing nine motifs required for helicase activity. The nine motifs named Q, I, Ia, Ib and II to VI which are involved in catalysis and substrate binding are arranged and spaced in a conserved manner within all members of the DEAD-box family (Tanner and Linder, 2001). Flanking the core region are divergent N- and C-terminal sequences that are proposed to confer specificity to the individual helicase enzymes (Tanner and Linder, 2001) by providing either protein or RNA binding domains or by specifying subcellular localization (de la Cruz *et al.*, 1999).

CrhR exhibits enzymatic activities characteristic of a RNA helicase, including RNA-dependent ATPase activity and ATP-stimulated RNA unwinding (Chamot *et al.*, 2005). Interestingly, CrhR also possesses ATP-dependent RNA annealing activity (Chamot *et al.*, 2005). CrhR is proposed to regulate expression of target RNAs at the translational level in response to the changing light/redox environment. CrhR's ability to both unwind and anneal complementary RNAs suggests it is capable of a wide range of structural rearrangements, perhaps allowing it to activate and repress translation of regulated RNAs (Kujat and Owttrim, 2000; Chamot *et al.*, 2005).

1.6 Thesis objectives

The primary objective of this thesis is to initiate investigation of the signal transduction pathway responsible for regulating the redox-responsive expression

of the CrhR DEAD-box RNA helicase. The research presented here focuses primarily on the identification of a LexA-related protein and its subsequent characterization in the regulation of gene expression in *Synechocystis*.

The first objective of the thesis was to identify the protein(s) previously shown to interact with upstream sequences of the *crhR* gene (Colvin, 2002). Using DNA affinity chromatography, this protein was identified as *Synechocystis* sp. strain PCC 6803 LexA. LexA regulation of *crhR* expression was investigated in Chapter 2. LexA interacts specifically with a sequence of DNA overlapping the translational ATG start codon. LexA binding at this site represses gene expression as demonstrated by decreased protein accumulation in a coupled *in vitro* transcription/translation system. Furthermore, *lexA* transcript accumulation under light (reducing) and dark (oxidizing) conditions was contrary to the patterns observed for *crhR* suggesting LexA is a repressor of *crhR* expression. Analysis of the classical association of LexA proteins with regulation of genes required to repair DNA indicated that DNA damaging agents did not induce accumulation of the *lexA* or *recA* transcripts in *Synechocystis*. The results indicate a divergence from the established LexA/RecA SOS repair system in *Escherichia coli* suggesting the *Synechocystis* LexA protein regulates redox responsive gene expression rather than DNA repair genes.

A better understanding of the LexA-DNA interaction was the second objective investigated in this study. This work was initiated by identifying the *lexA* gene as a target of its own protein. LexA interacts with the *lexA* gene downstream of the transcriptional start site as shown by primer extension and SI

nuclease protection assay. DNaseI footprinting identified protected sequences within the *lexA* and *crhR* genes. Similarities between the two regions were used to initiate site-directed mutagenesis studies to identify sequences essential for DNA recognition and complex formation. The combined analyses revealed that LexA binds as a dimer to a $CTA-N_9-CTA$ direct repeat conserved in the *lexA* and *crhR* genes.

The third objective was to determine the phenotypic effects of *lexA* and *crhR* mutations. Mutant constructs were generated by insertion of an antibiotic resistance cassette within each gene. A *crhR* homozygous mutant was obtained and phenotypically is unable to grow at low temperature. The lack of growth suggests an essential role for CrhR at 20°C, possibly a requirement for helicase activity to remove RNA secondary structures stabilized at low temperature. Heteroploid *lexA* mutants have been obtained suggesting *lexA* is an essential gene for survival. Reverse transcriptase PCR using the partial mutants showed increased accumulation of the *crhR* transcript in the *lexA* heteroploid ($lexA:Km^r/lexA^+$) under dark grown conditions. These results further support previous experiments suggesting LexA represses *crhR* expression under oxidizing conditions thereby regulating *crhR* expression in response to the redox poise of photosynthetic electron transport chain.

The surprising identification of LexA as the regulator of a redox-responsive gene and the observed lack of response to DNA damaging agents suggests *Synechocystis* LexA does not perform a physiological role similar to that shown for LexA homologues from *E. coli*, *Bacillus subtilis* and *Mycobacterium*

tuberculosis. Rather, dimerized *Synechocystis* LexA regulates expression of *crhR* and *lexA* through interaction with direct repeat sequences located downstream of the promoter and transcriptional start site. The identification of a novel role for LexA demonstrates the inherent problem with attributing protein function in the cell based solely on sequence similarities to known proteins and reveals further evidence for LexA-related proteins which have evolved to regulate different genes/responses in their host bacteria.

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Chapter 2: A LexA-related protein regulates redox-sensitive expression of the cyanobacterial RNA helicase, *crhR*

2.1 Introduction

The ability to adapt to a dynamic light environment is crucial for the survival of photosynthetic organisms and includes both short- and long-term responses. Light sensing occurs either via direct mechanisms involving photoreceptor proteins, or indirectly through light driven changes in the redox status of the electron transport chain between Q_A in photosystem II and Q_O in cytochrome b_6f (Gal *et al.*, 1997; Choi *et al.*, 1999; García-Domínguez *et al.*, 2000; Kujat and Owttrim, 2000). Electron carriers in this region of the inter-photosystem electron transport chain perform essential roles in redox sensing in higher plant chloroplasts, regulating expression of nuclear- and chloroplast-encoded genes involved in photosynthesis (Escoubas *et al.*, 1995; Maxwell *et al.*, 1995; Pfannschmidt *et al.*, 1999a; Pfannschmidt *et al.*, 1999b; Pfannschmidt *et al.*, 2001). For example, a direct link between the redox poise of plastoquinone and chloroplast gene expression has been shown for the *psbA* and *psaAB* genes, allowing rapid cellular response to the light environment via sensing of the redox status of the electron transport chain (Pfannschmidt *et al.*, 1999a). In contrast, the factors responsible for transduction of the electron transport redox poise to transcription regulation remain poorly characterized. Possible transducers identified in spinach chloroplasts include an unidentified 31 kDa dimeric protein shown to bind the *psaAB* promoter and the TSP9 thylakoid-associated protein (Cheng *et al.*, 1997; Carlberg *et al.*, 2003). Redox-mediated phosphorylation of TSP9 on three threonine residues releases the protein from the thylakoid membrane potentially allowing it to play a role as a signaling factor

responsible for transducing plastoquinone redox poise to gene expression (Carlberg *et al.*, 2003).

In prokaryotic cyanobacteria, the redox status of the electron transport chain carriers also regulates expression of a limited number of photosynthetic and non-photosynthetic genes. Expression of the RNA helicase, *crhR* (Kujat and Owttrim, 2000), glutamine synthetase, *glnA* (Reyes and Florencio, 1995), PII protein, *glnB* (García-Domínguez and Florencio, 1997), α and β subunits of phycocyanin, *cpcBA* (Alfonso *et al.*, 2000), photosystem proteins (Alfonso *et al.*, 2000; Li and Sherman, 2000; El Bissati and Kirilovsky, 2001) and a transcriptional regulator (Alfonso *et al.*, 2001) has been attributed to the redox poise of plastoquinone and/or cytochrome b_6f. Members of the signal transduction pathway(s) associated with sensing and transducing changes in redox status to the transcriptional machinery remain to be identified in cyanobacteria. Proposed mechanisms in cyanobacteria include a redox-responsive two-component signal transduction pathway (Li and Sherman, 2000), the redox-sensitive transcriptional regulators, NbIS, NtcA and PedR (Alfonso *et al.*, 2001; van Waasbergen *et al.*, 2002; Nakamura and Hihara, 2005) and alternative sigma factors (Imamura *et al.*, 2003; Yoshimura *et al.*, 2007).

As stated above, expression of the *Synechocystis* DEAD-box RNA helicase, *crhR* (Cyanobacterial RNA Helicase-Redox), is regulated by the redox poise of the electron transport chain (Kujat and Owttrim, 2000). *crhR* transcripts accumulate when the electron transport chain is reduced, either from light-driven electron flow or respiratory electron flow generated by the metabolism of

exogenously supplied glucose. In contrast, a reduction in electron flow, leading to oxidation of the electron transport chain, decreases *crhR* transcript accumulation. These results are corroborated by results obtained using electron transport inhibitors or alteration of light quality which alter *crhR* induction, confirming redox-regulated expression and identifying the redox poise of the electron transport chain between Q_A in photosystem II and Q_O in the cytochrome *b₆f* complex, as the potential sensor for redox-dependent regulation (Kujat and Owttrim, 2000). Biochemically, CrhR exhibits enzymatic activities characteristic of RNA helicases, including RNA-dependent ATPase activity and ATP-stimulated RNA unwinding (Fuller-Pace, 1994; Chamot *et al.*, 2005). In addition, CrhR also possesses ATP-dependent RNA annealing activity (Chamot *et al.*, 2005). Thus, CrhR has been proposed to regulate gene expression at the translational level through its ability to rearrange RNA secondary structures of RNA substrates, potentially of other redox-regulated gene transcripts (Kujat and Owttrim, 2000; Chamot *et al.*, 2005).

As an initial step to elucidate upstream factors involved in the redox regulated expression of the *crhR* gene, a LexA-related protein that controls *crhR* transcript accumulation was identified. Treatments known to enhance *crhR* transcript accumulation decrease *lexA* levels and vice versa. A direct effect of the recombinant His-tagged LexA protein (rLexA) on *crhR* expression was confirmed from the observation that rLexA represses *crhR* expression in an *in vitro* transcription/translation assay. LexA thus appears to function as a repressor of *crhR* transcription when *crhR* is not required i.e. under conditions which oxidize the electron transport chain. In concurrence with this conclusion, expression of

the *Synechocystis lexA* gene is not inducible by DNA-damage and its amino acid

sequence lacks two of three residues required for the self-cleavage activity of

prototypical LexA proteins (Little and Mount, 1982). The potential significance of

a LexA-related repressor in the regulation of redox-responsive gene expression

and, consequently, the implications of this novel role performed by LexA in

Synechocystis are discussed.

2.2 Material and Methods

2.2.1 Bacterial strains and growth conditions

Synechocystis sp. strain PCC 6803 was maintained on BG-11 agar

(Rippka *et al.*, 1979) solidified with 1% (w/v) Bacto-Agar (Difco Laboratories,

Detroit, MI) and grown photoautotrophically at 30°C under continuous illumination

at a constant intensity of 150 μmol photons m^{-2} s^{-1}. Liquid BG-11 cultures were

aerated by shaking at 150 rpm and continuous bubbling with humidified air. Dark

conditions were created by wrapping the flasks in aluminum foil. Glucose (5 mM)

was added where indicated. DNA damage was induced using short-wave

ultraviolet irradiation or mitomycin-C (Sigma). Cells were exposed to short-wave

ultraviolet light (UV-C; 254 nm) at a dose rate of 150 J/m^2, 300 J/m^2 or 600 J/m^2

using a XL-1000 UV crosslinker (Spectronics Corporation) or a constant dose

rate for 2 mins, 5 mins, 10 mins or 20 mins and subsequently incubated in the

dark for 1 h prior to harvesting. Alternatively, cells were exposed to mitomycin-C

at a dose rate of 1 μg/mL, 2 μg/mL or 5 μg/mL in the dark for 1 h.

E. coli strains DH5α and JM109 were used for propagation and protein expression of plasmid constructs, respectively. Cultures were grown in Luria-Bertani (LB) medium at 37°C and aerated by shaking at 200 rpm. Ampicillin (100 µg/ml) was added where appropriate.

2.2.2 PCR amplification

PCR (polymerase chain reaction) amplification was performed using the primer pairs listed in Table 2.1 in a volume of 50 µl, containing either 300 nM of each primer and 0.8 U of Expand High Fidelity enzyme mix (Roche) or 200 nM of each primer and 2.5 U of High Fidelity PCR enzyme mix (Fermentas). The PCR program consisted of 30 cycles of 30 s denaturation at 94°C, 30 s annealing and 1 min extension at 68°C; and terminated with a 4 min extension at 68°C. Optimal annealing temperatures for each primer pair were experimentally determined.

2.2.3 Plasmid constructs

A deletion series within the *crhR* promoter/ORF was created from a 3 kb EcoRI fragment encompassing the *crhR* promoter, 5'-untranslated region (UTR), and the *crhR* ORF (Kujat and Owttrim, 2000). Plasmid DNA was digested with *Not*I and *Sac*I, and a deletion series produced using the Erase-A-Base Kit (Promega) according to the manufacturer's instructions. Two additional deletion constructs were created by restriction enzyme digestion. *Spe*I removed a 328 bp fragment to construct KC+125. The KC+219 construct was created by *EcoRI/Xmn*I digestion to liberate a 2.6 kb fragment containing the *crhR* ORF

Table 2.1 Oligonucleotides used in this study. Relative location designates oligonucleotide position with respect to the transcriptional start site (+1). Restriction enzyme recognition sites are in bold.

Primer	Sequence 5' — 3'[a,b]	Relative location	Application
T7	GTATACGACTCACTATAGGGC		Sequencing
GWO-13	GTAAAACGACGGCCAGT	pBSKS+	Forward primer to obtain KC EMSA targets with GWO-45
KCO-4	GTAAAACGACGGCCAGT	pBSKS	Forward primer to obtain biotinylated KC+5 DNA with KCO-5
crhR			
GWO-45	AAGCCAATGTCGGCCAAGAG	189	Reverse primer to obtain KC EMSA targets with GWO-13
KCO-5[c]	AAGCCAATGTCGGCCAAGAG	189	Reverse primer to obtain KC+5 DNA with KCO-4
lexA			
LPF-1	CCTATAGG**AGATCT**TACATG	18	Forward primer for cloning LexA DNA binding domain with LPF-19
LPF-2	GAAACAAA**AAGCTT**AGGACG	789	Reverse primer for cloning full-length LexA with LPF-27
LPF-4	ATTTGCGTTCTCCGGCC	140	Forward primer to obtain LPF 4:5 EMSA target with LPF-5
LPF-5	CTTCGATTCCTCTCTC	452	Reverse primer to obtain LPF 4:5 EMSA target with LPF-4
LPF-19	TTTTGGTTATTGGTGGAGAA	256	Reverse primer for cloning LexA DNA binding domain with LPF-1
LPF-27	ACTGGT**GGATCC**GAACCTCTCACCCGAGCC	38	Forward primer for cloning full-length LexA with LPF-2

a. Restriction sites are shown in italics.
b. Position of 5' end of the oligonucleotide relative to the transcriptional start site.
c. 5' Bioitinylated primer.

downstream of +219 but lacking the promoter region. This fragment was blunt end ligated into *EcoR*V-digested pBluescript KS+ (Stratagene).

The amino-terminal DNA binding domain (NLexA) and full-length LexA (rLexA) were produced by expression of an in-frame translational fusion in *E. coli*. The full-length *lexA* insert was digested with *BamH*I and *Hind*III and cloned into *Bgl*II and *Hind*III-digested pRSETB plasmid DNA (Invitrogen). The resulting plasmid, pLexA, expresses a recombinant 29.3 kDa His6-tagged protein. The NLexA insert was digested with *Bgl*II and cloned into *Bgl*II and *Pvu*II digested pRSETB plasmid DNA (Invitrogen) producing pNLexA which expresses a recombinant 11.4 kDa His6-tagged protein. Both plasmids were sequenced to confirm successful in-frame insertion of the *lexA* gene or *lexA* gene fragment.

2.2.4 Generation of promoter fragments

Promoter fragments, KC-179 to KC+77, were generated by PCR amplification as described above. *BssH*II/*BssS*I digestion was used to generate fragments corresponding to KC+125 and KC+219. DNA fragments were purified from 1X TAE agarose gels using GENECLEAN®II (BIO 101).

2.2.5 DNA affinity column purification

To purify proteins binding to the *crhR* promoter region, μMACS Streptavidin magnetic separation was performed (Miltenyi Biotec). *Synechocystis* cultures (300 ml) were harvested and resuspended in 2 ml cyanobacterial protein extract buffer (20 mM Tris HCl pH 8, 10 mM NaCl, 1 mM EDTA pH 8, 5 mM DTT)

containing protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche). Cells were lysed by 8 cycles of sonication for 30 s followed by 30 s cooling in an ice-water bath. Lysed cells were clarified by centrifugation and the supernatant retained. Proteins were quantified by the Bradford assay (BioRad) using bovine serum albumin as a standard. Binding reactions were performed in 1X EMSA buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) containing 50 µg poly dI-dC, 28 µg biotinylated target DNA and 6.8 mg soluble protein extract. Biotinylated target DNA was prepared by PCR amplification using the KC04 forward primer and a biotinylated reverse primer, KC05 (Table 2.1) as described above. The binding reactions were gently shaken at 4°C for 80 min, 100 µl super-paramagnetic µMACS MicroBeads conjugated to streptavidin (µMACS Streptavidin kit, Miltenyi Biotec) added and incubation continued for 15 min. The µMACS column was prepared by rinsing consecutively with protein application buffer and 1X EMSA buffer. The binding reaction was applied to the µMACS column within the magnetic field of the µMACS separator, and washed consecutively with steps of increasing salt stringency (0.1 to 1 M KCl). Eluted proteins were concentrated by TCA precipitation, separated on a 10% (w/v) SDS polyacrylamide gel, and visualized by silver staining (BioRad).

Polypeptides of interest were identified by in-gel tryptic digestion and LC/MS/MS of the resulting peptides at the Institute for Biomolecular Design (University of Alberta). Generated LC/MS/MS data were used as queries for Mascot Daemon (Matrix Science, UK) searches of the National Center for

Biotechnology Information (NCBI) non-redundant databases. A protein score greater than 73 following Mascot searches was considered significant.

2.2.6 Electrophoretic mobility shift assays

Electrophoretic mobility shifts assays were performed using *Synechocystis* soluble protein extract, *E. coli* soluble protein extract or recombinant LexA protein, and the indicated PCR-generated promoter fragments end-labeled with $[\gamma^{32}P]$ ATP and T4 polynucleotide kinase (New England Biolabs). Binding reactions were performed for 20 min at 37°C in 1X EMSA buffer, 1 µg poly dI-dC, 2000 cpm end-labeled DNA (~ 0.006 pmol), and the indicated protein concentration in a final volume of 20 µl. Reaction products were separated on a 5% TBE non-denaturing polyacrylamide gel and subjected to autoradiography. Two non-specific competitor DNAs were prepared to control for non-specific protein binding. One control target was a 262 bp *EcoRV/Pvu*II fragment of the pBluescript KS+ plasmid containing its multiple cloning site. The second non-specific competitor DNA was a 321 fragment of the *Synechocystis lexA* gene generated by PCR using primers LPF-4 and LPF-5. LPF 4:5 encompass basepairs 105 to 427 of the *lexA* open reading frame.

2.2.7 Recombinant LexA expression and purification

E. coli JM109:pLexA and pNLexA cultures were grown at 37°C to OD600 = 0.6, and LexA expression induced by addition of IPTG (1.0 mM) and phage (M13/T7 DE3, 5 pfu/cell, Invitrogen). After induction for 3 h at 37°C, harvested

cells were resuspended in 1/10 volume lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole), lysed by sonication (6 x 30 s intervals), and clarified by centrifugation. The supernatant was loaded onto a Ni-NTA column (Qiagen) and incubated with gentle shaking for 60 min at 4°C. The column was washed consecutively with wash buffer (50 mM NaH_2PO4, 300 mM NaCl) containing increasing amounts of imidazole (20 to 50 mM), with bound rLexA eluting in buffer containing 250 mM imidazole. Imidazole was removed from the eluted rLexA buffer by dialysis against lysis buffer lacking imidazole as required.

2.2.8 Northern analysis

Total RNA was isolated from *Synechocystis* by mechanical lysis, separated on a 1.2% formaldehyde gel, and transferred to a nylon membrane (Hybond N+) as previously described (Chamot *et al.*, 1999). Blots were hybridized overnight at 65°C with the appropriate probe in aqueous buffer (5X SSPE, 5X Denhardt's, 0.5% SDS) and washed for 10 min at 65°C once in 1X SSPE, 0.1% SDS and once in 0.1X SSPE, 0.1% SDS. *lexA* and *crhR* DNA fragments were randomly labeled with $[\alpha^{32}P]$ dCTP using random hexanucleotide primers (Roche). The probes correspond to: *lexA*, a 750 bp *BglII/HindIII* fragment encompassing the entire ORF; *crhR*, a 784 bp internal *BstEII* fragment; and *recA*, a 1091 bp riboprobe encompassing the ORF labeled with $[\alpha^{32}P]$ UTP (Promega). Membranes were stripped by incubation in boiling 0.1% SDS and probed with the *Synechocystis rnaseP* gene as a control for RNA loading (Chamot and Owttrim, 2000).

2.2.9 In vitro transcription/translation assays

In vitro transcription and translation assays were performed using the Promega *Escherichia coli* S30 extract system for circular DNA in a final reaction volume of 25 μl. The plasmids pCrhR (IV) and pWM3-2 (Chamot *et al.*, 1999) were used for in vitro expression of the *crhR* and *crhC* genes respectively. p*CrhR* (IV) was prepared by ligating a 2.2 kb *BamHI/EcoRI* fragment of CS0096-9 (Kujat and Owttrim, 2000) into pBluescript KS+ to remove downstream sequences encoding the *argC* gene. The pCrhR (IV) and pWM3-2 plasmids contain 2.2 and 2.4 kb inserts respectively encoding the promoters, open reading frames, and 5' and 3' untranslated regions of *crhR* and *crhC*, respectively. Reactions were performed according to manufacturer's instructions using 1 μg plasmid DNA, corresponding to 0.29 and 0.28 pmol DNA for pCrhR (IV) and pWM3-2, respectively. Reaction products were separated on a 10% (w/v) polyacrylamide gel and subjected to autoradiography. Binding reactions containing rLexA were performed according to manufacturer's instructions with an initial 5 min incubation to allow protein binding to the *crhR* gene. Control reactions were performed to determine the effect of rLexA on *crhR* expression in the presence of BSA and expression of an unrelated RNA helicase, *crhC* (Chamot and Owttrim, 2000), from its own promoter.

2.3 Results

2.3.1 Promoter deletion series delineates the protein-binding site within the crhR gene

Identification of the *crhR* regulatory protein(s) was initiated by delineating the minimal region of the *crhR* promoter required for binding putative regulatory protein(s). A series of promoter cutbacks containing deletions of the *crhR* promoter were prepared and EMSA analysis performed to determine location of the putative binding site (Figure 2.1A). Intact *crhR* promoter (KC-179) and deletions up to position + 77 of the *crhR* transcript (KC+77) exhibited decreased mobility on a native PAGE gel upon incubation with *Synechocystis* protein extract (Figure 2.1B, lanes 1-14). The KC+125 DNA target, deleted to + 125 of the transcript, exhibited a decreased amount of shift (Figure 2.1B, lanes 15 and 16), while deletion to +219 completely abolished the mobility shift (Figure 2.1B, lanes 17 and 18). Together, these results indicate that the protein-binding site is located downstream of the translational start codon (+110) in the region of DNA surrounding an *Spe*I site (+125). Sequence specificity of binding was shown by competition assays in the presence of increasing amounts of either specific or non-specific competitor DNA (Figure 2.1C). Addition of specific competitor DNA (KC-179) progressively challenged formation of the shifted complex (Figure 2.1C, Specific competitor). Conversely, inclusion of a similar sized fragment containing the pBluescript KS+ multiple cloning site had no effect on the mobility shift (Figure 2.1C, Non-specific competitor). Taken together, these results indicate
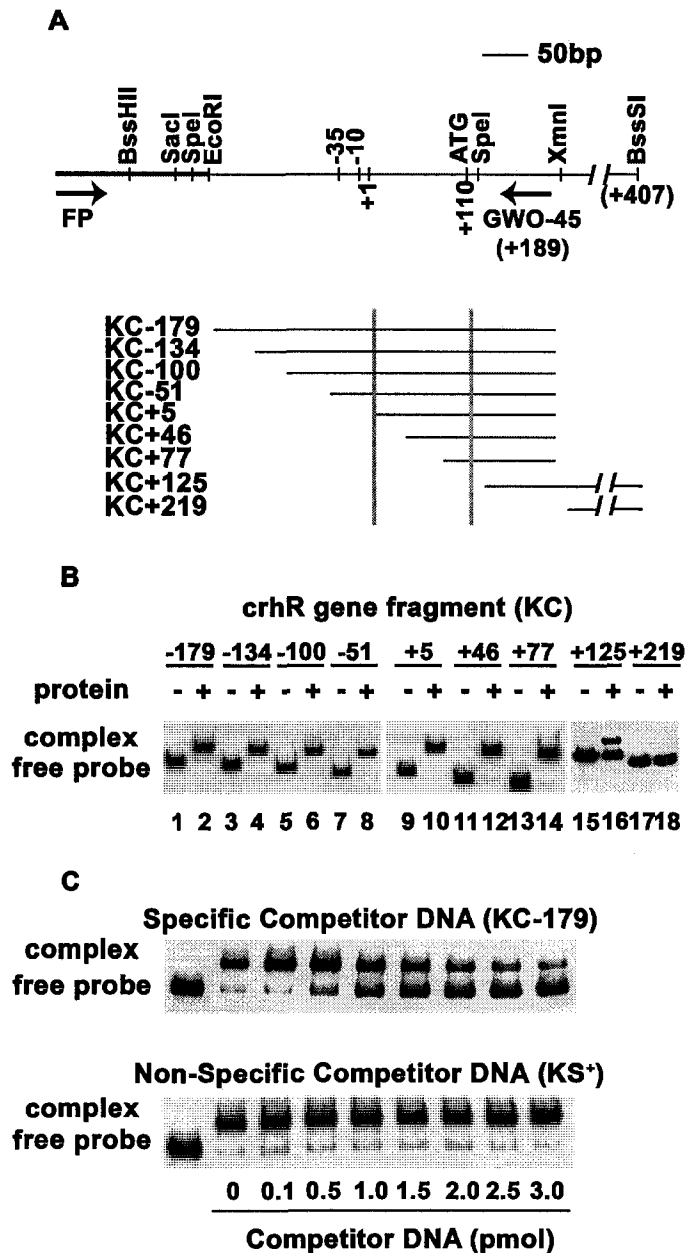
Figure 2.1 Localization of the *crhR* protein binding site.
A. *crhR* nested deletion series. DNA was deleted by directional digestion from the *SacI* site using Exonuclease III. Deleted clones are designated by their start site relative to the transcriptional start indicated as +1. DNA fragments corresponding to each deletion were generated by PCR using the M13 forward (FP) and GWO-45 primers, except KC+125 and KC+219 which were produced by restriction digestion (KC+125: *SpeI/BssSI*; KC+219: *XmnI/BssSI*). Plasmid and *crhR* insert sequences are indicated by thick and thin solid lines, respectively. Scale 50 bp = 1 cm.

B. Localization of the protein-binding region. ^{32}P-end-labelled DNA targets were incubated either alone (-) or with 30 μg *Synechocystis* soluble protein extract (+).
C. Competition assays. KC-179 ^{32}P-end-labelled target DNA, containing the entire crhR promoter, was incubated with no protein or 30 μg *Synechocystis* soluble protein extract. Increasing amounts (0-3.0 pmol) of either specific competitor DNA (unlabeled KC-179 fragment; top panel) or non-specific competitor DNA (unlabelled 262 bp *EcoRV/PvuII* fragment of pBluescript KS+; bottom panel) were included in the binding reaction to determine the specificity of the protein-DNA interaction.
(Work included in this figure done by Kimberley R. Colvin)

that at least one soluble *Synechocystis* protein interacts with the *crhR* gene in a sequence-specific manner.

2.3.2 Synechocystis *LexA-related protein binds within the* crhR *ORF*

To identify the protein responsible for altered mobility of the *crhR* gene, DNA affinity column chromatography was performed using light-grown *Synechocystis* soluble protein extracts and immobilized and biotinylated DNA. The biotinylated KC+5 DNA (239 bp) was prepared by PCR using the forward primer, KC04 and the biotinylated reverse primer, KC05. A single polypeptide with an apparent molecular weight of 28 kDa was recovered in the high stringency 1M KCl elution (Figure 2.2A). The single significant hit (score 92) identified by in-gel tryptic digestion and LC/MS/MS corresponded to the *Synechocystis* gene sll1626 which has been annotated as encoding the transcriptional repressor LexA (http://www.kazusa.or.jp/cyanobase/). Analysis of the deduced *Synechocystis* LexA amino acid sequence revealed that the sequence lacks the Ala-Gly self-cleavage site and the serine of the Ser-Lys dyad active site present in *E. coli* LexA, both of which are required for LexA self-cleavage (Figure 2.2B; Slilaty and Little, 1987). Furthermore, DNA sequences, similar to those identified as LexA binding sites in *E. coli* (Walker, 1984; Fernández de Henetrosa *et al.*, 2000), *Bacillus subtilis* (Miller *et al.*, 1996), and *Mycobacterium tuberculosis* (Davis *et al.*, 2002a) could not be identified withinthe upstream sequence of either *lexA* or *crhR*. However, a sequence related to the putative cyanobacterial SOS box (Mazón *et al.*, 2004), matching 7 of 9 essential

A
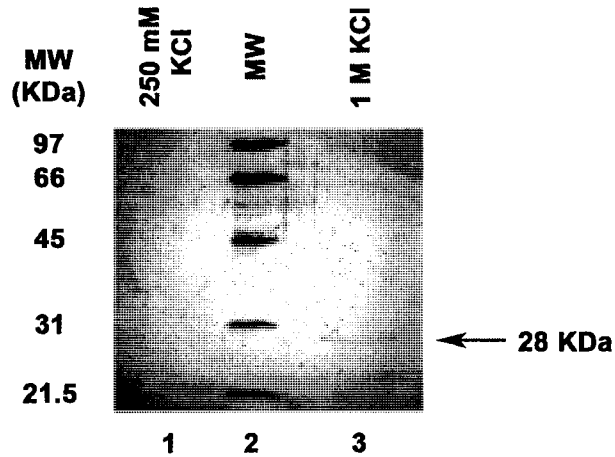
B

Syn.	MEPLTRAQKELFDWLVSYIDETQHAPSIRQMMRAMNLRSPAPIQSRLERLRNKGYVDW
E. coli	MKALTARQQEVFDLIRDHISQTGMPPTRAEIAQRLGFRSPNAAEEHLKALARKGVIEI

Syn.	TDGKARTLRILHQKPKGVSVIGELKGGELVEADAEEVEKIDFAP-LMKKSSVFALRVM
E. coli	VSGASRGIRLLQEEEEGLPLVGRVAAGEPLLAQQHIEGHYQVDPSLFKPNADFLLRVS
	*

Syn.	SNDLVDDFIVEGDMLILRSVTGEEEIEDGELVAASIKGGKIAIKRYYQDGTKVVLKAS
E. coli	GMSMKDIGIMDGDLLAVHK-TQDVRN--GQVVVARIDDE-VTVKRLKKQGNKVELLPE
	+ #

Syn.	NNKGPGQELKASDVEIQ--GILMGVWRNFQGV
E. coli	NSEFKPIVVDLRQQSFTIEGLAVGVIRNGDWLEFPGIRRPWRPLESTCSQANSGRISYD

C

crhR	**ATG**ACTAATACTTTGACT<u>AGTAC</u>CTTCGC<u>T</u>GACCTTGGTC
Cyanobacterial consensus	NRGTACNNN**DGTW**CBN

Figure 2.2 Isolation and characterization of a *crhR* regulatory protein by affinity chromatography and LC/MS/MS.
A. A 28 kDa polypeptide interacts with the *crhR* open reading frame. A single polypeptide was isolated by DNA affinity chromatography using KC+5 as the target. Non-specifically bound proteins were removed by increasing KCl washes. Silver staining of eluted proteins separated by a 10% SDS-PAGE reveals a single

polypeptide in the 1 M KCl elution. LC/MS/MS identified this polypeptide as the *Synechocystis* gene annotated as encoding LexA. Lane 1, 250 mM KCl wash; lane 2, low molecular weight standards (BioRad); lane 3, 1 M KCl wash.

B. Amino acid sequence analysis of the *Synechocystis* and *E. coli LexA* proteins. Residues essential for *E. coli* LexA self-cleavage in response to DNA damage are indicated as follows: * = Ala-Gly self-cleavage site; + = Ser and #= Lys indicate the Ser-Lys dyad active site.

C. Alignment of the putative LexA binding region of the *crhR* gene (*Spe*I site is underlined) with the consensus cyanobacterial LexA binding sequence (Mazón *et al.*, 2004). Conserved residues are bolded. The LexA binding sequence within the *crhR* matches at 7 of 9 conserved residues with appropriate spacing.

residues with required spacing between essential residues, was identified within the protein binding domain in *crhR* (Figure 2.2C). This sequence includes the *Spe*I site, possibly explaining the decreased shift observed with the *Spe*I generated KC+125 fragment (Figure 2.1B).

2.3.3 Synechocystis lexA, crhR *and* recA *transcript accumulation*

crhR transcript accumulation is regulated by the redox poise of the plastoquinone pool with treatments leading to reduction of plastoquinone correlating with an increase in *crhR* transcript accumulation, whereas conditions that lead to the oxidation of the plastoquinone pool result in decreased *crhR* accumulation (Kujat and Owttrim, 2000). Northern analysis was therefore performed to determine the relationship between *lexA* and *crhR* transcript accumulation under varying redox conditions (Figure 2.3A). Growth in the light (Figure 2.3A, lane 1), conditions favoring *crhR* transcript accumulation, correlate with a reduction in *lexA* transcript levels. Conversely, growth in the dark (Figure 2.3A, lane 2) reduces *crhR* while enhancing *lexA* transcript accumulation. The addition of glucose (5 mM) to light grown cells enhanced *crhR* and *lexA* transcript accumulation (Figure 2.3A, lane 3). *crhR* expression was significantly induced in response to cold stress (20°C; Figure 2.3A, lane 4), concomitant with the complete repression of *lexA* transcript accumulation. These data indicate differential regulation of *crhR* and *lexA* expression in response to alterations in the redox status of the electron transport chain, implying that LexA functions as a negative regulator of *crhR* expression.
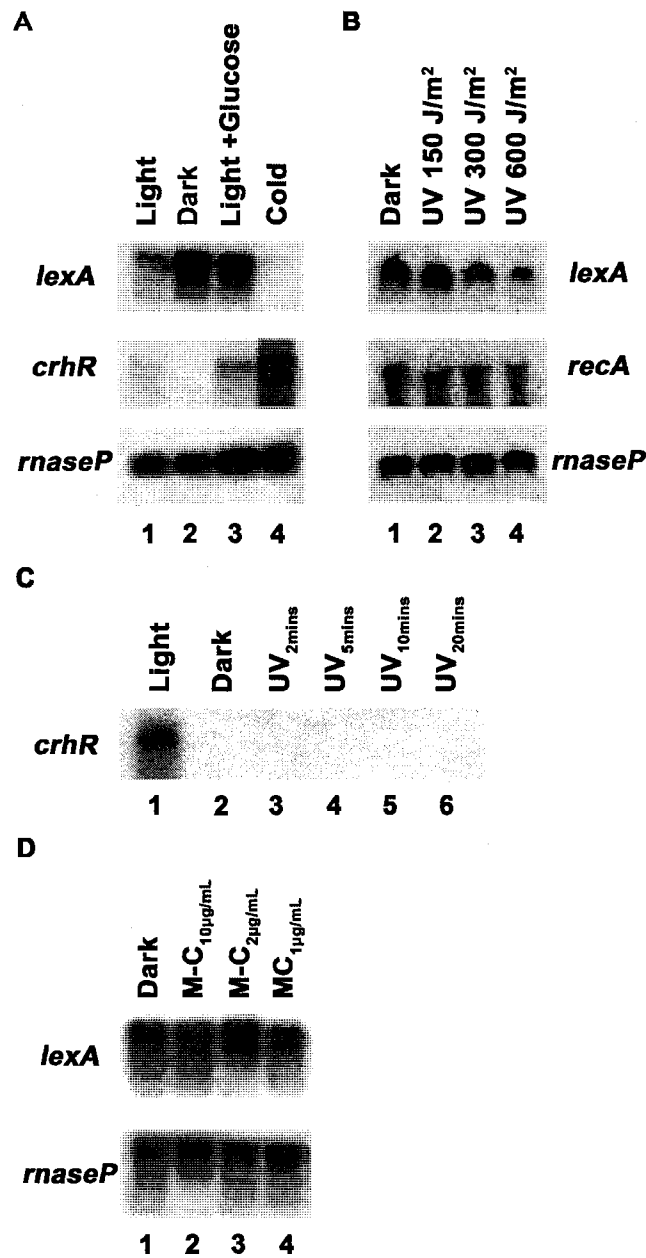
Figure 2.3 *crhR*, *lexA* and *recA* transcript analysis. Total RNA (30 μg) was isolated from *Synechocystis* cells grown as indicated. RNA was separated on a 1.2% formaldehyde agarose gel, transferred to Hybond N+ and hybridized with the indicated ^{32}P-labeled probe.

A. *lexA* and *crhR* transcript accumulation following incubation under different environmental conditions. Lane 1, 3 h light; lane 2, 3 h dark; lane 3, light plus 5 mM glucose; lane 4, cold stress for 3 h (20°C).

B. *recA* and *lexA* transcript accumulation in response to increasing levels of UV-irradiation. Lane 1, 1 h dark; lane 2, UV irradiated with 150; lane 3, 300; lane 4, 600 Joules/m^2 followed by a 1 h incubation in the dark.

C. *crhR* transcript accumulation in response to increasing exposure to UV irradiation. Lane 1, light; lane 2, 1 h dark, lane 3, 2 min UV exposure; lane 4, 5 min UV exposure; lane 5, 10 min UV exposure; lane 6, 20 min UV exposure.

D. *lexA* transcript accumulation in response to increasing levels of mitomycin-C. Lane 1, 1 h dark; lane 2, 10 μg/mL M-C; lane 3, 2 μg/mL M-C; lane 4, 1 μg/mL M-C.

rnaseP transcript accumulation was determined as a control for RNA loading.

LexA association with the *E. coli* SOS response and repression of DNA

damage repair gene expression warranted comparative analysis of

Synechocystis lexA, *recA* and *crhR* expression in response to DNA damage.

DNA damage was induced by ultraviolet (UV) irradiation, and the resulting

expression patterns were examined by Northern analysis (Figure 2.3B). In

contrast to *E. coli*, following UV irradiation neither *Synechocystis lexA* nor *recA*

expression (Figure 2.3B, lanes 2-4) was induced above basal levels detected in

dark grown cells (Figure 2.3B, lane 1). In fact, in contrast to other prokaryotic

systems, *recA* was expressed at very low levels under all conditions tested,

requiring riboprobe detection and extended exposure times. Similarly, expression

of *crhR* was not UV-inducible (Figure 2.3C); rather, it followed the expected

decrease in transcription that occurs in wild type cells in the dark (Kujat and

Owttrim, 2000). *lexA* transcript accumulation was also not altered by DNA

damage induced by mitomycin C (Figure 2.3 D). The lack of induction of the

Synechocystis recA, *lexA* and *crhR* genes following DNA damage suggests these

gene products are not required during the cellular response to DNA damage.

2.3.4 Synechocystis *LexA interacts with the* crhR *gene*

Recombinant His-tagged LexA (rLexA) was purified to near homogeneity

and used to test interaction with the KC+5 *crhR* promoter fragment (Figure 2.4).

Electrophoretic mobility shift assays reveraled that mobility of the KC+5 *crhR*

promoter DNA decreased by incubation with total *Synechocystis* protein extracts

(Figure 2.4A, lane 8 vs. lane 1). Mobility of the KC+5 DNA target was also altered

A

rLexA (nM)

0 10 20 40 60 80 100   6803 E. coli

complex

free probe

1   2   3   4   5   6   7   8   9

B

Specific   Non-specific

complex

free probe

0 25 50 100   0 25 50 100

Competitor DNA (fold excess)

1 2 3 4   5   6 7 8 9 10
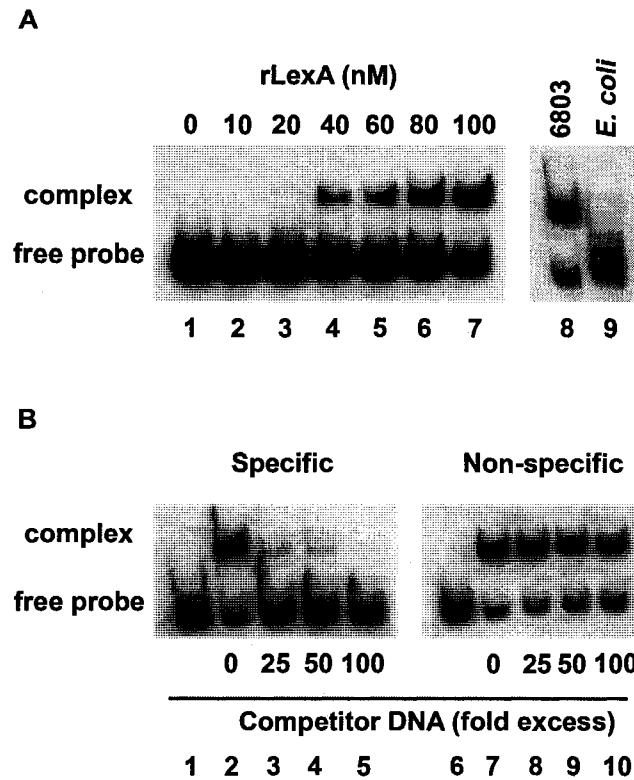
Figure 2.4 rLexA binding to the *crhR* gene. Electrophoretic mobility shift assays using recombinant LexA (rLexA) were performed to confirm interactions between LexA and the *crhR* gene.

A. rLexA concentration curve. Increasing concentrations of rLexA were incubated with 32P-labeled KC+5. As controls, rLexA was also incubated with 10 μg 6803 (lane 8) and 10 μg *E. coli* (lane 9) soluble protein extracts.

B. DNA competition assays. rLexA (100 nM) was incubated with ^{32}P-labelled KC+5 and the indicated fold excess of either specific competitor DNA (unlabelled KC+5; lanes 1-5) or non-specific competitor DNA (internal *lexA* fragment; lanes 6-10). Lanes 1 and 6 have no rLexA protein added.

by incubation with purified rLexA, with alteration of target DNA mobility exhibiting dependence upon rLexA concentration (Figure 2.4A, lanes 2-7). *E. coli* soluble protein extracts did not alter *crhR* DNA target mobility, indicating that *E. coli* proteins do not bind the *crhR* gene (Figure 2.4A, lane 9). Sequence specific binding was demonstrated by competition assays in the presence of increasing concentrations of either specific or non-specific competitor DNA. Addition of unlabelled specific competitor (KC+5) challenged formation of the shifted complex at all concentrations tested (Figure 2.4B, lanes 2-5), with addition of ≥50-fold excess of unlabelled target abolishing shift of the DNA target. In contrast, incubation with non-specific competitor DNA, an internal *lexA* fragment similar in size to the specific competitor, did not significantly alter mobility shift at comparable concentrations (Figure 2.4B, lanes 7-10).

Mobility shift assays were also performed using purified LexA protein encoding the amino-terminal DNA binding domain (NLexA) due to initial difficulties expressing the full-length protein in *E. coli*. In *E. coli*, the amino-terminal domain encodes DNA binding capabilities (Hurstel *et al.*, 1986; Bertrand-Burggraf *et al.*, 1987). Similarities between the *E. coli* and *Synechocystis* proteins within the amino-terminal domain suggested NLexA would also encode the DNA binding capabilities of the *Synechocystis* LexA protein. To test this possibility, EMSAs were performed using the KC+5 target and NLexA. Mobility of the KC+5 DNA target was altered by incubation with NLexA in a sequence specific manner (Figure 2.5A; specific, lanes 4-9; non-specific, lanes 10-15). NLexA was

A

	Specific	Non-specific

complex
free probe

competitor DNA (fold excess): 100 250 500 100 250 500

lanes: 1 2 3 4 5 6 7 8

B

KC target	-100	+77	+125	+219
NLexA	- +	- +	- +	- +

lanes: 1 2 3 4 5 6 7 8
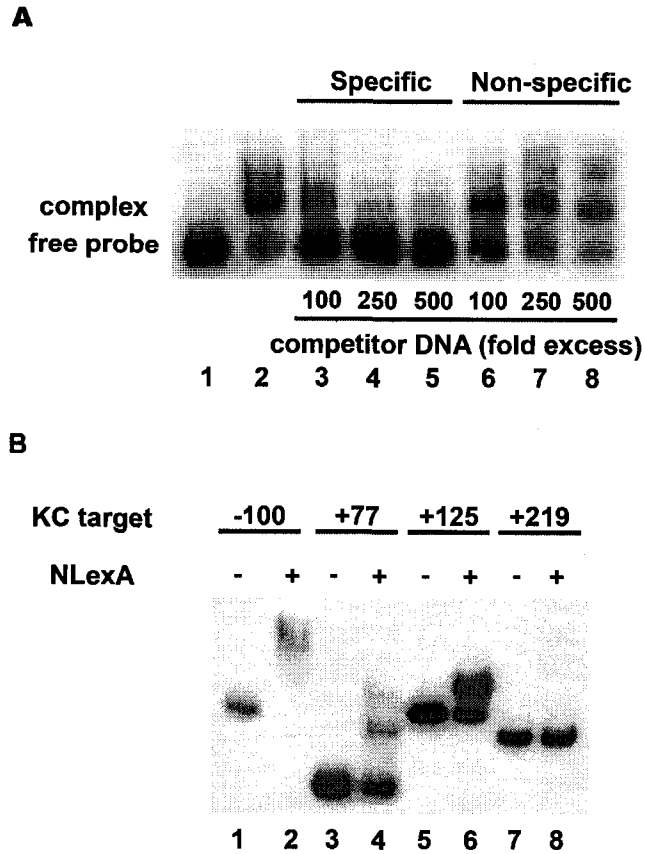
Figure 2.5 NLexA binding to the *crhR* gene. EMSA analyses using recombinant NLexA were performed to confirm interaction between NLexA and the *crhR* gene. **A.** Competition assays. ^{32}P-labeled KC+5 was incubated either alone (lane 1) or with 500 nM NLexA (lane 2) and the indicated fold excess of either specific competitor DNA (unlabeled KC+5; lanes 3-5) or non-specific competitor DNA (internal *lexA* fragment; lanes 6-8).
B. Localization of the protein-binding site. NLexA was incubated with ^{32}P-labeled KC targets either alone (-) or with 500 nM NLexA (+).

also shown to affect mobility of the KC -100, +77, +125, and +219 targets as expected based on previous EMSA results using total *Synechocystis* protein extract (Figure 2.5B). Unexpectedly, the KC+77 target exhibits reduced mobility compared to the mobility observed in the presence of soluble *Synechocystis* protein extract (compare Figure 2.1A, lanes 13 and 14 with Figure 2.5B, lanes 5 and 6). A reason for this discrepancy may be an unstable interaction between the DNA target and the NLexA protein due to loss of the carboxyl-terminal domain required for protein dimerization. No change in mobility of the KC+219 target is observed, as predicted due to loss of the putative binding site (compare Figure 2.1A, lanes 17 and 18 with Figure 2.5B, lanes 7 and 8). Together, the results using both proteins indicate that recombinant LexA interacts with the *crhR* gene in a sequence-specific manner.

2.3.5 LexA represses crhR *gene expression in vitro*

An *in vitro* transcription and translation system was used to confirm LexA regulation of *crhR* gene expression from its native promoter. As shown in Figure 2.6A, CrhR protein accumulation decreased in response to increasing rLexA concentration. Quantification of these results indicated that the rLexA inhibition of CrhR expression was linear with respect to rLexA concentration (Figure 2.6B). Expression of CrhR was confirmed by the absence of 55 KDa radiolabelled protein when the KS+ target lacking the *crhR* gene was used as target in the *in vitro* reaction (Figure 2.6C). The specificity of repression was demonstrated by

A

B

C
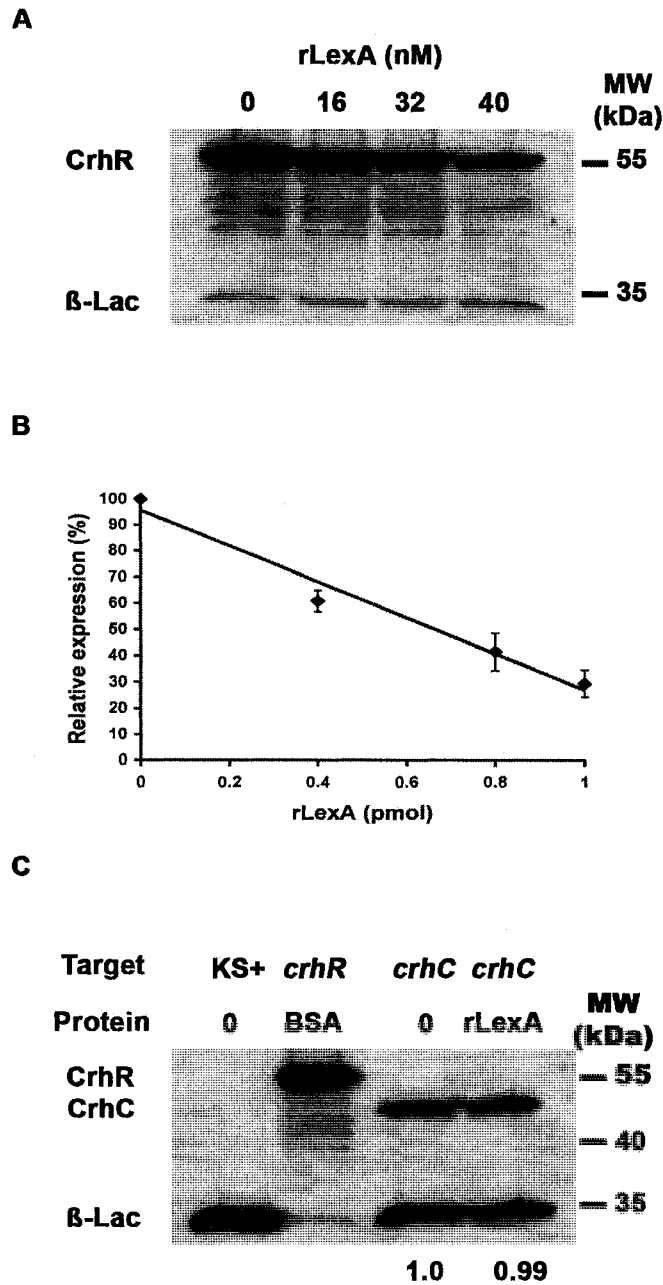
Figure 2.6 rLexA represses CrhR protein accumulation *in vitro*. *In vitro* transcription/translation reactions were used to investigate the nature of the *LexA* regulatory relationship with the *crhR* gene.

A. CrhR protein (55 kDa) accumulation in the presence of increasing rLexA concentration. A plasmid (pCrhR IV) containing the complete *crhR* gene, including 289 bp upstream of the translation start, was incubated in a transcription/translation mixture in the presence of increasing rLexA (0-40 nM).

B. Quantification of rLexA effect on CrhR expression. CrhR expression in the presence of increasing rLexA concentration was quantified from triplicate, independent replicates similar to the data shown in (A) using ImageQuant (Molecular Dynamics). Standard deviations from the means are shown.
C. Specificity of rLexA regulation. Specificity of the *in vitro* transcription/translation of CrhR was demonstrated by the absence of radiolabelled protein when KS+ lacking the *crhR* insert was used as target DNA. As control reactions, the transcription/translation efficiency of CrhR was evaluated in the presence of a non-specific protein, BSA (1 pmol). In addition, accumulation of the temperature-regulated cyanobacterial RNA helicase, CrhC (Chamot *et al.*, 1999; Chamot and Owttrim, 2001) was determined in the presence and absence of rLexA (1 pmol). The relative level of CrhC expression is shown below the figure, as determined using ImageQuant.

the lack of change in the levels of the plasmid-encoded β-lactamase protein, a

non-LexA regulated protein. Similarly, *crhR* expression was unaffected by

incubation in the presence of 1 pmol BSA (Figure 2.6C), a protein concentration

at which rLexA significantly altered *crhR* expression. Furthermore, in vitro

transcription and translation of a second cyanobacterial RNA helicase, *crhC*

(Chamot *et al.*, 1999; Chamot and Owttrim, 2000), was also unaffected by 1 pmol

r*LexA* (Figure 2.6C). Together, these results indicate that LexA specifically

regulates *crhR* transcription in a negative fashion.

2.4 Discussion

Characterization of the signal transduction pathway transducing the redox

poise of the electron transport system to the transcription apparatus in

cyanobacteria was investigated. In this chapter, a LexA-related protein was

shown toregulate expression of the redox-responsive RNA helicase, *crhR*. This

identification implies a novel function for LexA in *Synechocystis*, a conclusion

consistent with previous studies suggesting that LexA may regulate expression of

carbon metabolism and bidirectional hydrogenase genes in *Synechocystis*

(Domain *et al.*, 2004; Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005).

The LexA binding site is located downstream of the *crhR* transcription start

site, requiring sequences surrounding +125 of the *crhR* transcript. This

localization suggests a regulatory mechanism for the *Synechocystis* LexA-related

protein that differs from LexA regulation of DNA damage inducible genes in *E.*

coli and other bacteria, where the LexA binding site (SOS box) surrounds the

transcriptional start (Walker, 1984; Fernández de Henestrosa *et al.*, 2000).

Similar regulatory element arrangements, where transcription factors bind downstream of the transcription start site, have been observed in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (Li *et al.*, 1995). In fact, the light responsive transcription of the *psbA* and *psbD* gene families in *Synechococcus* requires enhancer elements located downstream of the transcription start (Li and Golden, 1993; Li *et al.*, 1995; Anandan and Golden, 1997). The LexA DNA binding site within the *crhR* open reading frame is therefore consistent with regulatory protein binding sites localized in other genes whose expression is known to be regulated by either light or redox signals. Unfortunately, the DNA binding proteins interacting with these other sites remain to be identified.

A combination of DNA affinity chromatography and mass spectrometry identified the protein interacting with the *crhR* gene as being related to LexA. Northern blot analysis showed that *Synechocystis lexA* transcripts accumulate when cells are grown under conditions correlating with the repression of *crhR* accumulation. Based on these results, it appears that LexA functions as a negative regulator of *crhR* expression. Negative regulation was confirmed using an in vitro transcription/translation assay, which demonstrated that LexA binding interferes with *crhR* expression possibly through interference with promoter recognition and/or transcription initiation. Based on these results, it appears that *Synechocystis* LexA functions as a repressor of *crhR* expression. LexA activity is well studied in *E. coli* and other prokaryotes where it regulates expression of ~20 unlinked genes associated with DNA damage repair, the SOS regulon, which

include *recA* and *lexA* (Little and Mount, 1982). Derepression occurs following DNA damage, and requires RecA-stimulated LexA autocleavage and subsequent derepression of *lexA*, *recA* and other regulon members (Little and Mount, 1982). Induction following DNA damage ranges between regulon members; *recA* and *lexA* are induced 10 and 2 to 5 fold, respectively (Little *et al.*, 1991; Courcelle *et al.*, 2001; Quillardet *et al.*, 2003). Similarly, DNA damage caused by UV-irradiation or mitomycin C treatment strongly induces *recA* transcript and protein accumulation in another cyanobacterium, *Anabaena variabilis* (Owttrim and Coleman, 1987; Owttrim and Coleman, 1989). Levels of both the *recA* transcript and its protein remain elevated until the damaging agents are removed and/or the DNA repaired, as observed for *E. coli recA* transcripts (Little and Mount, 1982). In contrast, expression of the *Synechocystis recA* and *lexA* genes was not induced by UV-irradiation. Rather, our results show that *recA* and *lexA* levels decrease following UV treatment, in agreement with other studies (Huang *et al.*, 2002; Domain *et al.*, 2004). These results indicate that the DNA damage induction of *recA* is variable among not only cyanobacteria but also prokaryotes in general. Our observation that *lexA* is not induced following UV-irradiation further suggests that *Synechocystis* LexA is not required for survival following DNA damage, and therefore potentially regulates expression of genes not associated with DNA repair. Therefore, the Synechocystis protein is LexA-related and not a homolog of the SOS-regulatory proteins observed in other bacteria systems.

The discrepancies in *lexA* regulation may also imply differences at the protein level, where LexA self-cleavage may not be required for derepression of gene expression. This appears to be the case, as *Synechocystis* LexA possesses modifications in two sites important for LexA function in *E. coli*; an altered cleavage site, and the absence of the nucleophilic serine of the Ser-Lys dyad. In *E. coli*, LexA self-cleavage and derepression of the SOS regulon requires a catalytic serine/lysine dyad and an Ala-Gly cleavage bond (Slilaty and Little, 1987). In the absence of these residues, as indicated by mutational studies, LexA self-cleavage in *E. coli* is defective (Slilaty and Little, 1987). These modifications to the *Synechocystis* protein have previously been noted as a potential explanation for the absence of a "cyanobacterial" SOS box within the upstream regions of *Synechocystis* DNA repair genes (Mazón *et al.*, 2004), and further imply an unique cellular function for the *Synechocystis* LexA protein.

Novel roles for LexA have been implied in other bacteria, including *Mycobacterium tuberculosis* (Davis *et al.*, 2002b; Rand *et al.*, 2003) and *Deinococcus radiodurans* (Narumi *et al.*, 2001), although the alternative function has not been identified. In *D. radiodurans*, RecA protein levels remain unchanged regardless of the *lexA* status (Bonacossa de Almeida *et al.*, 2002), which is unexpected if LexA is required to regulate *recA* expression and is similar to the results reported here. DNA damage induction of repair genes in *M. tuberculosis* also occurs predominately via a LexA- and RecA-independent mechanism as shown by mitomycin C induction of DNA repair gene expression in *recA* mutants (Davis *et al.*, 2002b; Rand *et al.*, 2003). Evidence for separation of

recA expression from *lexA* regulation may also exist in higher plant chloroplasts, which possess a DNA damage-induced *recA* homologue (Cerutti *et al.*, 1993), while *lexA* has not been reported to be encoded by plant genomes. This evidence suggests that conservation of the LexA/RecA regulation of the SOS response may be less widespread than previously anticipated, and furthermore, homologues of these proteins may fulfill different roles in their respective hosts.

Identification of a LexA-related protein as the regulator of *crhR* transcription provides insights into the mechanism by which redox-regulated gene expression is controlled in photosynthetic cyanobacteria. The observations suggest a regulatory role for *Synechocystis* LexA in regulating gene expression in response to environmental cues other than DNA damage. These insights also imply the ubiquitous nature of the LexA/RecA DNA repair dogma is not conserved in *Synechocystis*, raising questions regarding the mechanisms by which DNA repair gene expression is regulated in this organism.

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**Chapter 3: A *Synechocystis* LexA-orthologue binds direct repeats
in target genes**

3.1 Introduction

Escherichia coli LexA is a well-established transcriptional repressor of the SOS DNA damage repair response (Little and Mount, 1982). In *E. coli*, LexA represses expression of 31 unlinked genes, including itself and that of its co-protease RecA, under normal growth conditions by binding and blocking RNA polymerase access to regulated promoters (Little *et al.*, 1981; Little and Mount, 1982; Fernández de Henetrosa *et al.*, 2000). Conditions which damage DNA or inhibit DNA replication induce the expression of LexA-regulated genes whose products are required for DNA replication, DNA repair and control of cell division (Little and Mount, 1982; Luo *et al.*, 2001). Derepression of the LexA regulon proceeds via an intramolecular self-cleavage reaction requiring a catalytic serine nucleophile, a basic lysine residue and alanine-glycine bond cleavage (Slilaty and Little, 1987; Shepley and Little, 1996). ssDNA produced by DNA damage or replication inhibition activates RecA and in this form, RecA stabilizes the cleavable conformation of LexA allowing self-cleavage to proceed (Luo *et al.*, 2001). *E. coli* LexA self-cleavage inactivates its ability to bind a conserved inverted repeat sequence, CTGT $(TA)_4$ ACAG, found within the promoter regions of regulon members (Walker, 1984; Fernández de Henestrosa *et al.*, 2000)

LexA homologues have been characterized in a wide variety of bacterial species. These homologues are structurally related to the *E. coli* protein and are also involved in regulating cellular responses to DNA damage (Miller *et al.*, 1996; Davis *et al.*, 2002; Au *et al.*, 2005). Unlike many transcriptional regulators with conserved binding sites across various bacterial genomes (Makarova *et al.*,

2001; Rodioniv *et al.*, 2002; Khan *et al.*, 2006), LexA homologues exhibit

significant variation in both SOS box sequence, location and regulon members

both within and between species (reviewed in Erill *et al.*, 2007). Identified binding

motifs may be characterized as either symmetrical or asymmetrical containing

palindromic or direct repeats separated by spacer regions of variable length.

Similarities in the LexA binding motifs are evident in *Bacillus subtilis* (Miller *et al.*,

1996; Au *et al.*, 2005), *Mycobacterium tuberculosis* (Movahedzadeh *et al.*, 1997),

and *Anabaena* (Mazón *et al.*, 2004), which are closely related to the consensus

sequence GAAC-N_4-GTTC (Erill *et al.*, 2007). In contrast, sites containing

imperfect palindromes and odd numbered spacer regions have also been shown

to form complexes with the LexA protein in *Xanthomonas campestris*, *Xylella*

fastidiosa and *Myxococcus xanthus* (Campoy *et al.*, 2002; Yang *et al.*, 2002;

Campoy *et al.*, 2003). In the alpha-proteobacteria, the LexA binding site consists

of a direct repeat, GTTC-N_7-GTTC (Fernández de Henestrosa *et al.*, 1998;

Tapias and Barbé, 1998). The diversity among LexA homologues is also evident

in the identity of regulated genes. A minimal core of regulated genes is

suggested to include the *lexA*, *recA*, *uvrA*, *ssb* and *ruvAB* genes however, many

bacteria contain a LexA homologue, which does not regulate any of the core

genes except for *lexA* itself (Erill *et al.*, 2007). Furthermore, large scale

approaches to the identification of LexA regulon members have recently

identified regulated genes with no apparent role in DNA repair further

demonstrating the need to better understand the physiological role performed by

LexA in different bacterial species (Au *et al.*, 2005; Kelley, 2006).

Variability among regulon members and consequently LexA's

physiological role in the cell is no more evident than in cyanobacteria, where

novel roles for the LexA protein have recently been suggested in several species

(Domain *et al.*, 2004; Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005;

Patterson-Fortin *et al.*, 2006; Ferreira *et al.*, 2007; Sjöholm *et al.*, 2007; chapter

2). In the photosynthetic cyanobacterium, *Synechocystis* sp. strain PCC 6803,

LexA was identified as a negative regulator of redox responsive gene expression

(Patterson-Fortin *et al.*, 2006; chapter 2). Independently, LexA was also identified

as a regulator of the bidirectional hydrogenase *hoxEFUYH* (Gutekunst *et al.*,

2005; Oliveira and Lindblad, 2005) and carbon utilization genes (Domain *et al.*,

2004). Further divergence from the established LexA/RecA DNA repair dogma is

evident at both the RNA and protein levels. Accumulation of *lexA* and *recA*

transcripts decreased following UV irradiation treatment (Huang *et al.*, 2002;

Domain *et al.*, 2004; Patterson-Fortin *et al.*, 2006) unlike the scenario observed in

E. coli where DNA damage stimulates induction of regulon members from 2- to

10-fold (Little *et al.*, 1981; Courcelle *et al.*, 2001; Quillardet *et al.*, 2003). At the

protein level, *Synechocystis* LexA lacks the serine nucleophile and Ala-Gly

cleavage bond, sites important for LexA function in SOS regulated systems,

implying LexA self-cleavage is not involved in derepression of regulon expression

in this system (Patterson-Fortin *et al.*, 2006; chapter 2). The observed differences

between the *E. coli* and *Synechocystis* proteins suggest that the identified

Synechocystis protein is a LexA orthologue that has acquired new functional

domains to better suit its physiological role in the cell (Patterson-Fortin *et al.*, 2006; chapter 2).

In the present work, we demonstrate a specific interaction between the *Synechocystis* LexA protein and the *lexA* promoter and examine the nature of the LexA-target gene interaction. Our analysis identified sequences important for DNA binding by dimeric LexA.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

Synechocystis sp. strain PCC 6803 cultures were grown and maintained as previously described (Chapter 2). *Escherichia coli* strains DH5α and JM109 were used for propagation and protein expression as previously described (Chapter 2).

3.2.2 PCR amplification

PCR amplification was performed using the primer pairs listed in Table 3.1 as previously described (Chapter 2). Optimal annealing temperatures for each primer pair were experimentally determined. DNA fragments were purified using the QIA Quick PCR purification kit (QIAGEN) where appropriate.

Table **3.1** Oligonucleotides used in this study. Relative location designates oligonucleotide position with respect to the transcriptional start site (+1). Restriction enzyme recognition sites are in bold. Base pair changes within oligonucleotides LPF-53 and LPF-60 to -68 are underlined.

Primer	Sequence 5' – 3'[a,b]	Relative Location[c]	Application
T7	GTATACGACTCACTATAGGGC		Sequencing
GWO-13	GTAAAACGACGGCCAGT	pBSKS+	Forward primer to obtain KC+5 EMSA target with GWO-45
GWO-14	AACAGCTATGACCATG	pBSKS+	Forward primer to obtain EMSA targets with GWO-45
GWO-25	CGAACGGATCCATGACTA	99	Forward primer to obtain GW0 EMSA target with GWO-45
GWO-42	AGGGGAATGGCTTCAGTTTG	228	Sequencing
crhR			
GWO-45	AAGCCAATGTCGGCCAAGAG	189	Reverse primer to obtain EMSA targets with GWO-45 or LPF-55
LPF-53	TCCATGACTAATACTCCGCGCCTTGACTAGTACCTTC	107	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-54	TTTCCATGACTAATAACTAGTACCTTCGCTGA	105	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-55	TGTTATTGACGGTTGGTTCC	9	Forward primer to obtain DNaseI footprinting target with GWO-45
LPF-60	CGAAATATTTAACTTTCCGCTGCTAATACTTTGACTAGTACC	90	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-61	ATTATTTAACTTTCCATGAACGGTACTTTGACTAGTACCTTCG	94	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-62	TTTAACTTTTCCATGACTAACCTGTTGACTAGTACCTTCGCTGAC	98	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-63	ACTTTTCCATGACTAATACTCGCTCTAGTACCTTCGCTGACCTTG	102	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-64	TTCCATGACTAATACTTTGATGCTTACCTTCGCTGACCTTGGTC	106	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-65	TATCCGAAATTATTTAACTTGATGACTAATACTTTGACTAG	86	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-66	ATGACTAATACTTGACTAGGCGTTCGCTGACCTTGGTCTTTC	110	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-67	CTAATACTTTGACTAGTACCGATCCTGACCTTGGTCTTTCTGAA	114	Primer to introduce site-directed basepair mutations in the LexA binding site
LPF-68	TACTTTGACTAGTACCTTCGAGATCCTTGGTCTTTCTGAAAAAC	118	Primer to introduce site-directed basepair mutations in the LexA binding site
lexA			
LPF-1	CCTATAGGAGATCTTACATG	18	Forward primer for cloning LexA DNA binding domain with LPF-19
LPF-4	ATTTGCGTTCTCCGGCC	140	Forward primer to obtain LPF 4:5 EMSA target with LPF-5
LPF-5	CTTCGATTTCCTCTTCTC	452	Reverse primer to obtain LPF 4:5 EMSA target with LPF-4
LPF-3	CCATTTCTGAGATCTTCGGCG	-191	Forward primer to obtain *lexA* target for EMSA and DNaseI footprinting with LPF-6
LPF-6	GGCGGATGGGAGGGGCGTG	122	Reverse primer to obtain *lexA* target EMSA and DNaseI footprinting with LPF-3
LPF-19 LPF-56	TTTTGGTTATTGGTGGAGAA	256	Reverse primer for cloning LexA DNA binding domain with LPF-1 Forward primer to obtain DNaseI footprinting target with LPF-6
sll0359			
LPF-71	ACTCCTAGATCTCCAAACGCCTCC		Forward primer for cloning *sll0359* gene with LPF-59
LPF-59	GCGAAAAGCACTTAGTCCC		Reverse primer for cloning *sll0359* gene with LPF-71
hox			
ShopxrF	GCAATTGGGGTTGCGACTAT		Forward primer to obtain *hox* promoter with ShopxrR (Oliveira and Lindblad, 2005)
ShopxrR	CCTCCACAATCTTGCCCACAATAA		Reverse primer to obtain *hox* promoter with ShopxrF (Oliveira and Lindblad, 2005)

a. Restriction sites are shown in italics.   b. Site-directed basepair mutations are shown in bold.   c. Position of 5' end of the oligonucleotide relative to the transcriptional start site.

3.2.3 Generation, expression and purification of the NLexA, rLexA and rAbrB-like proteins

The NLexA and rLexA overexpression constructs were generated as previously described (Chapter 2). The *Synechocystis* AbrB-like protein was produced by expression of an in-frame translational fusion in *E. coli*. PCR amplified DNA was digested with *Bgl*II and ligated into *Bgl*II and *Pvu*II digested pRSETB vector DNA (Invitrogen) producing psll0359 which expresses a recombinant 17.08 KDa His-AbrB-like polypeptide. The psll0359 plasmid was sequenced to confirm successful in-frame insertion of the DNA. The AbrB-like protein was expressed and purified as previously described for rLexA and NLexA (Patterson-Fortin *et al.*, 2006; Chapter 2).

3.2.4 Electrophoretic mobility shift assays

EMSA were performed using the indicated recombinant proteins and PCR-generated DNA fragments as previously described (Patterson-Fortin *et al.*, 2006; chapter 2) with the addition of BSA (0.05 mg/mL). DNA targets were end-labeled with [γ-^{32P}]ATP using polynucleotide kinase (Fermentas) and purified from 5% TBE non-denaturing polyacrylamide gels, and eluted overnight at room temperature in 0.5M ammonium acetate, 1mM EDTA, and 0.1% SDS. A 321 bp non-specific competitor DNA of the *Synechocystis lexA* gene was prepared to control for non-specific protein binding to the *lexA* promoter (Patterson-Fortin *et al.*, 2006; chapter 2). The equilibrium dissociation constants (Kd) for rLexA and NLexA binding to the *crhR* and *lexA* targets were determined from LexA

saturation experiments, and the data quantified using ImageQuant™ v. 4.1 image analysis software (Molecular Dynamics).

3.2.5 DNaseI Footprinting

DNase I footprinting assays were performed using singly labeled DNA targets. Primers, GWO-45, LPF-6, LPF-55 or LPF-56 were end-labeled with [γ-^{32}P]ATP using polynucleotide kinase (Fermentas) and used for PCR amplification together with the specified unlabeled primer (Table 3.1). PCR amplification was performed using an annealing temperature of 45°C and products purified from a 5% non-denaturing polyacrylamide gel. Binding reactions were performed for 20 min at 37°C in 1X binding buffer (10 mM Tris-HCl pH 7.5, 50 mM KCl, 2 mM DTT, 10% glycerol), 1 μg ssDNA, 15 000 cpm labeled DNA, 2.5 mM $CaCl_2$ and 5 mM $MgCl_2$, and the indicated rLexA concentration in a final volume of 20 μl. DNaseI (Fermentas), 0.025 U, digestion was performed for 10 s at 37°C, and quenched by addition of 40 mM EDTA (Campoy *et al.*, 2003). Reaction products were separated on a denaturing (6M urea) 6% polyacrylamide sequencing gel and subjected to autoradiography. DNA sequencing ladders were synthesized with the appropriate primers and template DNA using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (USB Corporation) and 33P-dideoxy nucleotide terminators (Amersham) according to manufacturer's instructions.

3.2.6 Primer extension and SI nuclease protection assay

Total RNA was isolated from *Synechocystis* by mechanical lysis as described previously (Chamot *et al.*, 1999). Primer annealing reactions contained 1X aqueous hybridization buffer (1 M NaCl, 0.167 M HEPES-KOH pH 7.5, 0.33 M EDTA), 50 μg total RNA, 6×10^5 cpm ^{32}P-labelled LPF-6 and 10 U RNaseOUT (Invitrogen). Annealing was performed at 85°C for 10 min, 65°C for 90 min followed by slow cooling to 30°C. The solution was ethanol-precipitated, and the pellet resuspended in reverse-transcriptase mix [0.55 mM of each dNTP, 10 U RNase inhibitor, 1X M-MuLV reverse transcriptase buffer (New England BioLabs) and 20 Units M-MuLV reverse transcriptase (New England BioLabs)]. DNA synthesis proceeded for 1 h at 37°C. The reaction was stopped by RNaseA digestion of the RNA template.

S1 annealing reactions contained 1X aqueous hybridization buffer (1 M NaCl, 0.167 M HEPES-KOH pH 7.5, 0.33 M EDTA), 25 μg total RNA, 1.2×10^5 cpm ^{32}P-labelled LPF 3:6 and 10 U RNaseOUT (Invitrogen). LPF 3:6 was generated by PCR amplification as described above using the primers LPF-3 and LPF-6 and an annealing temperature of 60°C. Gel purified product was end-labeled with [γ-^{32}P]ATP using polynucleotide kinase (Fermentas). Annealing was performed at 85°C for 10 min, slowly cooled to 37°C followed by a 37°C incubation for 1 h. S1 (Fermentas), 10 U, digestion was performed for 30 min at 37°C in 1.4X S1 nuclease buffer (Fermentas) and 20 μg/ml ssDNA and quenched by addition of S1 stop solution (0.8 M NH_4OAc, 8 μg/ml tRNA, 4 mM EDTA).

Reaction products were separated on a denaturing (6M urea) 6% polyacrylamide gel and subjected to autoradiography. DNA sequencing ladders were synthesized with the appropriate primers and template DNA as described above.

3.2.7 Binding site mutagenesis

Oligonucleotides used for mutagenesis are listed in Table 3.1; changes to the DNA sequence are in underlined. Primers were phosphorylated with ATP and T4 polynucleotide kinase (Fermentas). The mutants 53 and 54 were prepared by Kunkel mutagenesis (Kunkel *et al.*, 1991). Annealing reactions were performed at 85°C in 1X annealing buffer (0.2 M Tris pH 7.5, 20 mM $MgCl_2$, 0.5 NaCl), 0.3 μg ssDNA and 0.05 μg primer. DNA synthesis reactions containing 1X synthesis buffer (4 mM dNTPs, 7.5 mM ATP, 175 mM Tris pH 7.5, 37.5 mM $MgCl_2$, 15 mM DTT), 40 U T4 DNA ligase (New England Biolabs) and 0.3 U T7 DNA polymerase (New England Biolabs) were incubated for 5 min at 0°C, 5 min at room temperature (RT) , and 2 h at 37°C. Reaction products were transformed into *E. coli* DH5α cells. Mutants 60 t0 68 were prepared by thermal cycling plasmid mutagenesis as described by Sawano and Miyawaki (2000). Mutagenesis reactions contained 1.1X PfuTaq buffer (1X PT Buffer (0.05M KCl, 0.01M Tris-Cl, pH 8.5, 1.5 mM $MgCl_2$ and 0.1 mg/ml BSA) and 0.2 mM dNTPs), 10 mM NAD, phosphorylated primer, 0.55 μg template DNA, DMSO, 12U *Taq* DNA ligase (New England Biolabs) and 5U *Pfu* DNA polymerase. The PCR program consisted of an initial 2 min denaturation at 95°C followed by 30 cycles of 1 min

denaturation at 95°C, 1 min annealing at 55°C and 10 min extension at 65°C. Reactions were incubated with 10 U *Dpn*I (Fermentas) for 2 h at 37°C prior to transformation into *E. coli* XL1-Blue cells. The mutant *crhR* binding sites were confirmed by sequencing. The 5' and 3' constructs containing sequences upstream and downstream of the *Spe*I restriction site, respectively were generated by restriction digestion. The 3' construct was generated previously (Colvin, 2002; Patterson-Fortin *et al.*, 2006, chapter 2). The 5' construct was generated by *EcoRI/Spe*I restriction enzyme digestion of CS0096-9 (Kujat and Owttrim, 2000) and ligation into *EcoRI/Spe*I digested pBluescript KS⁺ (Stratagene). A 180° rotation of the binding site was generated by *EcoRI/Hinc*II digestion of CS0096-9 (Kujat and Owttrim, 2000) and ligation into *EcoRI/Hinc*II digested pBluescript KS+ (Stratagene). Mutant *crhR* EMSA targets were generated by PCR as previously described (Table 3.1).

3.2.8 RNA EMSAs

Radioactive ssRNA targets were generated using the Promega Riboprobe T7 system according to manufacturer's instructions. Linearized plasmids KC+5 and JW-8 were used to generate the 173 nt *crhR* and 162 nt *crhC* (D. Chamot and G. Owttrim, unpublished data) transcripts respectively. RNA EMSAs were performed as previously described (Sparanese and Lee, 2006). Labeled RNAs were heated to 50°C for 5 min and cooled to RT before addition to the binding reaction. Binding reactions contained 1X RNA EMSA buffer (5 mM Tris-HCl pH 7.4, 2.5 mM EDTA pH 8.0, 2 mM DTT, 5% glycerol, 0.1 mg/mL BSA, 0.5 mg/mL

yeast tRNA), 2 x 10^4 cpm ^{32}P labeled RNA, 10 U RNaseOUT (Invitrogen) and rLexA as indicated in a final volume of 20 μl. Binding was performed at 30°C for 10 min followed by 5 min at 0°C. This sequence of incubation was repeated twice. Heparin (Sigma, 5 mg/mL) was added and reactions incubated for 5 min at 0°C. Reaction products were separated on a denaturing (6M urea) 5% polyacrylamide gel and subjected to autoradiography.

3.2.9 Determination of rLexA oligomeric state

Size exclusion chromatography was used to determine the oligomeric state of rLexA in solution. Purified rLexA was separated on a Superose 12 FPLC gel filtration column calibrated with five protein standards in 300 mM NaCl; 50 mM NaH$_2$PO$_4$ buffer. Fractions containing rLexA were determined by colloidal Coomassie Brilliant Blue G-250 staining and Western analysis. The oligomeric state of rLexA and NLexA when bound to DNA was determined using the method developed by Orchard and May (1993). The PCR-amplified DNA fragments, KC+5 (Chapter 2) and GWO (Table 3.1) were end-labled with [γ-^{32}P]ATP and gel purified. Protein-DNA complexes and low molecular weight non-denatured protein standards (Bio-Rad) were separated on 4.5%, 5%, 6%, 7%, 8%, 9% and 10% TBE non-denaturing polyacrylamide gels. The lanes containing the protein standards were stained with colloidal Coomassie Brilliant Blue R-250 while the lanes containing the DNA-protein complexes were subjected to autoradiography. The Rf of each protein standard was plotted against the acrylamide concentration and the slope determined to give a retardation coefficient (Kr). Plotting the Kr for

each protein standard against its molecular weight produces a standard curve from which the molecular weight of the various protein-DNA complexes and the DNA target can be determined.

3.3 Results

3.3.1 Synechocystis LexA interacts with its own promoter

Recombinant LexA (rLexA) was used to test interaction with the *lexA* gene. The LPF 3:6 target (313 bp) encompasses the promoter, the 5' untranslated region and 100 bp of open reading frame. Mobility of this *lexA* fragment was decreased upon incubation with rLexA in a concentration dependent manner (Figure 3.1A). The specificity of binding was demonstrated by competition assays using increasing concentrations of either specific or non-specific competitor DNA. Addition of unlabelled specific DNA targets, either LPF 3:6 (313 bp) or the binding site in *crhR*, KC+5 (167 bp), challenged formation of the shifted complex (Figure 3.1B, lanes 3-6). Specifically, specific competitor shift assays suggest rLexA has a stronger affinity for its own promoter than for the *crhR* target (Figure 3.1B. compare lanes 4 and 6). In contrast, addition of a non-specific internal *lexA* fragment (321 bp) did not significantly alter mobility of the shifted target (Figure 3.1B, lanes 7 and 8). These results suggest that rLexA interacts specifically with its own promoter.

A

rLexA (nM)

0 25 50 75 100 125 150

complex

free probe

B

| | Specific | Non-specific |

complex

free probe

0 10 25 10 25 10 25

competitor DNA (fold excess)

1 2 3 4 5 6 7 8
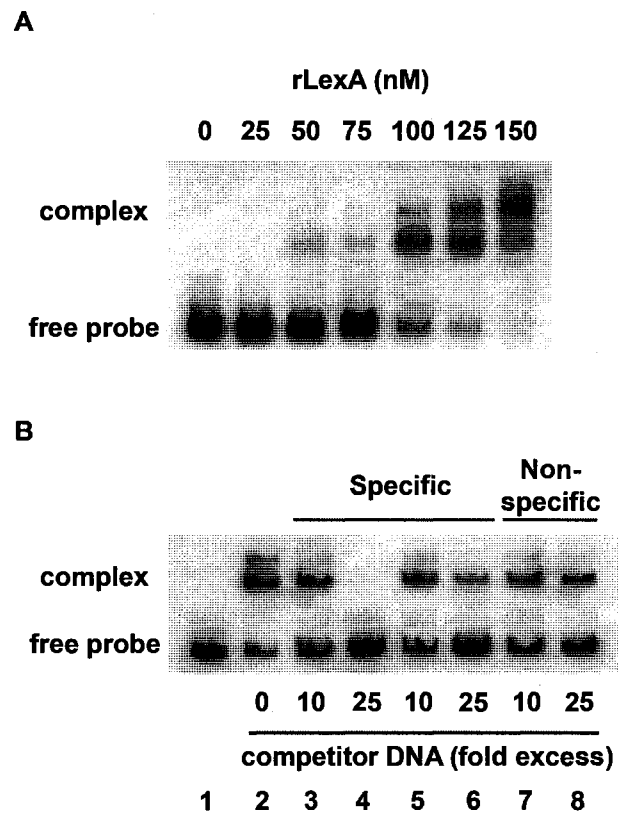
Figure 3.1 rLexA binding to the *lexA* promoter.

A. rLexA concentration curve. Increasing concentrations of rLexA were incubated with ^{32}P-labeled LPF 3:6. LPF 3:6 (313 bp) encompasses the promoter, the 5' untranslated region and 100 bp of the open reading frame.

B. DNA competition assays. rLexA (100 nM) was incubated with ^{32}P-labeled LPF 3:6 and the indicated fold excess of either specific competitor DNAs from the *lexA* and *crhR* genes (unlabeled LPF 3:6, lanes 3-4; unlabeled KC+5, lanes 5-6) or non-specific competitor DNA (321 bp internal *lexA* fragment, lanes 7-8).

3.3.2 Dissociation constants for rLexA and NLexA binding to the crhR *and* lexA *targets*

Initial observations suggesting differential binding affinities by rLexA for its targets, *lexA* and *crhR*, were further investigated (Figure 3.1) using LexA saturation experiments. Equilibrium dissociation constants (Kd) were determined for rLexA and NLexA binding to the *crhR* and *lexA* targets (Figure 3.2). The determined Kd values for rLexA binding to the *crhR* and *lexA* targets are 57.8 ± 1.2 nM and 70.8 ± 1 nM, respectively. The low binding affinities of rLexA for both the *crhR* and *lexA* targets may be an indication that LexA regulation in *Synechocystis* is directed towards a fine-tuning mechaninsm. The lower affinity binding sites ensures the *lexA* and *crhR* are always expressed at basal levels with increased or decreased expression under certain environmental conditions. Truncated NLexA, containing the amino-terminal DNA binding domain, binds to the *crhR* target with a Kd of 891 ± 1.2 nM, 15.4-fold weaker than binding by the full-length protein.

3.3.3 Synechocystis lexA *transcription initiates at a T residue*

Primer extension identified the transcriptional start site of the *lexA* gene (Figure 3.3A). *lexA* transcription initiates at a T residue located 35 nucleotides upstream of the translational start codon. The transcript start point is not altered by variation in light conditions. Identical results were observed when SI nuclease protection assays were used to determine the *lexA* transcription start site (Figure 3.3B).

A. rLexA/KC+5
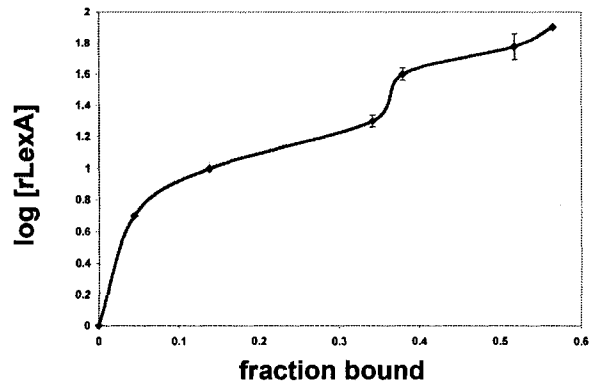
B. NLexA/KC+5
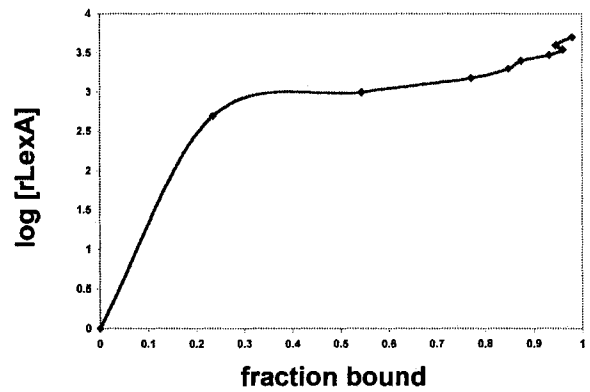
C.rLexA/*lexA*
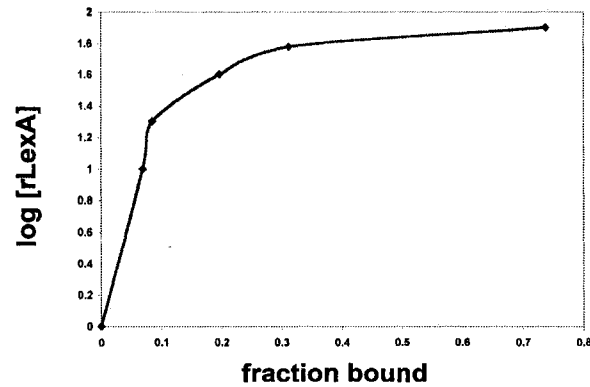
Figure 3.2 LexA equilibrium dissociation constants. LexA saturation experiements were performed to determine Kd values for rLexA and NLexA binding to the *crhR* and *lexA* targets. Kd values were determined from duplicate, independent EMSA replicates using ImageQuant (Molecular Dynamics).

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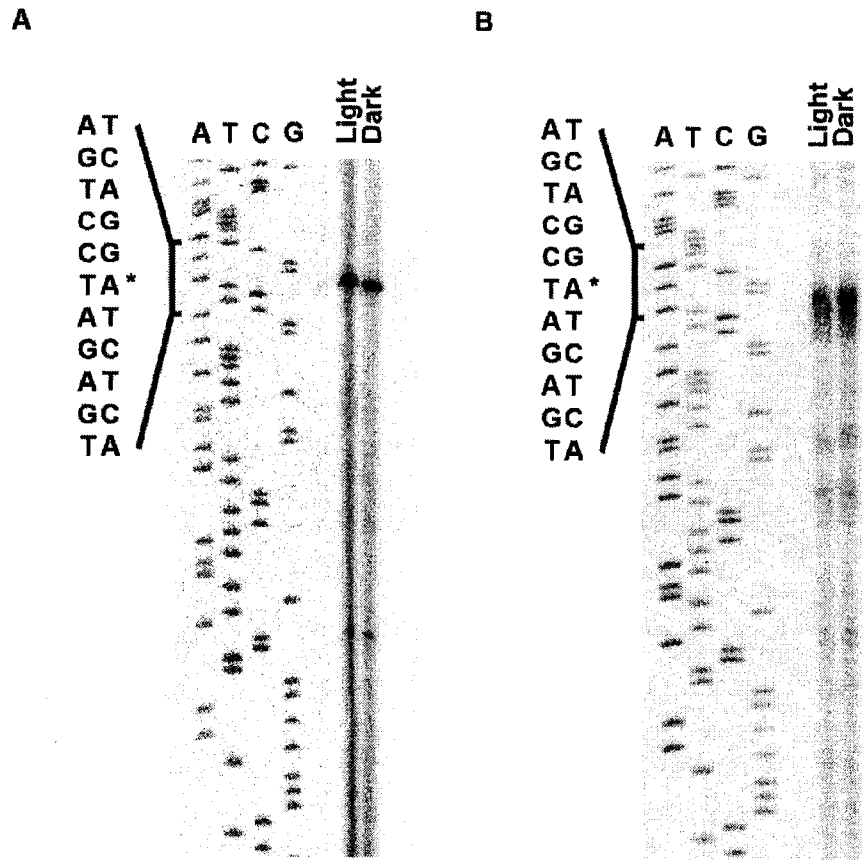
Figure 3.3 *lexA* transcription start site determination. Primer extension **(A)** and S1 nuclease protection assay **(B)** were used to identify the *lexA* transcript start site under light and dark growth conditions (3 hours). Products were separated on a denaturing (6M Urea) 6% polyacrylamide gel alongside sequencing reactions.

3.3.4 rLexA protected DNA contains direct repeat sequences

To further characterize the rLexA binding site on the *crhR* and *lexA*

targets, DNaseI footprinting was performed to identify the sequences protected

from endonuclease digestion by rLexA binding. Binding of rLexA to the *crhR*

gene protects the DNA from DNaseI digestion with the degree of protection being

protein concentration dependent (Figure 3.4A). rLexA binding protects 31

nucleotides from +108 to +139 in *crhR*. Similarly, rLexA binding to the *lexA* gene

protects 34 nucleotides from -5 to +29 (Figure 3.4B). Identical results were

observed in both cases when binding was assayed on the opposite strand

(Figure 3.5). Alignment of the *crhR* and *lexA* protected sequences revealed 12 bp

direct repeats, the half sites containing two and three mismatches, respectively

(Figure 3.4C). Spanning these direct repeats is a conserved sequence consisting

of $CTA-N_9-CTA$ within which the spacer region is A/T rich (7 of 9 bp) (Figure

3.4D).

The existence of direct repeats together with sequence similarities

between the two targets was used to initiate further study of the *Synechocystis*

rLexA binding site. Initially, the *Spe*I restriction site was used to split the

protected sequence in half (5' and 3' targets). As shown in Figure 3.6B, loss of

sequences either 5' or 3' to the *Spe*I site lowered rLexA binding efficiency

(compare lanes 2, 4 and 6). Similarly, the addition (LPF-53; CCGCC) or deletion

(LPF-54) of 5 bp decreased rLexA-*crhR* complex formation (compare lanes 2, 10

and 12). A 180° rotation of the binding site had minimal effect on rLexA binding

capabilities (compare lanes 2 and 8). To identify which sequences are important

A *crhR*

rLexA (µM)

0 0.13 0.25 0.50 1.0   A T C G

+80

+90

+100

+111 (ATG)

protected sequence

+120

+130

+140

B *lexA*

rLexA (µM)

0 0.13 0.25 0.50 1.0   A T C G

−10

+1

+10

+20

+30

ATG

+40
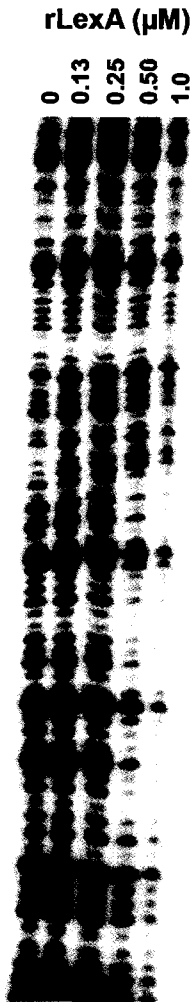
protected sequence
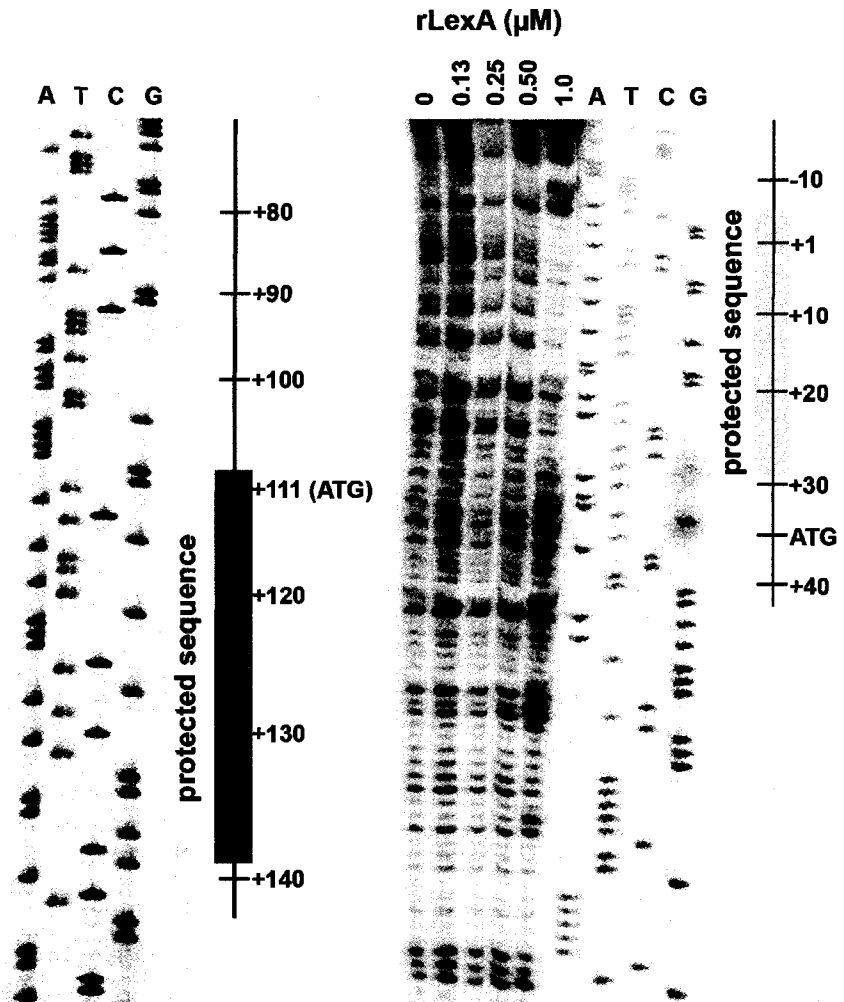
C Synechocystis LexA protected sequences

1   2

crhR   CCATGA [████████████] GTACCTTCGC

3   4

lexA   AGTCCTAGAGTC [████████████] TAGGAGA
              +1
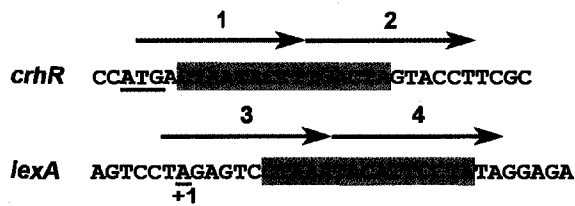
D Synechocystis LexA direct repeat alignment

Figure 3.4 DNaseI footprint analysis of the crhR and lexA non-coding strands.

A. KC+5 footprinting (*crhR* non-coding strand). ^{32}P-labeled KC+5 was incubated with increasing concentrations of rLexA and subject to DNaseI cleavage.

B. LPF 3:6 footprinting (*lexA* non-coding strand). ^{32}P-labeled LPF 3:6 was incubated with increasing concentrations of rLexA and subject to DNaseI cleavage. The boxed regions represent protected sequences.

C. Schematic of rLexA protected sequences on the *crhR* and *lexA* targets. The transcription (+1) and translation start sites are underlined. The direct repeat sequences are indicated by arrows and numbered sequentially. The CTA-N$_9$-CTA motifs are boxed in blue.

D. Sequence logo of the CTA-N$_9$-CTA direct repeat using "Weblogo" (Crooks *et al.*, 2004).

Figure 3.5. DNasel footprint analysis of the *crhR* and *lexA* coding strands.
A. LPF 55:GWO 45 footprinting (*crhR* coding strand). ^{32}P-labeled KC+5 was incubated with increasing concentrations of rLexA and subject to DNasel cleavage.
B. LPF 56:6 footprinting (*lexA* coding strand). ^{32}P-labeled LPF 3:6 was incubated with increasing concentrations of rLexA and subject to DNasel cleavage. The boxed regions represent protected sequences

A *crhR* LexA binding site mutants
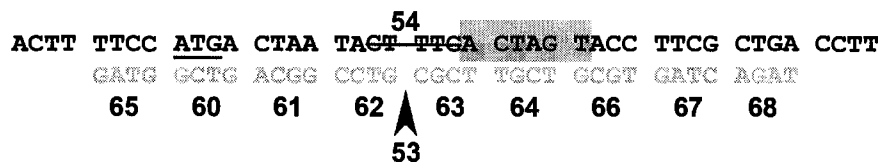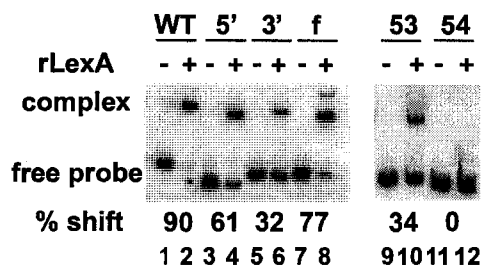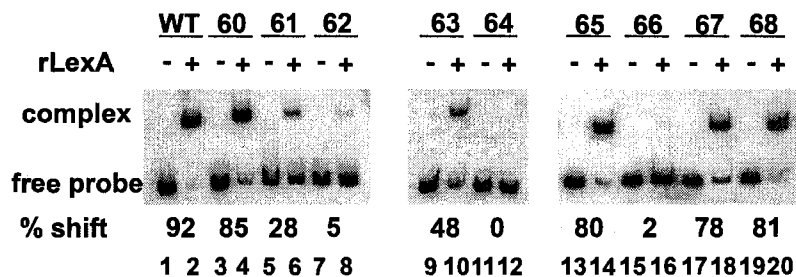
Figure 3.6 Characterization of the *crhR* rLexA binding site.
A. Schematic of binding site mutants. The translation start site is underlined and the *Spe*I restriction site shaded. The position of bp addition (53) is indicated by an arrowhead. Deleted bp are designated by a strikethrough (54). Bp changes to the putative LexA binding site are shown in grey beneath the region of sequence mutagenized (60-68). One set of 4 basepair changes corresponds to 1 mutant *crhR* binding site.
B and C. ^{32}P-labeled targets were incubated either in the absence (-) or the presence (+) at subsaturating concentrations of rLexA. "f" refers to a 180° rotation of the binding site. The relative level of binding for a representative gel is shown below each lane, as determined using ImageQuant.

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for rLexA binding to the *crhR* gene, a total of nine mutated sequences were constructed (Figure 3.6A, numbered 60-68). EMSA analysis using the mutated targets identified sequences important for rLexA recognition (Figure 3.6C, compare lane 2 to lanes 6, 8, 10, 12 and 16) and those which did not significantly alter rLexA's binding activity (Figure 3.6C, compare lane 2 to lanes 4, 14, 18 and 20). The results confirm that the direct repeats and specifically the CTA-N_9-CTA motif are important for LexA binding to the *crhR* gene.

3.3.5 rLexA does not bind the crhR transcript

Due to the location of the LexA repressor binding site, we were interested in determining whether rLexA binds the *crhR* transcript and exerts its effect on expression post-transcriptionally. RNA gel shifts were performed using a T7-generated transcript encompassing the complete rLexA binding site. rLexA does not specifically interact with the *crhR* transcript as heparin progressively reduces rLexA binding (Figure 3.7A). Non-specific binding by rLexA to RNA was also shown using a *crhC* transcript, whose expression is not expected to be regulated by LexA (Figure 3.7B). Taken together, these results suggest rLexA binds non-specifically to RNA and consequently does not appear to exert its effect on gene expression at the post-transcriptional level.

3.3.6 rLexA binds the crhR gene as a dimer

Since the LexA-protected sequences contain direct repeats, we were interested in determining the oligomeric state of *Synechocystis* rLexA in the presence and absence of DNA target. In solution, rLexA exists predominately as

A *crhR*

heparin (μg/μl)	0	0	5	10
rLexA	-	+	+	+

complex

free probe

B *crhC*

heparin (μg/μl)	0	0	5
rLexA	-	+	+

complex

free probe
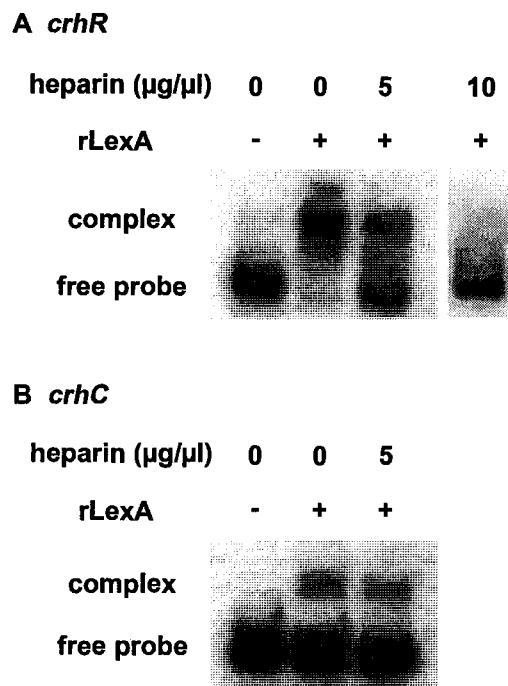
Figure 3.7 rLexA interactions with the *crhR* and *crhC* RNAs.
A. rLexA-*crhR* RNA interaction. *rLexA* was incubated with a T7-generated ^{32}P-labeled *crhR* transcript in the absence or the presence of increasing concentrations of heparin.
B. Specificity of rLexA-RNA interactions. As a control reaction, rLexA was incubated with *crhC* transcript in the absence and the presence of heparin . *crhC* expression is not known to be regulated by LexA.

a monomer at low protein concentration (Figure 3.8A). We then determined the molecular masses of the rLexA-*crhR* and NLexA-*crhR* complexes using the EMSA-based method described by Orchard and May (1993) which assumes the analysed proteins have a similar shape to the protein standards used. Two DNA targets were tested both encompassing the LexA binding site on *crhR* as demonstrated by DNaseI footprinting. The molecular masses of the rLexA-GWO DNA and rLexA-KC+5 DNA complexes were 91 KDa and 104 KDa respectively (Figure 3.8B). The calculated molecular mass of the DNA targets themselves were 32.5 KDa for GWO and 45 KDa for KC+5. Subtracting the contributions of the target DNA, the molecular masses for the two complexes formed were 58.5 KDa and 59 KDa, respectively. rLexA has a predicted molecular weight of 29.3 KDa indicating that the complexes formed on the two DNA targets are composed of dimeric rLexA. In contrast, NLexA (11.4 KDa) exhibited a molecular mass of 9.3 KDa, consistent with binding of a monomer (Figure 3.8B). Taken together, these results show LexA exists predominately as a monomer in solution and dimerizes upon binding to its DNA targets. Furthermore, the C-terminal domain is important for protein dimerization as shown by monomer binding to the DNA when the NLexA protein encoding only the amino-terminal domain was used (Figure 3.8B).
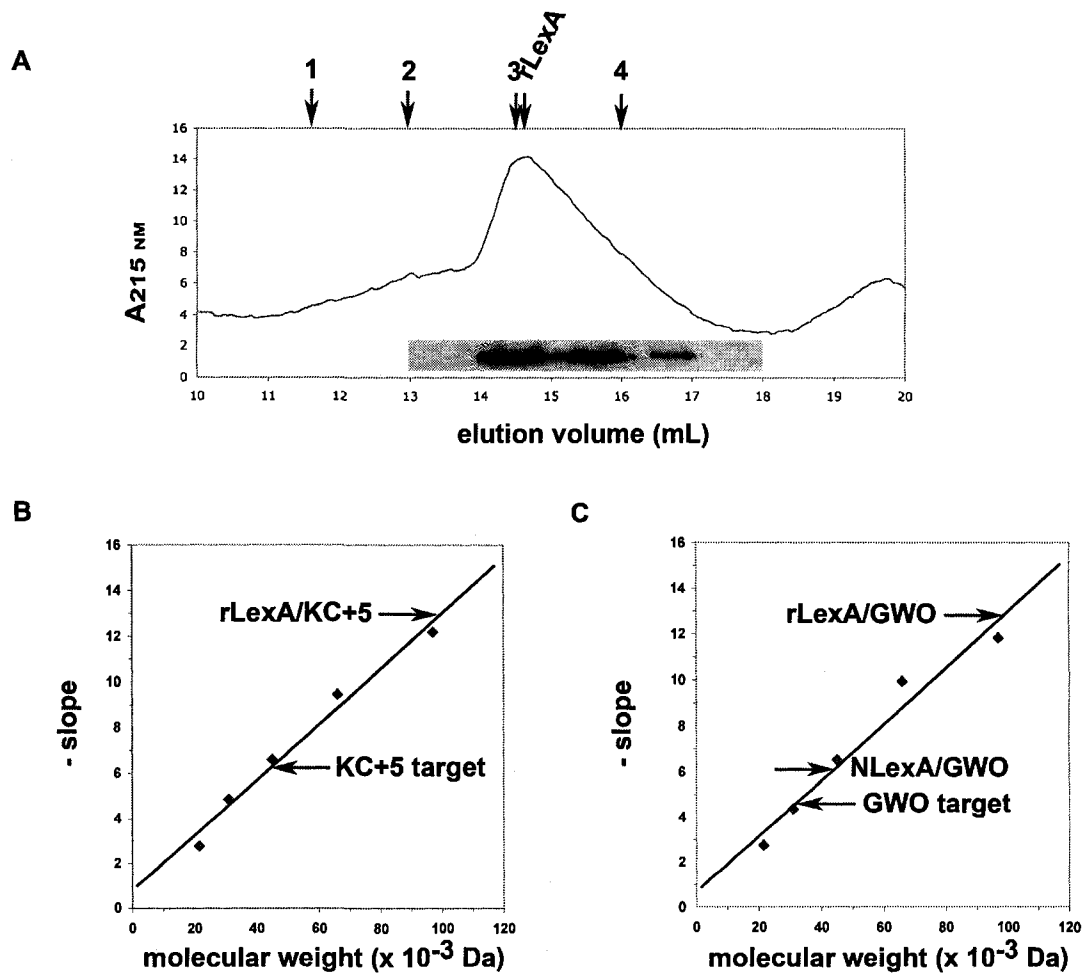
Figure 3.8 rLexA oligomeric state in solution and when bound to DNA.
A. Superose 12 FPLC gel filtration column elution profile. The elution volume for the protein standards: 1:β-amylase (200 kDa), 2:albumin (66 kDa), 3:carbonic anhydrase (29 kDa), 4: cytochrome-C (12.4 kDa) and rLexA (26.7 kDa) are indicated by arrows. Western blot analysis column fractions containing rLexA is included as an insert.
B. An EMSA-based protocol (Orchard and May, 1993) was used to determine the oligomeric states of rLexA and NLexA when bound to the different DNA targets encompassing the *crhR* binding site (GWO and KC+5). Molecular weight standards: phosphorylase B (97.5 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa) are indicated by small diamonds (◆).

3.3.7 An AbrB-like protein does not bind the crhR *or* lexA *targets*

Expression of the *Synechocystis hoxEFUYH* genes has recently shown to be regulated by an AbrB-like protein in addition to the previously demonstrated positive regulation by LexA (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005; Oliveira and Lindblad, 2008). I was interested in determining whether the AbrB-like protein also interacts with the *crhR* and *lexA* targets. Previously performed DNA affinity chromatography (Patterson-Fortin *et al.*, 2006; chapter 2) did not identify any proteins other than LexA interacting with the *crhR* target. In accordance, EMSA analysis demonstrates that the recombinant *Synechocystis* AbrB-like protein does not alter mobility of the KC-179 and LPF 3:6 targets under conditions where mobility of the *hox* Shopx target is decreased (Oliveira and Lindblad, 2005) (Figure 3.9, compare lane 3, 7 and 11). The KC-179 target (368bp) encompasses the promoter, 5' untranslated region and 79 base pairs of open reading frame of the *crhR* gene. Addition of both the rLexA and rAbrB-like proteins in a 1:1 ratio to the reaction mixture did not result in supershift of the protein-DNA complex relative to complexes formed in the presence of a LexA alone (Figure 3.9, compare lanes 4, 8 and 12). The absence of a supershift suggests rLexA and rAbrB-like proteins do not form a complex that synergistically binds DNA. Taken together, these results indicate the rAbrB-like protein does not interact with the *crhR* and *lexA* targets nor enhances LexA binding to these targets.

	crhR				hox				lexA			
rAbrB-like	-	-	+	+	-	-	+	+	-	-	+	+
rLexA	-	+	-	+	-	+	-	+	-	+	-	+

complex

free probe

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
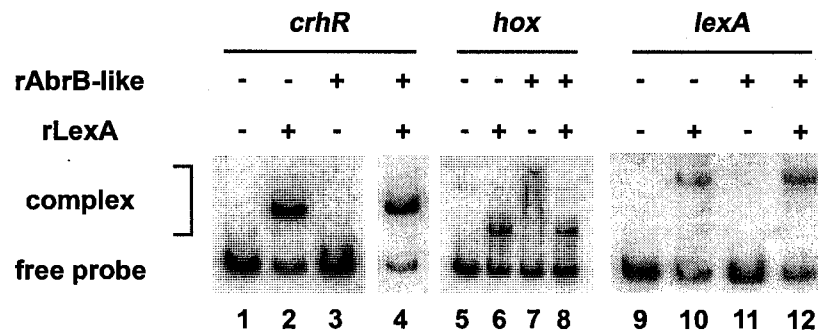
Figure 3.9 AbrB-like protein binding to the crhR, lexA and hox targets. EMSAs using recombinant AbrB-like and/or rLexA proteins were performed to determine the ability to bind to the crhR, lexA, and hox targets. ^{32}P-labelled targets were incubated in the absence of protein (lanes 1, 5, 9), in the presence of 50 pmol rLexA (lanes 2, 6, 10), 350 ng rAbrB-like protein (lanes 3, 7, 11) and 30ng rLexA and rAbrB-like (lanes 4, 8, 12).

3.4 Discussion

In this chapter, LexA was shown to interact with its own promoter in addition to the previously identified targets *crhR* (Patterson-Fortin *et al.*, 2006; Chapter 2) and *hoxEFUYH* (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2006). Furthermore, the LexA-DNA interaction was characterized by analyzing DNA binding affinity, oligomeric state and sequences important for protein binding. The insights obtained regarding the LexA binding site improve our understanding of the mechanism by which LexA regulates gene expression and provide information by which additional members of the LexA-regulon can be identified, aiding in our ability to define the physiological role LexA performs in *Synechocystis*.

Our analysis has demonstrated rLexA interaction with its own gene in a sequence specific and concentration dependent manner, binding to a sequence covering +1 and extending into the 5' untranslated region of *lexA* and a region covering the ATG translation initiation codon in *crhR*. LexA binding sites within the 5' UTR and close to the translational start site have also been identified for the *lexA-recA* operons of *X. fastidiosa* and the *M. tuberculosis* Rv3074 and Rv3766 genes (Campoy *et al.*, 2002; Davis *et al.*, 2002). The existence of alternative locations for the LexA binding motif in other bacterial species suggest LexA may not exert transcriptional control solely through steric hindrance of RNA polymerase binding. These observations imply a different mechanism of regulating LexA from the *E. coli* system. In addition, evidence presented here indicates that while rLexA binds RNA transcribed from the promoter region of the

crhR gene, it does so non-specifically. These results suggest that *Synechocystis* LexA is unable to regulate gene expression at the post-transcriptional level by binding to target RNA transcripts and inhibiting translation initiation.

The mechanism(s) by which LexA function is altered to regulate binding and derepression of the *Synechocystis* LexA regulon remain to be investigated. The lack of both the Ala-Gly bond and the serine nucleophile suggest protein self-cleavage is not responsible for LexA derepression in *Synechocystis* (Mazón *et al.*, 2004; Patterson-Fortin *et al.*, 2006). Self-cleavage experiments performed under conditions shown to catalyze *E. coli* LexA and *Anabaena* HetR cleavage did not alter the size of the *Synechocystis* LexA protein (Little, 1984; Zhou *et al.*, 1998; L.M. Patterson-Fortin and G.W. Owttrim, unpublished data). In addition, the potential for redox-based regulation of LexA DNA binding activity apparently does not occur in *Synechocystis* due to the absence of redox active amino acid residues. The AbrB-like protein has recently been shown to regulate expression of the *hoxEFUYH* genes in *Synechocystis,* which have also been identified as LexA targets (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005; Oliveira and Lindblad, 2008). Under our conditions, the AbrB-like protein does not interact with the *crhR* or *lexA* promoter regions nor does it appear to function synergistically with LexA to enhance DNA binding. The link between the AbrB-like and LexA proteins in regulating regulon members is not understood. The combined observations provide evidence that regulation of LexA binding does not involve self-cleavage and suggest the potential for LexA-regulation of gene expression by more than one mechanism in cyanobacteria.

Mutagenic analysis of LexA protected regions within the *crhR* gene

identified sequences important for protein recognition and binding to target DNA

sequences. Similarly, the distance and the orientation of the direct repeats on the

DNA helix were shown to be important for proper rLexA-*crhR* complex formation.

Together with comparative sequence analysis, site-directed mutagenesis

identified a CTA-N_9-CTA sequence in the *crhR* and *lexA* targets as important for

LexA binding. The spacer region is AT rich with 7 of 9 bp being A or T residues.

Our analysis of sequences within the *hox* promoter region, previously shown to

interact with the LexA protein (Oliveira and Lindblad, 2005), reveals several CTA-

N_9-CT(A/T) repeats further suggesting their importance in LexA recognition of its

DNA targets. LexA binding to direct repeats has also been shown for other

bacterial species, including *Rhodobacter sphaeroides*, *Rhizobium etli* and

Paracoccus denitrificans (Fernández de Henestrosa *et al.*, 1998; Tapias and

Barbé, 1998). *Anabaena* LexA whose binding site is GTAC-N_4-GTWC could also

be described as encoding repeats (Mazón *et al.*, 2004; Erill *et al.*, 2007). Taken

together with the results presented here, direct repeats may be a characteristic

feature of cyanobacterial LexA binding sites.

The 2-fold symmetry of the binding site suggests *Synechocystis* LexA

binds DNA targets as a dimer. Gel-exclusion chromatography revealed rLexA

exists predominately as a monomer in solution while EMSA-based analysis

indicated LexA dimerization upon interaction with the *crhR* binding site. The

inability to detect LexA dimers during size exclusion chromatography may be

related to the low rLexA concentrations employed (< 20 pM), suggesting that

Synechocystis LexA may be a dimer in solution, as observed in *E. coli* (Mohana-Borges *et al.*, 2000). The importance of dimerization in LexA-DNA's interactions is emphasized by comparison of the binding constants for the full-length protein and the amino terminal LexA proteins. The truncated protein binds with significantly lower affinity compared to the full-length protein. The weaker affinity may be attributed decreased dimerization capacity due to loss of the carboxyl dimerization domain, as observed for binding of the amino-terminal *E. coli* LexA to the *uvrA* SOS box (Bertrand-Burggraf *et al.*, 1987; Schnarr *et al.*, 1988).

Our previous data (Patterson-Fortin *et al.*, 2006; Chapter 2) combined with the current results provide further evidence that the *Synechocystis* gene annotated as LexA is functionally divergent from the canonical regulator of SOS gene expression in other bacteria. The *Synechocystis* LexA protein shares 27%, 48% and 38% identity with the *E. coli* LexA over the entire protein, the N-terminal (DNA binding) and the C-terminal (dimerization and self-cleavage), respectively. Thus, the two proteins are distantly, but obviously related, indicating the potential for functional divergence with respect to both domains.

In conclusion, characterization of the *Synechocystis* LexA-orthologue has revealed additional insights into the mechanism by which LexA interacts with two target genes. The sequence conservation between the *crhR* and *lexA* binding sites suggests a functional significance for the CTA-N$_9$-CTA motif which will assist identification of additional LexA target genes, providing greater insight into the physiological role performed by LexA in *Synechocystis*. These results demonstrate the evolutionary divergence of bacterial LexA proteins specifically

with respect to regulon members and recognition binding sequence illustrating the ability of regulatory circuits to evolve to best suit the environmental niche inhabited by an organism.

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Chapter 4: *crhR* and *lexA* mutant analysis

4.1. Introduction

Synechocystis sp. strain PCC 6803 is a model organism for the study of

photosynthesis (Thiel, 1994). Several features make Synechocystis an amenable

genetic model including availability of the sequenced genome, natural

competency, and the ability to grow photoheterotrophically on exogenously

provided glucose (Grigorieva and Shestakov, 1982; Labarre et al., 1989;

Anderson and McIntosh, 1991; Kaneko et al., 1995). The natural competency

and high frequency of transformation in Synechocystis allows for the generation

of mutants by insertion of an antibiotic cassette within the coding sequence or

replacement of gene sequences by the cassette (Grigorieva and Shestakov,

1982; Golden et al., 1987). Gene conversion proceeds through homologous

recombination between the wild type chromosome copy of the gene and the

cassette mutagenized version commonly introduced on a suicide vector. A

suicide vector lacks an origin of replication recognized by the cyanobacterial

replication machinery which results in a bias towards chromosomal integration

(Labarre et al., 1989; Golden et al., 1987). Sufficient sequence homology

between the targets is necessary for successful recombination (Labarre et al.,

1989). The genetic nature of Synechocystis complicates the generation of strains

homozygous for the mutated gene (Labarre et al., 1989; Thiel, 1994).

Synechocystis cells contain on average 12 copies of their genome with the intial

recombination event occurs on a single chromosome resulting in a strain

heterozygous for the gene of interest (Herdman et al., 1979; Labarre et al.,

1989). Wild type and inactivated versions of the gene coexist on the genome for

several generations with segregation requiring several rounds of replication in the presence of increasing selection (Labarre *et al.*, 1989; Thiel *et al.*, 1994). The nature of the mutation, neutral or deleterious, determines whether complete segregation may be achieved. Complete segregants can be generated in non-essential genes and are stable in the absence of selection. In contrast, deleterious mutations occur within genes essential for survival. Under these circumstances, only heteroploid mutants encoding both the wild type and mutant versions of the gene can be generated. The heterozygous nature of the gene complement ensures cells persist even in the presence of decreased levels of the wild type gene (Labarre *et al.*, 1989). Heteroploid mutants have been extensively used in *Synechocystis* studies with some success to identify the phenotypic effects of inactivation of genes encoding transcriptional regulators (García Domínguez *et al.*, 2000; Domain *et al.*, 2004; Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2008).

The high frequency of recombination in *Synechocystis* was exploited in this study to generate mutants in the *crhR* and *lexA* genes. *crhR* (Cyanobacterial RNA Helicase-Redox) encodes a DEAD-box RNA helicase whose expression is regulated by light-driven changes in the redox poise of the electron transport chain (Kujat and Owttrim, 2000). Biochemically, CrhR exhibits RNA-dependent ATPase activity, ATP-stimulated RNA unwinding, and ATP-dependent RNA annealing (Chamot *et al.*, 2005). CrhR has been proposed to regulate expression of genes associated with photosynthesis at the translational level through its ability to rearrange RNA secondary structure (Kujat and Owttrim, 2000; Chamot

et al., 2005). LexA has been identified as the transcriptional regulator of the *crhR*,

hoxEFUYH, and carbon utilization genes in *Synechocystis* (Domain *et al.*, 2004;

Oliveira and Lindblad, 2005; Gutekunst *et al.*, 2005; Patterson-Fortin *et al.*,

2006). *In vitro* transcription/translation and Northern blot analyses suggests that

LexA is a negative regulator of *crhR* expression under oxidizing conditions

(Patterson-Fortin *et al.*, 2006; Chapter 2). To date, the characterized targets

suggest a novel role for LexA in *Synechocystis* unrelated to the physiological role

played by other bacterial LexA proteins in the DNA damage repair response

(Little and Mount, 1982; Miller *et al.*, 1996).

In this chapter, we describe the preliminary results of our *crhR* and *lexA*

mutant analyses. Complete segregation of the *crhR* mutation was achieved as

demonstrated by PCR and Southern analyses. The *crhR* mutant is unable to

grow at 20°C suggesting CrhR is required for growth at low temperature. *lexA* is

an essential gene as demonstrated by our inability to achieve complete

segregation of the generated mutants. The heteroploid strain maintains at least

one copy of the wild type gene as demonstrated by PCR analysis. Nevertheless,

the *lexA*: $Km^r/lexA^+$ strain was used for RT-PCR analyses to determine the

effects of *lexA* gene depletion on accumulation of the *crhR* transcript under

oxidizing conditions. *In vivo* results confirmed previous *in vitro* data

demonstrating that LexA is a negative regulator of *crhR* expression. We discuss

our initial results from mutational analysis and the clues they provide about the

physiological roles of the CrhR and LexA proteins in *Synechocystis*.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions

Synechocystis sp. PCC 6803 was grown and maintained as previously described (Chapter 2). To attempt to achieve complete segregation of the *lexA* mutant, cells were grown in the light under high CO_2 conditions (750-900 ppm CO_2). Medium additions, trycine-KOH pH 8 (25 mM), sodium thiosulfate (12 mM), glucose (5 mM), NaCl (0.5 M) and antibiotics were made where appropriate.

Escherichia coli strain DH5α was used for propagation of plasmid constructs (Chapter 2).

4.2.2 PCR amplification

PCR amplification was performed using the primer pairs listed in table 4.1 as previously described (Chapter 2; Chapter 3) using High Fidelity PCR enzyme mix (Fermentas). Optimal annealing temperatures for each primer pair were determined experimentally. Where appropriate, DNA fragments were purified using the QIA Quick purification kit (QIAGEN). DNA fragments were separated by agarose or polyacrylamide gel electrophoresis, stained with ethidium bromide (10 µg/mL) and visualized under ultraviolet light.

4.2.3 lexA and crhR inactivation constructs

Cassette mutagenesis was used to create insertional inactivations of the *crhR* and *lexA* genes. The *crhR* construct was generated by *PmlI/HpaI* restriction enzyme digestion of pBRcrhR (Kujat-Choy, 2001). The Sp[r] cassette was blunt end ligated into the digested pBRcrhR DNA. The resulting plasmid,

Table 4.1 Oligonucleotide primers used in this study. Relative location designates oligonucleotide position with respect to the transcriptional start site (+1).

Primer	Sequence 5' – 3'	Relative location	Application
LPF-1	CCTATAGGAGATCTTACATG	+ 18	Forward primer for *lexA* mutant analysis with LPF-2 or LPF-21
LPF-2	GAAACAAAAAGCTTAGGACG	+ 789	Reverse primer for *lexA* mutant analysis with LPF-1, LPF-4, LPF-20 or LPF-22
LPF-20	AGCCCAAAGATTATTAACCG	- 635	Forward primer to obtain *lexA* target for inactivation with LPF-21 and for *lexA* mutant analysis with LPF-2 or LPF-22
LPF-4	ATTTGCGTTCTCCGGCC	+ 140	Forward primer for *lexA* mutant analysis with LPF-2 or LPF-21
LPF-21	TCCCCATCTGACGGTTTTGC	+ 1357	Reverse primer to obtain *lexA* target for inactivation with LPF-20 and for *lexA* mutant analysis with LPF-1 or LPF-4
LPF-22	GCGGATCAGTGAGGGTTTGC	Km^r	Forward primer for *lexA* mutant analysis with LPF-2 and for *lexA* mutant analysis with LPF-20
LPF-47	CGAAACCGTCTGAAACAACAAA	+ 1189	Forward primer for *crhR* RT-PCR with LPF-48
LPF-48	TTCCTTGAGTTGTTCCTGCAGTT	+ 1288	Reverse primer for *crhR* RT-PCR with LPF-47
LW-19	GAAAACAGCAACCAGTA		Forward primer for *rnaseP* RT-PCR with LW-20
LW-20	GAAAAGACCAACCTTT		Reverse primer for *rnaseP* RT-PCR with LW-19
GWO-39	TGACGATGTGAAAACC	+ 607	Forward primer for *crhR* mutant analysis with GWO-40 or GWO-41
GWO-40	TGTGTGGCTTCAGGC	Sp/Sm^r	Reverse primer for *crhR* mutant analysis with GWO-39
GWO-41	CCGGGGGAGGTAGAGA	+ 2230	Reverse primer for *crhR* mutant analysis with GWO-39

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pBRcrhRΔ::Sp, is a deletion mutant which removes the last three conserved amino acid domains characteristic of the DEAD-box family of RNA helicases (Kujat-Choy, 2001; Tanner and Linder, 2001). The *lexA* inactivation constructs were created by insertion of a kanamycin resistance cassette within the *lexA* open reading frame. Amplified DNA was cloned into pGem-T Easy (Promega) according to manufacturer's instructions producing pGemLexA which contains the *lexA* gene with 670 bp upstream sequence and 710 bp downstream sequence. The Km^r cassette was generated by *EcoRV* restriction enzyme digestion of pBSL128 (Alexeyev *et al.*, 1995). The insertion and deletion mutants were created by *BsaBI* and *BglII/BsaBI* restriction enzyme digestion of pGemLexA, respectively. The Km^r cassette was blunt end ligated into the digested pGemLexA DNA. The resulting plasmids, pGemLexA::Km and pGemLexAΔ::Km, were sequenced to confirm insertion and orientation of the Km^r cassette.

4.2.4 Synechocystis transformation

Plasmid DNA was introduced by transformation into the naturally competent *Synechocystis* (Grigorieva and Shestakov, 1982). For each transformation, 4.8 x 10^8 cells in mid-log phase were harvested and resuspended in Tricine-buffered BG-11 without selection. Cells were incubated with 1-5 µg plasmid DNA, at 30°C, in the dark with shaking at 150 rpm for 6 h. The transformation tube was covered with a thin layer of KimWipes and incubated at 30°C for 24 h followed by a 24 h incubation with constant illumination at 30°C.

Aliquots were plated onto buffered BG-11 plates containing glucose (5 mM) and the appropriate antibiotic(s), and incubated at 30°C with constant illumination. Colonies were detected after 10-14 days and individual colonies re-streaked onto fresh selective BG-11 plates. Colonies were restreaked every 14 days onto selective BG-11 plates containing increasing concentrations of the appropriate antibiotics to promote chromosome segregation.

4.2.5 Genomic DNA isolation

Genomic DNA was isolated from *Synechocystis* by mechanical lysis. Cells were vortexed in the presence of an equal volume of glass beads (0.20-0.30 mm, Dyno-mill) and phenol, in STE buffer containing 0.2% SDS. Lysed cells were clarified by centrifugation, the supernatant retained and treated with RNaseA (0.1 mg/mL). DNA samples were purified by phenol/chloroform extraction and ethanol precipitation.

4.2.6 Southern Blot Analysis

Genomic DNA (5 μg) was cleaved with the indicated restriction endonucleases, separated on a 0.8% agarose (0.5X TBE) gel and transferred to a nylon membrane (Hybond N$^+$) as previously described (Sambrook *et al.*, 1989). The transferred DNA was UV crosslinked to the membrane using a XL-1000 UV crosslinker (optimal crosslink program; Spectronics Corporation). Blots were hybridized overnight at 65°C in aqueous hybond buffer (5X SSPE, 5X Denhardt's, 0.5% SDS) containing sheared salmon sperm DNA (10 mg/mL) and

the appropriate radioactively labeled probe. Blots were washed for 10 min once at 65°C in 2X SSC, 0.1% SDS, once at 65°C in 0.2X SSC, 0.1% SDS and once at RT in 0.2X SSC, 0.1% SDS. The crhR probe used for Southern blot analysis corresponds to a 784 bp internal BstEII fragment. crhR DNA fragments were randomly labeled with [α-^{32}P]dCTP using random hexanucleotides primers (Roche). Blots were subjected to autoradiography to detect hybridization.

4.2.7 Western Blot Analysis

Total protein was isolated from Synechocystis as previously described (Patterson-Fortin et al., 2006; Chapter 2). Polypeptides (30 μg) were separated on 10% (w/v) SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Hybond ECL) using a semidry apparatus (Tyler). Blots were blocked in 1X Blotto (1X TBS, 5% skim milk powder), hybridized overnight at RT with rabbit anti-LexA (1:2500 dilution) or anti-CrhR (1:5000 dilution) antiserum in 1X Blotto (1X TBS, 5% skim milk powder) and washed for 10 min at RT twice in 1X TBS and once in 1X TBST. Blots were incubated with goat anti-rabbit immunoglobulin G horseradish perodixase (1:20000, Sigma) in 1X TBS, washed as previously described and visualized by chemoluminescence (ECL, GE Healthcare). Polypeptide size was determined by comparison with PageRuler Prestained protein molecular weight standards (Fermentas).

4.2.8 Reverse Transcriptase PCR

Total RNA was isolated from *Synechocystis* by mechanical lysis as previously described (Chamot *et al.*, 1999). Total RNA was treated with RNase-free DNaseI (Invitrogen) to remove genomic DNA contamination. Two-step RT-PCR was performed to determine accumulation of the *crhR* transcript in dark-grown wild type *Synechocystis* and in the *lexA* heteroploid cells (*lexA$^+$:lexA*:Km^r). cDNA primer annealing reactions contained 50 ng/µl random primers (Roche), 0.5 mM dNTPs and total RNA (1 µg) in a final volume of 30 µl. Annealing reactions were incubated for 10 min at 70°C, 10 min at 25°C, and cooled quickly on ice. cDNA synthesis reactions contained the RNA/primer hybridization mix, 1X M-MuLV RT buffer (New England Biolabs) and 40 U M-MuLV reverse transcriptase (New England Biolabs). Synthesis reactions were incubated for 10 min at 25°C, 1 hour at 37°C and 1 hour at 42°C. cDNA samples were purified by phenol/chloroform extraction and ethanol precipitation. PCR was performed as previously described (section 4.2.2) using primers LPF-47/-48 to amplify the *crhR* cDNA and primers LW-19/-20 to amplify the *maseP* cDNA.

4.3 Results

4.3.1 Inactivation of the crhR *and* lexA *genes*

A deletion mutant of the *crhR* gene was generated as depicted in Figure 4.1A. *crhR* sequence between the *Pml*I and *Hpa*I restriction sites was replaced with an antibiotic cassette encoding streptomycin resistance. Deletion and

A

B
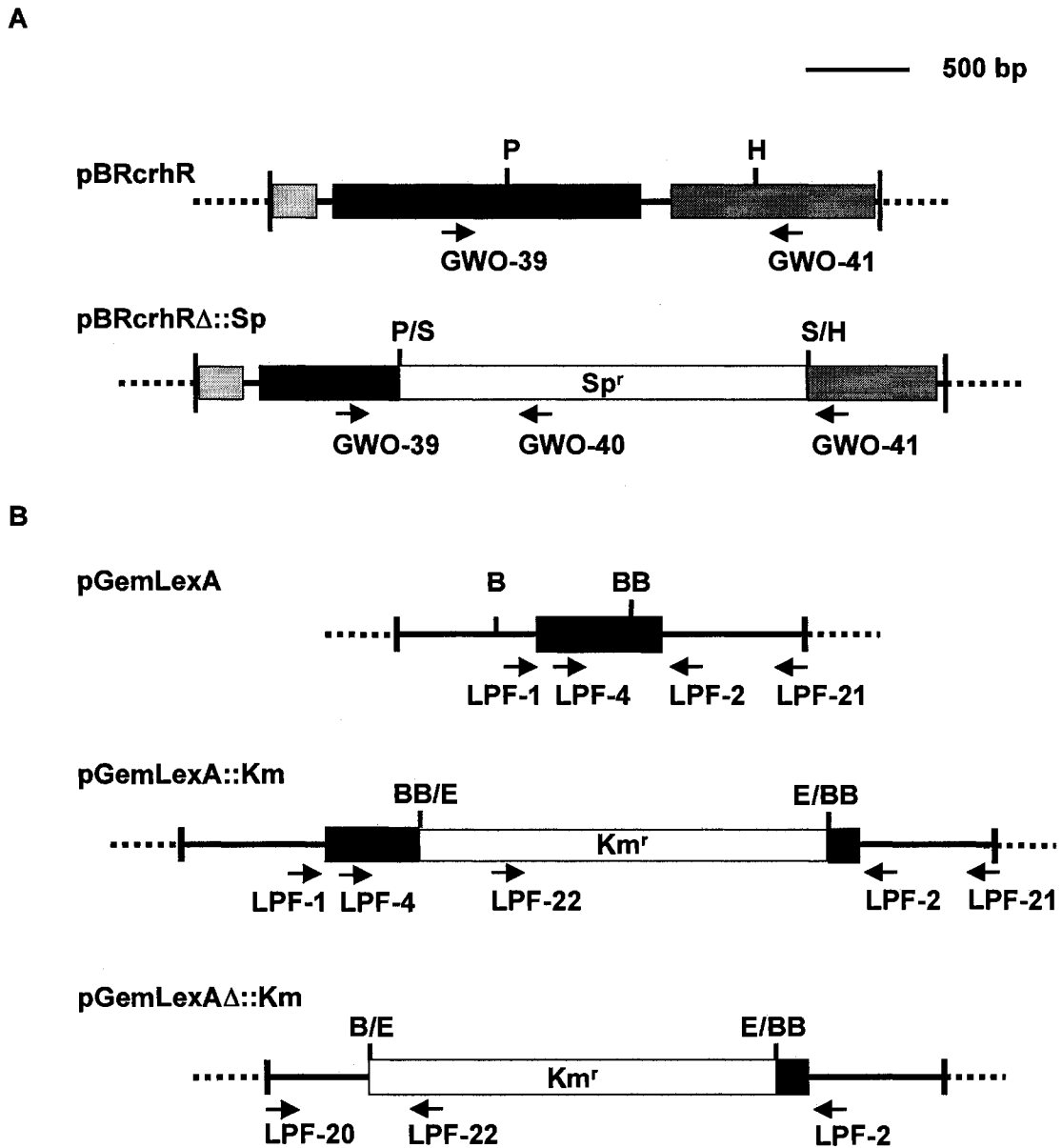
Figure 4.1 Schematic of the *crhR* and *lexA* mutant constructs.
A. The *crhR* mutant was generated by replacement of sequence between the *Pml*I and *Hpa*I restriction sites with an antibiotic cassette encoding streptomycin resistance. The Sp^r cassette was blunt end ligated into the digested pBRcrhR plasmid.
B. The *lexA* mutants were generated by insertion at the *BsaB*I restriction site of an antibiotic cassette encoding kanamycin resistance or replacement of sequence between the *Bgl*II and *BsaB*I restriction sites with the kanamycin

cassette. The Km^r cassette was blunt end ligated into the digested pGemLexA plasmid.

The *crhR* and *lexA* genes are represented by black boxes, respectively. The pBRcrhR construct also encodes the *argC* gene (dark grey box) and the 3' end of the slr0082 gene (light grey box). Oligonucleotides used in mutant analysis are indicated by arrows. Restriction sites are indicated by single letters. P, *Pml*I. H, *Hpa*I. S, *Sma*I. E, *EcoR*V. B, *Bgl*II. BB, *BsaB*I.

insertion mutants were prepared for the *lexA* gene (Figure 4.1B). The insertion

mutant (pGemLexA:Km) was generated by insertion of a kanamycin resistance

cassette at the *BsaB*I site. The deletion mutant (pGemLexAΔ::Km) was

generated by replacement of *lexA* sequence between the *Bgl*II and *BsaAB*I

restriction sites with a kanamycin cassette. Deletion of *lexA* sequence removed

the amino-terminal DNA binding domain.

4.3.2 crhR *is a non-essential gene*

Successful inactivation of all genomic copies of the *crhR* gene was

demonstrated by a combination of PCR, Southern and Western analyses (Figure

4.2). PCR amplification of genomic DNA isolated from wild type *Synechocystis*

and two *crhR* mutants, 2-73 and 2-76 demonstrated successful inactivation of all

crhR gene copies in the 2-76 mutant (Figure 4.2A). The primers GWO-39 and

GWO-40 are located within the *crhR* coding sequence and the Sp^r cassette

respectively. Product (1200 bp) obtained with this primer pair indicates

successful recombination of the cassette mutagenized *crhR* gene into the

genome. As predicted, no product is detected when WT genomic DNA is used as

template (lane 1) while product is detected for the *crhR* mutants, 2-73 and 2-76

(lanes 4 and 6). The primers GWO-39 and GWO-41 flank the Sp^r cassette

insertion site and amplify the *crhR* gene plus any intervening sequence from both

wild type and *crhR* mutant genomic DNAs. The size of the resulting PCR product

indicates the presence (2205 bp) or absence (1624 bp) of the Sp^r cassette. In

Figure 4.2A, WT (lane 1) and 2-73 (lane 3) contain the 1623 bp PCR product
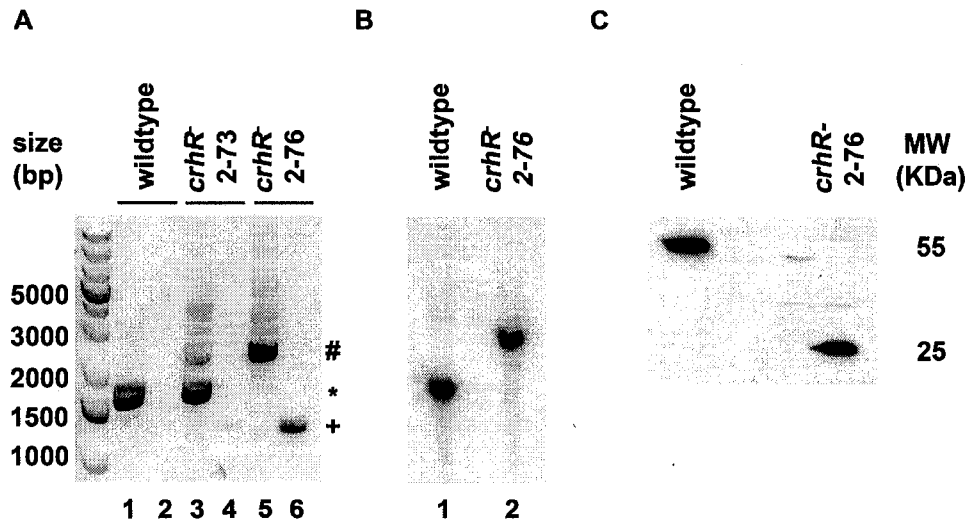
Figure 4.2 Analysis of the *crhR* mutant. PCR, Southern and Western analysis was performed to confirm complete segregation of *crhR* in the 2-76 mutant.
A. PCR reactions were performed on genomic DNA (100 ng) isolated from wild type *Synechocystis* and 2 *crhR* strains (2-73 and 2-76). Products were separated on a 0.8% TBE agarose gel, stained with ethidium bromide and visualized under ultraviolet light. PCR primer pairs: GWO 39:41 (see Figure 4.1), lanes 1, 3 and 5 showing the 1623 bp wildtype gene (*) or the 2205 bp inactivated gene (#); GWO 39:40 (see Figure 4.1), lanes 2, 4 and 6 showing the 1200 bp inactivated gene (+). Note the absence of a PCR product corresponding to the WT gene in the 2-76 mutant.
B. Southern analysis. *EcoR*I digested genomic DNA (5 µg) isolated from wild type *Synechocystis* and *crhR* mutant cells was separated on a 0.8% TBE agarose gel, transferred to Hybond N⁺ and hybridized with a ^{32}P-labeled probe corresponding to a 784 bp internal *BstE*II fragment of the *crhR* gene. Note the lack of a detected wildtype fragment in the 2-76 mutant.
C. Western Analysis. Total protein (30 µg) isolated from wild type *Synechocystis* and *crhR* mutant cells was separated on a 10% (v/v) SDS polyacrylamide gel, transferred to Hybond XL and hybridized with anti-CrhR and anti-rabbit immunoglobulin G horseradish perodixase antibodies. Note the absence of the 55 kDa wild type CrhR polypeptide in the 2-76 mutant.

indicating the presence of wild type copies of the *crhR* gene. Amplification of 2-73 genomic DNA also produces a faint band of ~ 2200 bp indicating that 2-73 is heterozygous for the mutation with its genomic DNA encoding both wild type and mutated version of the gene. The *crhR* mutant 2-76 lacks product corresponding to the wild type gene (lane 5) only producing product consistent with mutated *crhR* (2205 bp). Taken together, the PCR results indicate that 2-76 is homozygous for the cassette mutagenized version of the *crhR* gene; all wild type copies of the gene have been lost. Southern analysis, using a 784 bp ^{32}P-labeled probe encompassing +618 to +1402 of the *crhR* ORF, was performed to confirm the homozygous nature of 2-76. The *crhR* hybridizing band in *EcoR*I digested 2-76 genomic DNA is larger than the *crhR* hybridizing band in *EcoR*I digested wild type genomic DNA consistent with the addition of the Sp^r cassette and complete deletion of WT *crhR* sequences as indicated by the absence of hybridization to a ~ 3000 bp fragment (Figure 4.2B, compare lanes 1 and 2). Finally, the *crhR* mutant, 2-76, does not produce full-length CrhR protein as demonstrated by Western analysis (Figure 4.2C). As expected from the inactivation construct, the mutant produces a truncated protein, 25 kDA in size. Taken together, the results suggest that the *crhR* gene is not essential for *Synechocystis* growth under standard conditions as demonstrated by successful generation of a homozygous mutant of the *crhR* gene.

4.3.3 CrhR is required for growth at low temperature

Growth experiments were performed to determine the phenotypic effects of the *crhR* mutation. Wild type and *crhR* mutant strains were treated to six different conditions (a) 30 μE light, (b) 20°C, (c) 80 μE light, (d) 30 μE light plus high salt (0.5M) and (e) 80 μE light plus high salt (0.5 M) and (f) high CO_2 (850-1000 ppm) (Figure 4.3). Low temperatures and high salt were investigated due to previous reports demonstrating induction of *crhR* transcript accumulation at 20°C or by high salt treatment (Kujat-Choy, 2001, Vinnemeier and Hagemann, 1999). Growth of the cultures was followed by OD 750 measurements at 24 hour intervals for a period of 7-13 days. The *crhR* mutant was unable to grow at 20°C as shown by minimal change in cell density over the course of 13 days (Figure 4.3B, red line). Unlike the mutant, wild type *Synechocystis* was capable of exponential growth at lower temperatures following a short lag period (Figure 4.3B, black line). However, growth at 20°C was slowed with a doubling time of 36.0 h, 2.2X longer than wild type *Synechocystis* grown at 30°C. A single replicate is shown for growth of the wild type and mutant at 20°C. Duplicate growth curves were prepared and differ only in the final OD 750 values achieved for the wild type *Synechocystis* culture. Under all other conditions examined no difference in growth of the wild type *Synechocystis* and *crhR* mutant strains was detected. At 30 μE and 80 μE illumination, cell density of the wild type and mutant strains increased exponentially during the first 4-5 days, after which the cells entered stationary phase (Figure 4.3A, C). The addition of NaCl (0.5 M) similarly slowed growth of both wild type and mutant cultures (Figure 4.3D, E).
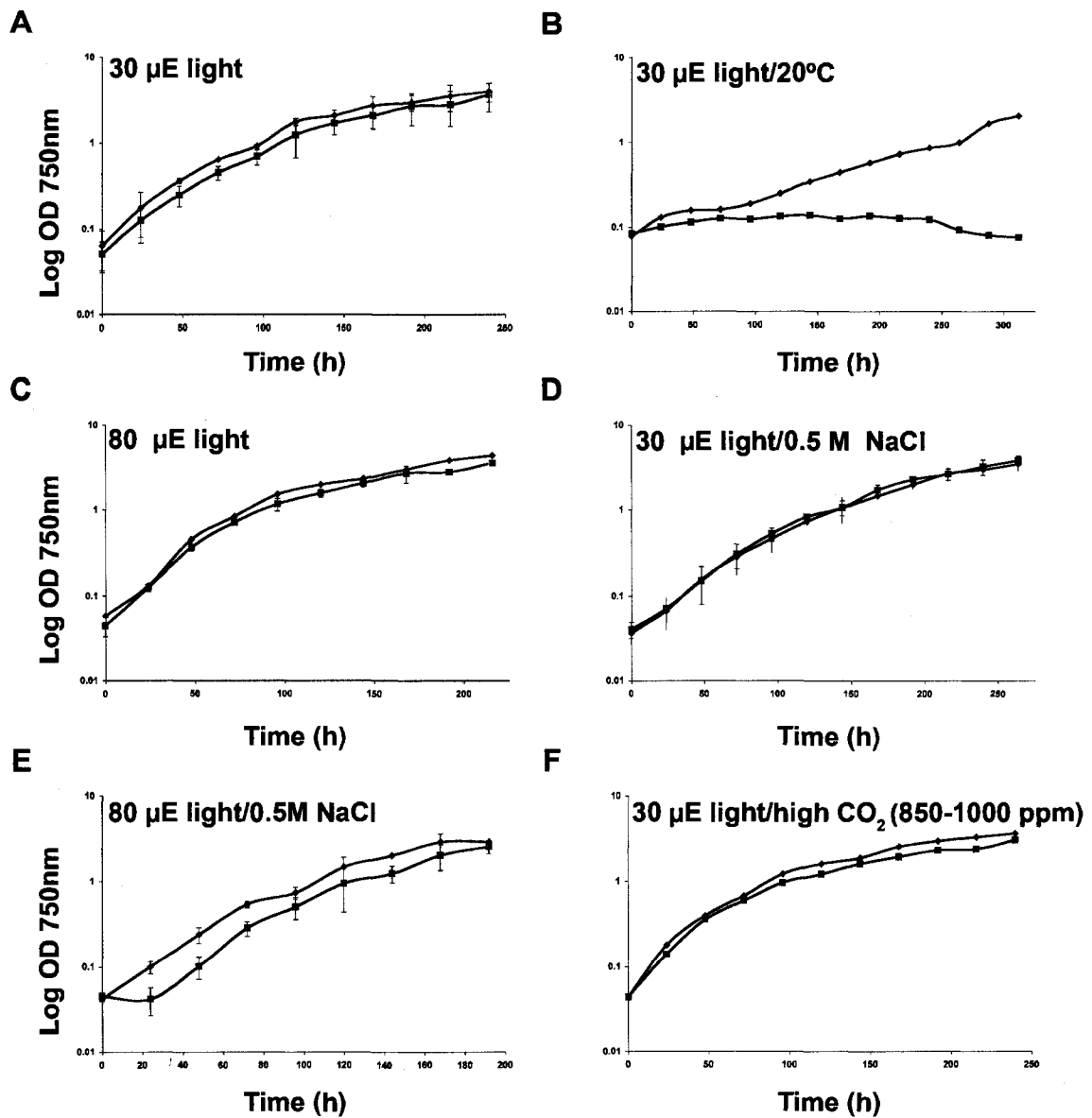
Figure 4.3 Growth of wild type *Synechocystis* and the *crhR* mutant under six different conditions (a) 30 μE, (b) 20°C, (c) 80 μE light, (d) 30 μE light/high salt (0.5 M), (e) 80 μE light/high salt (0.5M) and (f) high CO2 (850-1000 ppm). Growth was followed by OD 750 measurements taken at 24 h intervals up to a 13 day period. Growth curves represent average data of two different experiments (blue line- WT *Synechocystis*; red line- *crhR* mutant).

The generation time in the presence of salt was lengthened by 6-7 hours. At 80 μE light in the presence of high salt, the *crhR* mutant exhibited a longer lag phase following inoculation when compared to the wild type under the same growth conditions but reached a similar density over the course of the experiment. Similar, to the addition of salt to the medium, bubbling with CO_2 (850-1000 ppm) slowed the growth rate of both cultured lengthening their generation time by 6-7 hours (Figure 4.3 F). The determined growth kinetics under the conditions examined are shown in Table 4.2. Taken together, the growth studies suggest that the CrhR RNA helicase is required for growth at low temperature.

4.3.4 lexA *is an essential gene*

The *lexA* gene is essential for *Synechocystis* survival as demonstrated by the heterozygous nature of the *lexA* insertion mutant after greater than 1000 generations on selective media. A series of PCR amplification reactions on wild type and numerous mutant genomic DNAs were performed to demonstrate the heteroploid nature of the *lexA* insertion mutant strain (*lexA*:Km^r/*lexA*$^+$) (Figure 4.4). The primers LPF-1, LPF-2, LPF-4, and LPF-21 are located external to the Km^r cassette insertion site and allow detection of both the wild type and mutant versions of the *lexA* gene. The size of the resulting PCR product in clone 25-1 indicates the nature of the *lexA* gene copies, wild type vs. mutant (Table 4.3). A single PCR product is detected when wild type genomic DNA is used as template (Figure 4.4A, lanes 1, 3, 5 and 7). A larger PCR product consistent with addition

Table 4.2 Growth kinetics of wildtype *Synechocystis* and the *crhR* mutant under six different growth conditions. Cultures were treated as described and growth followed by OD 750 measurements taken at 24 h intervals for up to 13 days.

Strain and Condition	Doubling time (h)
Light (30 μE m^{-2} s^{-1}) Wild type	18.5 ± 3.6
Light (30 μE m^{-2} s^{-1}) $\Delta crhR$	18.4 ± 2.1
Light (30 μE m^{-2} s^{-1})/ 20°C Wild type	36.0
Light (30 μE m^{-2} s^{-1}) 20°C $\Delta crhR$	no growth
Light (30 μE m^{-2} s^{-1})/ CO$_2$ (850-1000 ppm) Wild type	22.96 ± 2.2
Light (30 μE m^{-2} s^{-1})/ CO$_2$ (850-1000 ppm) $\Delta crhR$	25.0 ± 1.97
Light (30 μE m^{-2} s^{-1})/ salt (0.5 M) Wild type	27.9 ± 5.0
Light (30 μE m^{-2} s^{-1})/ salt (0.5 M) $\Delta crhR$	27.1 ± 0.43
Light (80 μE m^{-2} s^{-1}) Wild type	17.3 ± 2.1
Light (80 μE m^{-2} s^{-1}) $\Delta crhR$	18.0 ± 2.1
Light (80 μE m^{-2} s^{-1})/ salt (0.5 M) Wild type	25.4 ± 4.6
Light (80 μE m^{-2} s^{-1})/ salt (0.5 M) $\Delta crhR$	21.7 ± 1.4
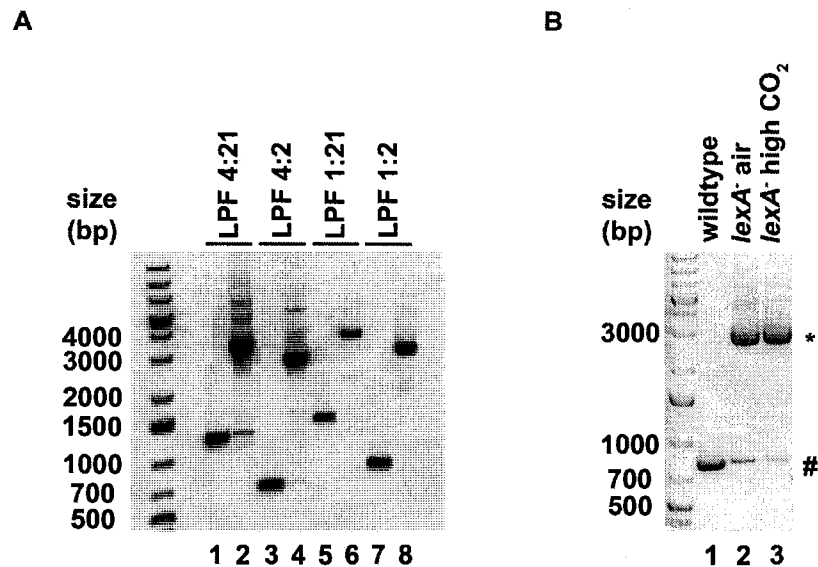
Figure 4.4 *lexA* insertion mutant analysis. PCR was performed to confirm the heterozygous nature of the *lexA* mutant (*lexA*: $Km^r/lexA^+$).

A. PCR reactions were performed on genomic DNA (100 ng) isolated from wild type *Synechocystis* and *lexA* heteroploid cells grown in CO_2 (850-1000 ppm) using primers flanking the Km^r cassette insertion site (see Figure 4.1; Table 4.3). Lanes 1, 3, 5 and 7 wild type genomic DNA; lanes 2, 4, 6 and 8, *lexA* heteroploid genomic DNA (25-1). The primer pairs used for DNA amplification are noted above the lanes. Note the presence of a wild type *lexA* gene copy using each primer pairs LPF 4:21 and LPF 4:2.

B. PCR reactions using primer pair LPF 1:2 were performed on genomic DNA (100 ng) isolated from lane 1, wild type *Synechocystis*; lane 2, air-grown *lexA* heteroploid cells; lane 3, high-CO_2 grown *lexA* heteroploid cells showing 772 bp wild type gene (#) or 2772 bp inactivated gene (*). Products were separated on a 0.8% TBE agarose gel, stained with ethidium bromide and visualized under ultraviolet light. Note the presence of a wild type band in the mutant lanes (#).

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Table 4.3 Expected size of PCR products amplified from wild type and *lexA* heteroploid genomic DNAs.

LPF primer pair	Wild type	pGemLexA::Km	pGemLexAΔ::Km
1:21	1339 bp	3339 bp	n/a
4:21	1217 bp	3217 bp	n/a
1:2	772 bp	2772 bp	n/a
4:2	649 bp	2649 bp	n/a
22:2	n/a	827 bp	n/a
20:2	1424 bp	n/a	2740 bp
20:22	n/a	n/a	827 bp

n/a: not applicable

of the Km^r cassette was obtained using primer pairs, LPF 4:2, LPF 1:21 and LPF

1:2 and *lexA* mutant genomic DNA as template (lanes 4, 6, and 8) suggesting

complete inactivation of the *lexA* gene. Unfortunately, a PCR product consistent

in size with the wild type *lexA* gene was detected when the PCR was performed

using primer pair LPF 4:21 (compare lanes 1 and 2). The wild type nature of this

DNA fragment was confirmed through its gel purification, amplification and

sequencing (data not shown). The relative intensities of the two bands suggest

the presence of relatively few wild type copies of the *lexA* gene. Successful

inactivation of the *lexA* gene has been suggested to require growth under high

CO_2 conditions (A. Kaplan, personal communications). To determine if these

conditions would allow complete segregation of *lexA*, single colonies of clone 25-

1 were grown at high CO_2 for approximately five generations. Similar to previous

results, growth at high CO_2 did not lead to complete segregation of the *lexA*

mutant. Comparison of high CO_2 and air grown cells (Figure 4.4B, compare lanes

1, 2 and 3) suggests the high levels of CO_2 lowered the number of wild type *lexA*

gene copies but were insufficient to allow complete segregation and achievement

of a homozygous mutant. Similar results were obtained for the *lexA* deletion

mutant (*lexA*Δ:Km^r/*lexA*$^+$) demonstrating maintenance of wild type *lexA* gene

copies (Figure 4.5). PCR using the primer pair LPF 20:2, located external to the

Km^r cassette, detected a single band when wild type genomic DNA was used as

template DNA (lane 1). When genomic DNA isolated from the *lexA* heteroploid

cells was used two different PCR products were obtained corresponding to the

WT *lexA* and *lexA* mutagenized genes (lanes 2 and 3). The size of the mutant
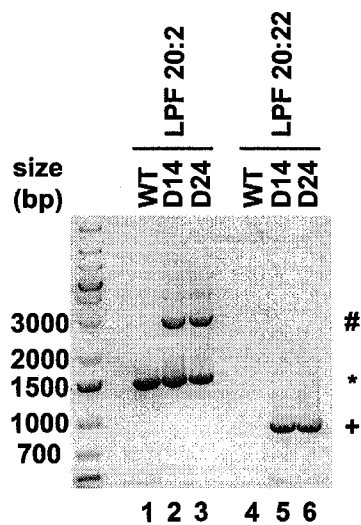
Figure 4.5 *lexA* deletion mutant analysis (*lexAΔ*:Km^r/*lexA*$^+$). PCR was performed to confirm the heterozygous nature of the lexA deletion mutant. PCR reactions were performed on genomic DNA (100 ng) isolated from wild type Synechocystis and lexA depleted cells. Products were separated on a 0.8% TBE agarose gel, stained with ethidium bromide and visualized under ultraviolet light. LPF 20:2, lanes 1-3 showing the 1424 bp wild type gene (*) or the 2740 bp inactivatied gene (#), LPF 20:22, lanes 4-6 showing the 827 bp inactivated gene (+). Note the presence of both wild type (*) and inactivated (#) lexA genes in the D14 and D24 mutants.

PCR product is consistent with addition of the antibiotic cassette and deletion of the amino-terminal DNA binding domain. The primer LPF-22 is located within the Km^r cassette and amplification with LPF-20 was performed to demonstrate successful insertion of the cassette into the genome as demonstrated by its absence in the wild type control (compare lanes 4, 5 and 6). Together, the PCR analysis of the generated *lexA* insertion and deletion mutants demonstrates the essential nature of the *lexA* gene under the conditions examined.

4.3.5 LexA represses crhR gene expression in vivo

crhR expression analysis was performed using the *lexA* depleted strain to confirm previous *in vitro* results demonstrating LexA repression of CrhR protein accumulation (Patterson-Fortin *et al.*, 2005; Chapter 2). Semi-quantitative reverse transcriptase PCR was used to determine *crhR* transcript levels in wild type *Synechocystis* and the *lexA* heteroploid (*lexA*: Km^r/*lexA*$^+$) during growth in the dark (oxidizing conditions), conditions where *crhR* transcripts do not accumulate. The optimal number of PCR cycles was experimentally determined for both the *crhR* and *rnaseP* reactions for each experimental primer pair. *crhR* transcripts accumulate to lower levels in wild type cells (Figure 4.6A) with higher levels of the *crhR* transcript detected in the *lexA* depleted strain. Transcript levels of the internal control, *rnaseP*, varied by less than 1% confirming specificity of the response in *crhR* transcript accumulation to the depletion in the number of wild type *lexA* gene copies in the cell. Quantification of these results demonstrates a significant difference in *crhR* transcript accumulation in the heteroploid strain
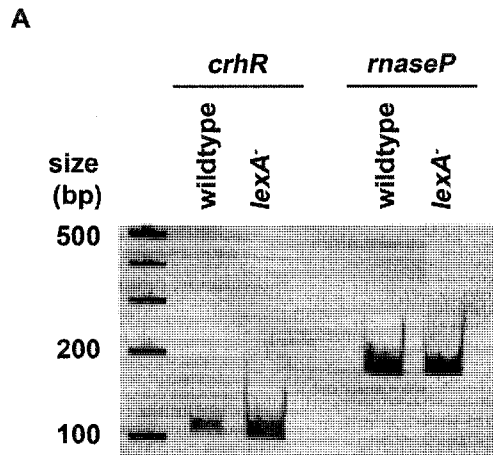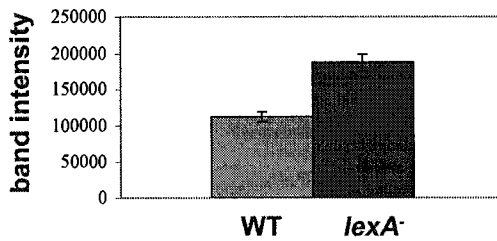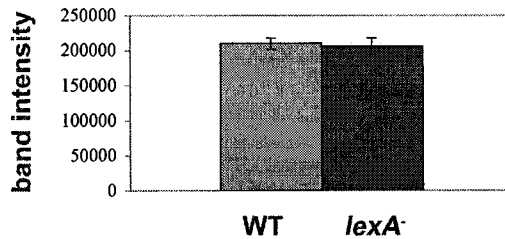
Figure 4.6 *crhR* transcript accumulation in the *lexA* heteroploid (*lexA*:Km^r/*lexA$^+$*). RT-PCR was performed to investigate the *in vivo* effects of *lexA* depletion on *crhR* and *rnaseP* transcript accumulation in the dark.

A. RT-PCR was performed on cDNAs prepared from DNaseI-treated RNA isolated from dark grown (1 hour) wild type *Synechocystis* (WT) and *lexA* heteroploid cells (*lexA$^-$*)

B.,C. Quantification of the effects of *lexA* depletion on *crhR* and *rnaseP* transcript accumulation. Transcript accumulation was quantified from triplicate,

independent replicates similar to the data shown in (A) using ImageQuant (Molecular Dynamics). Standard deviations from the means are shown.

versus wild type (Figure 4.6B). No significant difference in *rnaseP* transcript

accumulation was detected (Figure 4.6C). Taken together, these results support

previous *in vitro* analysis suggesting LexA is a negative regulator of *crhR*

expression under oxidizing conditions (Patterson-Fortin *et al.*, 2006; chapter 2).

4.4 Discussion

This chapter reports the initial investigation into the phenotypic effects of

inactivating the *Synechocystis crhR* and *lexA* genes. The results show that

complete segregation of a *crhR* deletion mutant is achievable under normal

growth condtions and that a functional CrhR protein is required for cell growth at

low temperature. In contrast, *lexA* appears to be an essential gene as

demonstrated by the continual maintenance of wild type copies of the gene after

greater than 1000 generations on both high concentrations of selective agent and

in the presence of high levels of CO_2. Depletion of *lexA* levels in the cell has a

phenotypic effect as shown by increased accumulation of the *crhR* transcript

under conditions previously shown to repress its expression (Kujat and Owttrim,

2000).

A functional CrhR protein is required for growth at low temperatures as

demonstrated by growth analysis of the *crhR* mutant and wild type strains at

20°C. The *crhR* mutant was unable to grow at low temperatures unlike wild type

Synechocystis cultures which entered exponential growth following a short period

of acclimation to the low temperature. Northern analysis has previously

demonstrated that *crhR* transcript accumulation is induced at low temperature

(Kujat-Choy, 2001). Taken together, the lack of growth at low temperature and

the cold induced accumulation of the *crhR* transcript suggest CrhR may be involved in the *Synechocystis* cold-shock response. Cold-induced bacterial RNA helicases have previously been identified in *Anabaena* sp. strain PCC 7120, *Escherichia coli* and *Methanococcoides burtonii* where they play important roles in ribosome biogenesis, mRNA processing and translation initiation (Jones *et al.*, 1996; Chamot *et al.*, 1999; Chamot *et al.*, 2000; Lim *et al.*, 2000; Charollais *et al.*, 2003; Charollais *et al.*, 2004). The increased stability of RNA secondary structures at low temperature may be responsible for the crucial need for a RNA helicase under these conditions as established by the absence of growth in the *crhR* mutant and corroborated by the cold-sensitive phenotype exhibited by *E. coli csdA* and *srmB* mutants (Thieringer *et al.*, 1998; Charollais *et al.*, 2003; Charollais *et al.*, 2004). The differential temperature requirement for the CrhR RNA helicase and likewise for the *E. coli* SrmB and CsdA cold-induced RNA helicases suggests thermodynamic breathing of RNA secondary structure under normal growth conditions may be sufficient to permit cellular functions for example, ribosome biogenesis to proceed even in the absence of helicase activity (Charollais *et al.*, 2003; Charollais *et al.*, 2004).

The requirement for a functional CrhR RNA helicase was also investigated under two other environmental conditions; high salt and CO_2. High salt has also been shown to induce expression of *crhR* (Vinnemeier and Hagemann, 1999; Kujat-Choy, 2001). Therefore, the effects of high salt on growth of the *crhR* mutant were investigated. No differences in growth kinetics could be detected between the wild type and *crhR* mutant strains in response to salt (0.5 M NaCl).

Likewise, Vinnemeier and Hagemann (1999) did not detect any difference from wild type when their *crhR* mutant was grown at salt concentrations up to 684 nM. The disparity between *crhR* expression patterns and a functional requirement for the protein at high salt concentrations was unexpected. Interestingly, *crhR* was not identified following genome wide analysis of expression patterns during salt or hyperosomotic stress (Kanesaki *et al.*, 2002). Taken together with our results, this suggests CrhR is not essential for the *Synechocystis* response to increased salt concentrations. Wild type and mutant cultures bubbled with CO_2 exhibited no difference in growth rate indicating CrhR is also non-essential for growth in high CO_2 environments. Thus the physiological function of CrhR remains unanswered.

Knowledge of the RNA targets of CrhR may allow further characterization of the role of CrhR in the cell. Putative targets identified to date, using a strategy to co-immunoprecipitate CrhR and its interacting RNAs, are related to translation and protein turnover (L. Wu and G.W.Owttrim, unpublished data). Furthermore, the *crhR* mutant cells exhibit diminished photosynthetic capabilities when transferred from 30°C to 20°C (G. Espie, unpublished data). The rapid loss of photosynthetic activity observed by Dr. Espie suggests that the CrhR protein may prevent translation of mRNA (s) whose protein products turnover rapidly and are required for the maintenance of photosynthetic capabilities. A possible target is the *psbA* gene encoding the D1 protein of the photosystem II reaction center. The DI protein is required for maintaining photosynthetic electron flow, however its sensitivity to oxidative damage necessitates continual turnover to ensure any damage is repaired (Singh 2000). A potential involvement of the CrhR RNA

helicase in DI turnover may be responsible for the observed defect in photosynthetic activity of the *crhR* mutant at low temperature.

lexA is an essential gene required for *Synechocystis* survival under the conditions examined as a variety of inactivation constructs and growth conditions were unsuccessful in obtaining complete segregation of the *lexA* gene. The essential nature of the *lexA* gene was previously suggested by Domain *et al.* (2004). It was, however, possible to use the *lexA* heteroploid strain (*lexA*: Km^r/*lexA$^+$*) to investigate LexA's regulatory role towards the *crhR* gene with depletion of the *lexA* gene resulting in increased accumulation of the *crhR* transcript under oxidizing conditions. The increased level of the *crhR* transcript under conditions previously shown to repress transcript accumulation (Kujat and Owttrim, 2000) reinforces the conclusion that LexA is a negative regulator of gene expression (Patterson-Fortin *et al.*, 2006). Independently, studies using *lexA* depleted strains have also demonstrated LexA's regulatory role in *Synechocystis*. These studies demonstrated a bifunctional role for LexA as both an activator and a repressor of *Synechocystis* gene expression (Domain *et al.*, 2004; Gutekunst *et al.*, 2005). The combination of the data presented here and in the published literature implies *Synechocystis* LexA does not regulate DNA repair responses in *Synechocystis* and rather is implicated in cellular responses to changing environmental conditions.

Characterization of the *crhR* and *lexA* mutants has provided additional insights into their role in the cyanobacterium *Synechocystis*. The *in vivo* confirmation of LexA regulation of *crhR* expression confirms our previous

conclusions drawn regarding its role in the cell as a regulator of redox-responsive gene expression. Similarly, the essential requirement for CrhR at low temperature suggests the helicase annealing and unwinding activities are important during cold shock and insinuates *crhR* expression may be responsive to temperature in addition to redox status (Kujat and Owttrim, 2001; Chamot *et al.*, 2005). This information clearly implies that both CrhR and LexA play important roles in ensuring growth and survival of the cell in a changing environment.

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Chapter 5: Conclusions

Cyanobacteria fill diverse roles in our environment as important players in among others the ocean food webs, as producers of toxic blooms and as potential sources of microbial H_2 (Stanier and Cohen-Bazire, 1977; de Figueiredo *et al.*, 2004). These significant functions in a changing environment make it important for us to understand how cyanobacteria respond and adapt to their surroundings. Their ultimate dependence on the light environment for growth and survival implies that cellular responses to fluctuating light conditions are crucial. Light quality and quantity may be sensed directly using photoreceptor proteins or indirectly through light induced changes in cellular redox poise (Mullineaux, 2001; Chen *et al.*, 2004). The mechanisms of redox-sensing in the cyanobacteria including, *Synechocystis* sp. strain PCC 6803, remain poorly characterized. The primary objective of this thesis was to investigate redox-responsive gene expression using the *crhR* RNA helicase as my model. Identification of a LexA-related protein as the regulator of *crhR* has both provided crucial first clues to elucidating light/redox sensing networks and their evolution.

5.1 *Synechocystis* sp. Strain PCC 6803 LexA

The 611 bp *Synechocystis lexA* gene encodes a 22 kDa polypeptide composed of two distinct domains (Figure 5.1A) based on homology to known LexA proteins. The similarities between the *E. coli* and *Synechocystis* proteins are depicted as % identity between the full-length protein and the amino- and carboxyl-terminal domains (Figure 5.1B). The *E. coli* amino-terminal domain encodes DNA binding capabilities while proteolytic and dimerization functions are

A *Synechocystis* LexA structure

━━ 10 amino acids

DNA binding domain
AA 1-65

Peptidase domain
AA 108-184
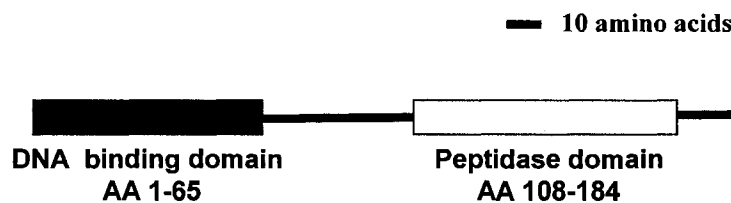
B

	% similarity to *E. coli* LexA
full-length protein	27.1
DNA binding domain	47.8
peptidase domain	38.0

Figure 5.1 *Synechocystis* sp. strain PCC 6803 LexA.
A. Schematic of LexA protein domains as determined by BLAST analysis.
B. Percent similarity between the full-length, amino-terminal and carboxyl-terminal LexA proteins of *Synechocystis* and *Escherichia coli*.

determined by the carboxyl-terminus (Hurstel *et al.*, 1986; Bertrand-Burggraf *et al.*, 1987; Schnarr *et al.*, 1988). To determine if the two domains in the *Synechocystis* LexA protein performed similar functions, mobility shift assays using the NLexA recombinant protein, containing the DNA binding domain were performed and indicated that the amino terminus of the *Synechocystis* LexA is required for sequence-specific interactions between protein and DNA. Likewise, EMSA-based analysis established that protein dimerization requires the C-terminal domain of the protein. Protein-DNA interaction was significantly enhanced by the presence of of the dimerization domain as the full-length protein exhibited 15.4 fold greater affinity for the *crhR* gene.

The carboxyl-terminal peptidase domain is required for LexA self-cleavage and thus deregulation of the SOS regulon in *E. coli* (Slilaty and Little, 1987; Shepley and Little, 1996). Sequence analysis revealed that *Synechocystis* LexA lacks two of three essential carboxy-terminal residues required for proteolytic activity of the *E. coli* protein (Mazón *et al.*, 2004; Patterson-Fortin *et al.*, 2006; Chapter 2). The alanine-glycine cleavage bond and the nucleophilic serine residues required for self-cleavage of the prototypical LexA in *E. coli* are absent (Slilaty and Little, 1987). Furthermore, the *Synechocystis* LexA does not undergo self-cleavage *in vitro* under conditions known to inactivate the *E. coli* protein. These observations strongly suggest that the biochemical capabilities of the *Synechocystis* LexA protein differ significantly from those exhibited by canonical LexA proteins associated with DNA repair regulons (Little and Mount, 1982; Miller *et al.*, 1996). Interestingly, sequence alignment revealed that the *Synechocystis*

LexA is the only identified bacterial LexA lacking the amino acid residues required for proteolytic activity (John W. Little, personal communication). Furthermore, sequence alignments of LexA proteins from other cyanobacterial species revealed that the lack of proteolytic capabilities is limited to *Synechocystis* and is not a common feature of cyanobacterial LexA proteins (Mazón *et al.*, 2004). Therefore, it is not unexpected that phylogenetic analysis reveals that the *Synechocystis* LexA protein is unrelated to the other cyanobacterial LexAs (Figure 5.2). Taken together, bioinformatic analyses suggesting that the *Synechocystis* LexA is an orthologue of the *E. coli* protein has been expanded by the experimental analyses at the DNA, RNA and protein levels reported in this thesis.

5.2 Transcriptional regulation by LexA binding in *Synechocystis*

The LexA-orthologue binds as a dimer to a direct repeat, CTA-N_9-CTA, located within the *lexA* 5' UTR and overlapping the *crhR* translational start site, respectively (Patterson-Fortin and Owttrim, 2008; Chapter 3). Analysis of the LexA target, *hoxEFUYH*, revealed a potential CTA binding site located upstream of the transcriptional start site, in a region of DNA encompassing the promoter -35 element (Oliveira and Lindblad, 2005). The identification of several binding sites for one protein located at divergent sites within target genes and their respective upstream sequences points towards to a complex mode of gene regulation.

Cyanothece sp. ATCC 51142
Cyanothece sp. CCV 0110
Crocosphaera watsonii WH 8501
Cyanothece sp. PCC 7424
Synechococcus sp. PCC 7002
Microcoleus chthonoplastes PCC 7420
Cyanothece sp. PCC 7425
Synechococcus sp. RCC307
Synechococcus sp. WH 8102
Synechococcus sp. CC9605
Synechococcus sp. BL107
Synechococcus sp. CC9902
Synechococcus sp. WH 7803
Synechococcus sp. WH 7805
Prochlorococcus marinus str. MIT 9313
Prochlorococcus marinus str. MIT 9303
Synechococcus sp. RS9916
Synechococcus sp. RS9917
Synechococcus sp. CC9311
Synechococcus sp. WH 5701
Prochlorococcus marinus str. NATL1A
Prochlorococcus marinus str. NATL2A
Prochlorococcus marinus subsp. marinus str. CCMP1375
Prochlorococcus marinus str. MIT 9211
Prochlorococcus marinus str. MIT 9515
Prochlorococcus marinus str. MIT 9215
Prochlorococcus marinus str. AS9601
Prochlorococcus marinus subsp. pastoris str. CCMP1986
Prochlorococcus marinus str. MIT 9301
Prochlorococcus marinus str. MIT 9312
Synechococcus sp. PCC 7335
Synechococcus sp. WH 7805
Synechococcus sp. WH 7803
Synechococcus sp. WH 8102
Synechococcus sp. RS9917
Synechococcus sp. CC9311
Synechococcus sp. RS9916
Synechococcus sp. WH 5701
Prochlorococcus marinus subs
Prochlorococcus marinus str. MIT.
Prochlorococcus marinus subsp. mar
Prochlorococcus marinus str. NA.
Gloeobacter violaceus PCC 7421
Acaryochloris marina MBIC11017
Nodularia spumigena CCY 9414
Nostoc punctiforme PCC 73102
Anabaena variabilis ATCC 29413
Nostoc sp. PCC 7120
Microcystis aeruginosa NIES-843
Microcystis aeruginosa PCC 7806
Cyanothece sp. PCC 8801
Cyanothece sp. ATCC 51142
Cyanothece sp. ATCC 51142
Cyanothece sp. CCV 0110
Cyanothece sp. CCV 0110
Cyanothece sp. CCV 0110
Synechocystis sp. PCC 6803
Synechocystis sp. PCC 6803
Euhalothece sp. BAA001

Figure 5.2 Phylogenetic tree of LexA protein sequences in cyanobacteria. The tree was derived from Blast pairwise alignments using the *Synechocystis* LexA sequence as query.

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While no evidence as to the mechanism of regulation was determined in this thesis, it is possible to propose at least two different ways by which transcriptional regulators bound at sites external to the promoter could influence gene expression. The data obtained suggests that a simple steric hindrance model as observed for *E. coli* LexA is not responsible for differential expression of target genes in *Synechocystis* (Little and Mount, 1982; Fernández de Henestrosa *et al.*, 2000). Furthermore, LexA is not a bi-functional protein binding to both the DNA and RNA as demonstrated by the lack of specific interaction between rLexA and the *crhR* RNA. Therefore post-transcriptional regulation can also be discounted as a possible means of gene regulation.

Two proposed mechanisms of LexA regulated gene expression in *Synechocystis* are described below. Firstly, protein-protein interactions between two transcriptional regulators bound at distant sites can loop the DNA, influencing RNA polymerase activity and or promoter structure (Dunn *et al.*, 1984; Su *et al.*, 1990; Choy and Adhya, 1996). In *E. coli*, repression at both promoters of the *gal* operon involves GalR bound at two distant sites and GalR protein-protein interactions to loop the DNA. The looped DNA encompasses the promoter and blocks RNAP initiation of transcription. Single site occupancy by GalR permits expression to proceed from one of the two promoters demonstrating the importance of DNA looping for repression of the *gal* operon (Mandal *et al.*, 1990; Choy and Adhya, 1996). Similarly, DNA looping is important for AraC repression of regulated targets (Choy and Adhya, 1996). Sequence analysis of the *crhR* upstream sequences identified a second putative CTA-N_9-CTA sequence which

may allow for DNA looping. This second CTA motif is located 608 bp upstream of the previously identified motif within the *crhR* gene. Interestingly, a CTA-N$_{10}$-CTA motif was also found at a similar distance upstream (599 bp) of the previously identified *lexA* CTA motif within the 5' UTR suggesting DNA looping may be important for LexA-mediated regulation of *crhR* and *lexA* expression. Loop formation by the distantly bound LexAs may impact RNAP activity and consequently gene expression. Mobility shift assays performed using DNA fragments containing these putative motifs would reveal whether rLexA specifically interacts with these upstream CTA-N$_9$-CTA sites within the *lexA* and *crhR* genes and therefore the potential for DNA looping as a mechanism of gene regulation.

Secondly, structural alteration of promoter architecture by proteins bound at sites either upstream or downstream of the promoter regulate gene expression (Dunn *et al.*, 1984). In cyanobacteria, protein induced changes in DNA structure are important for expression of the *secA* and *gap2* genes. DNA binding proteins act to remodel the promoter, reducing the distance between the -10 and -35 elements improving *secA* and *gap2* promoter activity (Mazouni *et al.*, 1998; Figge *et al.*, 2000). Similarly, in *E. coli*, MerR and SoxR reduce suboptimal promoter spacing to improve activity of regulated promoters (Heltzel *et al.*, 1990; Ansari *et al.*, 1992; Hidalgo and Demple, 1994; Ding *et al.*, 1996; Brown *et al.*, 2003). It is suggested that LexA bound to the *lexA* and *crhR* targets may induce architectural changes to the DNA which are subsequently transferred to the promoter changing its affinity for RNA polymerase. For example, promoter remodeling may

prevent formation of an important RNAP-DNA contact (Su *et al.*, 1990; Choy and Adhya, 1996). The *Synechocystis lexA* promoter is typical of cyanobacteria encoding only an *E. coli*-like -10 element (Curtis and Martin, 1994; Domain *et al.*, 2004). Sequence alignments suggest a similar promoter composition for the *crhR* gene. The lack of the -35 element suggests structural features of the DNA possibly induced by bound protein may be important for favorable RNA polymerase-DNA contacts and optimal promoter activity.

5.3 A model for LexA-regulated expression of the *crhR* gene

Expression of the *crhR* RNA helicase is regulated by the redox poise of the photosynthetic electron transport chain (Kujat and Owttrim, 2000; Kujat-Choy, 2001). The previous model proposed that redox-regulated transcription of the *crhR* gene involves a signal transduction cascade initiated by reduction of the electron transport chain carriers, plastoquinone and cytochrome b_6f. Changes in redox poise are coupled to activation of a thylakoid-associated kinase and a phosphorylation cascade regulating binding activity of regulatory protein(s) involved in *crhR* expression (Kujat and Owttrim, 2000). *Synechocystis* LexA may be a member of the proposed signal transduction pathway allowing a revised model for redox-responsive gene expression (Figure 5.3A). LexA regulates expression of the *crhR* RNA helicase in response to photosynthetic electron transport. Under conditions promoting reduction of the electron transport chain, e.g. during photoautotrophic or photoheterotrophic growth, LexA has a lower affinity for its binding site within the *crhR* ORF and *crhR* transcripts accumulate.
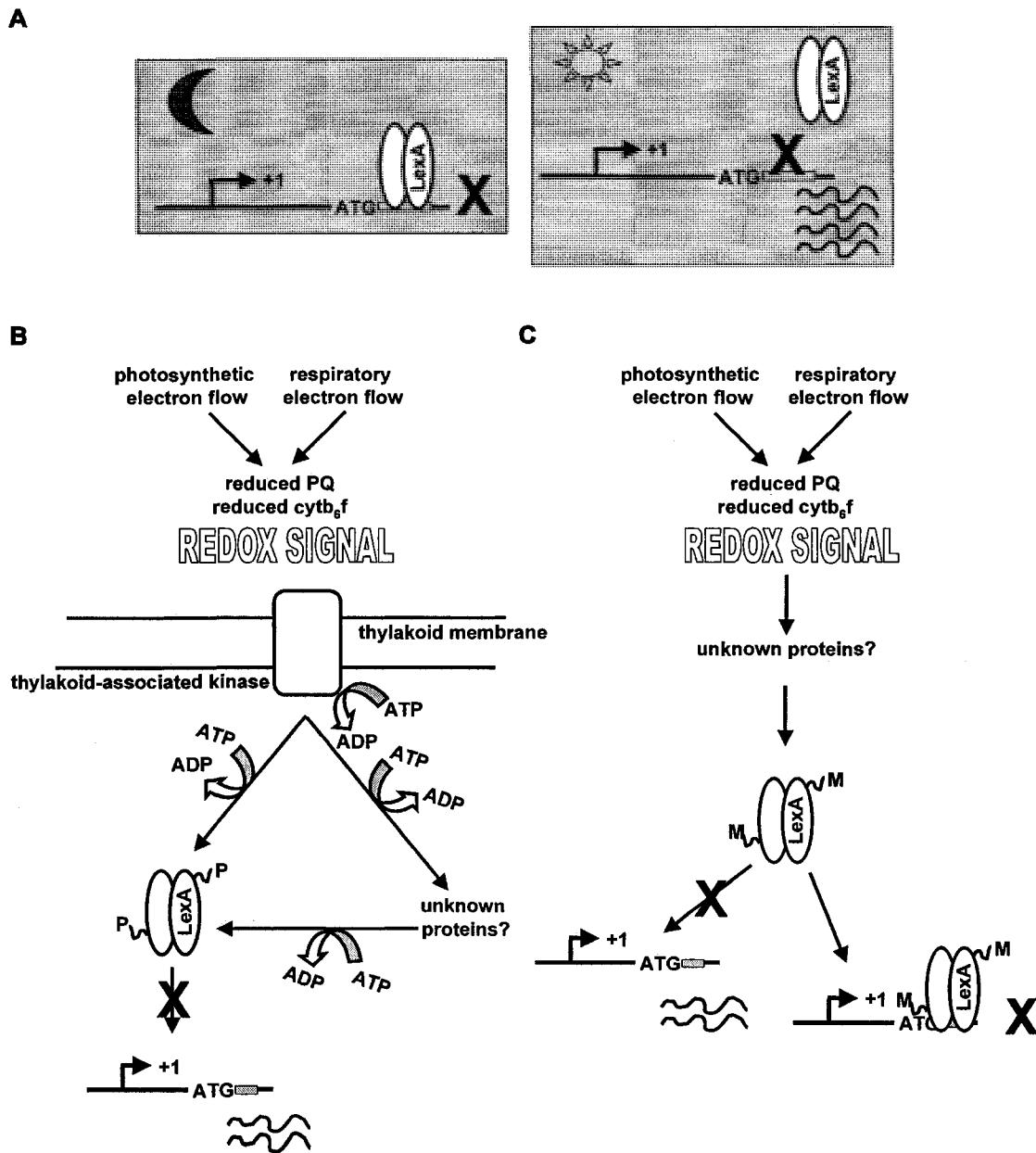
Figure 5.3 Proposed model of LexA-regulated expression of the CrhR RNA helicase.

A. Light/Dark regulation. In the dark, under oxidizing conditions, LexA binds the CTA-N9-CTA motif within the *crhR* ORF, blocking *crhR* transcript accumulation. In the light, under reducing conditions, LexA is unable to bind the *crhR* gene, permitting accumulation of the *crhR* transcript.

B. LexA phosphorylation. The proposed redox signal could be transduced to LexA binding capabilities via a phosphorylation cascade. A proposed thylakoid-associated kinase senses redox poise, autophosphorylating under reduced conditions. The phosphate group may be directly transferred to LexA or through

other yet undetermined members of a phosphor-relay system. Phosphorylated LexA is unable to bind the CTA motif within the *crhR* gene permitting gene expression under reducing conditions.

C. Other post-translational modifications. The proposed redox signal could also be transduced to LexA binding capabilities via other post-translational modifications (M) for example, methylation or acetylation. Post-translational changes to LexA may influence its affinity for the DNA, either reducing or increasing DNA binding affinity and consequently *crhR* transcript accumulation.

In contrast, reduced electron flow and oxidation of the electron transport chain signals LexA to bind the *crhR* gene and repress transcription. LexA has been reported to be a peripheral thylakoid membrane protein (Wang *et al.*, 2000; Srivastava *et al.*, 2005) making it suitably located to receive redox signals initiated by changes in the poise of the photosynthetic electron transport chain. Direct regulation of LexA binding activity, by redox induced changes in LexA structure, is unlikely due to the absence of redox-sensitive cysteine residues. Furthermore, mobility shift assays performed in

different redox environments had no effect on rLexA interaction with the *crhR* gene (L.M. Patterson-Fortin and G.W Owttrim, unpublished data). Changing redox environments have previously been shown to alter *in vitro* binding capabilities of the redox sensitive proteins, NtcA and PedR, in cyanobacteria (Jiang *et al.*, 1997; Alfonso *et al.*, 2001; Nakamura and Hihara, 2005).

The DNA binding capabilities of the prototypical LexA are regulated by a proteolytic self-cleavage reaction occurring under DNA damaging conditions. ssDNA produced by DNA damage or replication inhibition activates RecA. The RecA nucleoprotein stabilizes the cleavable conformation of LexA allowing self-cleavage in *E. coli* (Little and Mount, 1982; Luo *et al.*, 2001). Both the expression and the activity of *E. coli* LexA and RecA are DNA damage inducible. In contrast in *Synechocystis*, expression of the *lexA* and *recA* genes is not induced by DNA damage inducing treatments. Furthermore, self-cleavage of purified rLexA does not occur under conditions known to induce the cleavage of *E. coli* LexA suggesting *Synechocystis* LexA must regulate its DNA binding capabilities via an

unconventional mechanism (Little, 1984). As direct redox regulation does not appear to regulate LexA binding, it is possible that LexA binding is modulated by alternative post-translation modifications for example, phosphorylation, methylation or acetylation. The relatively low affinity of recombinant LexA for its targets as determined by LexA saturation experiments suggests post-translational modifications may be required for optimal DNA-protein interactions (e.g. the LexA protein purified from *E. coli* may not have these modifications). The proposed modifications may influence LexA structure and in turn its affinity for target binding sites on the DNA. The lower binding affinities exhibited by the LexA protein may also be an indication that its regulatory role is directed towards fine-tuning gene expression rather than functioning solely as an off-on switch. As observed for other repressor proteins, LexA levels in the cell will dictate the magnitude of gene expression based on target site binding affinities. Binding sites with low affinities will be expressed at higher levels while those binding sites with higher affinity will remain inactive longer therefore exhibiting less induction. The potential involvement of a post-translation modification modulating LexA binding capabilities would further influence expression levels such that conditions where fewer LexA molecules are modified to allow DNA binding, only those genes with high affinity binding sites will successfully compete for available protein and exhibit altered expression.

Protein phosphorylation has been implicated in cyanobacterial, green algae and higher plant responses to the redox environment (Li and Sherman, 2000). Thylakoid membrane associated kinases are proposed sensors linking

redox poise of the electron transport chain to cellular activity and and/or gene

expresssion (Escoubas *et al.*, 1995; Snyders and Kohorn, 1999; Snyders and

Kohorn, 2000; Depège *et al.*, 2003; Bellafiore *et al.*, 2005). Kinases required for

state transitions have been identified in *Chlamydomonas reinhardtii* and

Arabidopsis (Depège *et al.*, 2003; Bellafiore *et al.*, 2005) with suggested roles in

initiating a phosphorylation cascade linking redox status within the chloroplast to

nuclear gene expression (Escoubas *et al.*, 1995, Durnford and Falkowski, 1997).

A plausible phosphorylation scheme regulating LexA binding capabilities

involving a thylakoid-associated kinase is depicted in Figure 5.3B. The reduction

of the photosynthetic electron transport chain initiates a phosphorylation cascade

ultimately ending in phosphorylation of LexA (LexA~P) and an inability to bind the

DNA. Under oxidizing conditions, the thylakoid-associated kinase and the

associated phosphorylation cascade would be less active therefore LexA would

not be modified allowing interaction with target genes. Preliminary mobility shift

assays performed under conditions to mimic different phosphorylated states of

the LexA protein were unable to detect differences in binding to the *crhR* target.

Therefore, to determine the involvement of protein phosphorylation in the LexA

redox response, it will be necessary to firstly show whether or not LexA is

differentially phosphorylated in response to the cellular redox poise and secondly

to identify potential members of the proposed phosphorelay system. Alternative

post-translational modifications, such as acetylation or methylation, could also be

responsible for differential binding of LexA to its DNA targets (Figure 5.3C). DNA

affinity of the *Aphanizomenon ovalisporum* AbrB-like protein is proposed to be

regulated by reversible post-translational acetylation and methylation reactions (Shalev-Malul *et al.*, 2008). The AbrB-like protein is involved in the differential regulation of the toxin biosynthesis genes, *aoaA* and *aoaC* in response to both nitrogen availability and the redox/light environment (Shalev-Malul *et al.*, 2008). The potential involvement of acetylation and methylation in regulating genes responsive to the redox/light environment suggests further investigation is warranted into the potential role of these reactions in LexA regulated gene expression. Mass spectrometry of the native LexA protein would reveal whether or not the protein is post-translationally modified. The exact nature of the post-translational modification will help elucidation of the upstream events coupling LexA's DNA binding and regulatory activities.

An unrelated alternative to explain the apparent low affinity of LexA for DNA targets is the involvement of accessory proteins. In *E. coli*, optimal regulation of several promoters requires the concerted activity of two or more proteins (Browning and Busby, 2004). Recently, the AbrB-like protein was shown to regulate both expression and hydrogenase activity of the *Synechocystis hoxEFUYH* bidirectional hydrogenase, a known LexA target (Oliveira and Lindblad, 2008). The authors did not investigate the relationship between the LexA and AbrB-like proteins in regulating *hox* gene expression only indicating this analysis is necessary to better understand the interplay between the two proteins (Oliveira and Lindblad, 2008). Under our experimental conditions, the recombinant AbrB-like protein, alone or together with rLexA did not interact with

either the *crhR* or *lexA* targets suggesting that, at least *in* vitro, the AbrB-like protein is not involved in LexA regulation of these targets.

5.4 The *Synechocystis* LexA regulon

To date, the *Synechocystis* LexA regulon is composed of three genes, *crhR*, *lexA* and *hoxEFUYH* (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005; Patterson-Fortin *et al.*, 2006; Patterson-Fortin and Owttrim, 2008). Knowledge of a CTA-N_9-CTA direct repeat important for LexA binding was used to identify other potential members of the regulon. This analysis was performed using the online Regulatory Sequence Analysis Tool (RSAT) (van Helden *et al.*, 1998; van Helden *et al.*, 2000; van Helden, 2003) and the CTA-N_9-CTA sequence as query. Both the experimentally determined binding sites within the *crhR* and *lexA* genes were identified using this program. One hundred and eighty-six annotated genes containing the CTA motif within their upstream sequence or the first 100 bp of their open reading frame are shown in Tables 5.1 and 5.2. One hundred and thirty hypothetical genes also encoded the query sequence within their upstream or open reading frame sequences. The identified genes are putative members of a group of genes whose expression is regulated by LexA. However, it will be necessary to confirm the regulatory relationship between LexA and these putative targets through mobility shift assays using rLexA and gene expression profiles in wild type and *lexA* heteroploid cells.

Analysis of the putative targets revealed gene products required for a variety of different cellular processes including energy metabolism,

photosynthesis, gene regulation and DNA/RNA metabolism (Tables 5.1 and 5.2). Among the putative targets are three genes, *ruvA*, *mutS* and *sll1457*, involved in DNA repair and recombination. Northern analysis revealed that *Synechocysits lexA* and *recA* expression is not induced by DNA damage unlike their *E. coli* counterparts (Courcelle *et al.*, 2001; Huang *et al.*, 2002; Quillardet *et al.*, 2003; Damon *et al.*, 2004; Patterson-Fortin *et al.*, 2006). Likewise, the three putative LexA targets were not identified as transcripts induced following ultraviolet irradiation (Huang *et al.*, 2002). In fact, RuvA and the sll1547 gene product may be required during homologous recombination (West, 1997) while MutS is required for mismatch repair (Li, 2008). Both processes occur independent of DNA damage (West, 1997; Li, 2008), therefore their DNA damage independent expression may suggest involvement in responding to normal cellular replication and recombination rather than specifically to external DNA damage.

Not unexpectedly due to the proposed role of LexA in redox-responsive gene expression, the CTA-N9-CTA motif could also be found upstream of genes associated with energy metabolism, photosynthesis and respiration, for example, ATP synthase subunits, ferredoxin and photosystem subunits. Interestingly, none of the identified genes were directly related to or involved in carbon metabolism even though LexA has also been implicated in regulating their expression (Domain *et al.*, 2004). In several cyanobacteria, LexA regulates expression of the bidirectional hydrogenase and hydrogenase accessory proteins (Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005; Ferreira *et al.*, 2007; Sjöholm *et al.*, 2007). In

Table 5.1 Annotated genes containing the CTA-N$_9$-CTA motif within their upstream sequences

ID	Gene	Location[a]	CTA motif[b]
	Energy metabolism		
sll0020 (atpA)	ATP synthase subunit, alpha	-65, -51	gcgaCTAAATTAACTGCTAgagg
sll1323 (atpG)	ATP synthase subunit beta	-31, -17	tccgCTAATCCCTGACCTAgttt
sll1327 (atpC)	ATP synthase subunit, epsilon	-45, -31	ctaaCTATTTCCTAACCTAgacc
slr2135 (hupE)	hydrogenase accessory protein	-54, -40	ccccCTACAGCTAAGGCTAcctg
slr0822	cation-transporting ATPase	-24, 10	gaacCTAAATTTAAGCCTActtt
	Photosynthesis and respiration		
sll0813 (ctaC)	cytochrome C oxidase subunit II	-43, -29	tggtCTAATGGGGAAGCTAgatt
sll0199 (petF)	Plastocyanin	-42, -28	gaaaCTAGCAAATTAACTActaa
sll0248 (isiB)	Flavodoxin	-87, -73	actaCTAAACCAAGCCCTActta
sll0819 (psaF)	PSI subunit III	-46, -32	aagaCTAAAAAGGCGGCTAaata
slr1125 (crtX)	zeaxanthin glycosyl transferase	-56, -42	ttggCTATGGACAGGACTAtagg
slr1138 (ctaE)	cytochrome C oxidase subunit III	-29, -15	taagCTAGCCATCCCCCTAagca
ssl1417 (ycf33)	Ycf33	-94, -80	aatcCTAGGTCTCTTGCTAcact
ssr0390 (psaK)	PSI subunit X	-52, -38	attaCTAATTAGTGGACTAaggg
ssr3383 (apcC)	phycobilisome linker protein	-99, -85	tcagCTAAAACCCCGGCTAtcc
smr0008 (psbJ)	PSII protein	-50, -36	ccagCTATTTCTTTAACTAaact
	Transcription/Translation		
sll1216 (Tsf)	elongation factor TS	-96, -82	ccagCTATATTTTCCCCTAgtac
sll1615 (thdF)	tRNA modification	-74, -60	gttaCTACGGCAGGTACTAcaaa
sll1816 (rps13)	30S ribosomal protein S13	-87, -73	atcaCTAGGAGAACTGCTAacct
sll1682	alanine dehydrogenase	-87, -73	tggaCTAGAACCCCAACTAtgga
slr1105 (Fus)	EF-G	-102, -88	gcttCTAAAACTGAACCTAagag
slr1550 (lysS)	lysyl-tRNA synthetase	-60, -46	aataCTAATAAACTATCTActtc
	Regulation		
sll0396	OmpR subfamily protein	-24, -10	tttgCTATGCGATCGCCTAgttt
sll1626 (lexA)	LexA repressor	-30, -16	agtcCTAAATACATTCCTAtagg
sll1670 (hrcA)	heat inducible transcription repressor	-23,-9	tttcCTAAATTGCGGGCTAaaga
slr1037	CheY subfamily protein	-101-87	gtttCTATTCGTACTGCTAatat
slr1147	histidine kinase	-76, -62	tggtCTACTTATGGCCCTAgggg

slr1489 (*PchR*)	regulatory protein	-43, -29	ctgt**CTAGCAAGGATGCTA**ggca
slr1489 (PchR)	regulatory protein	-42, -28	ctgc**CTAGCATCCTTGCTA**gaca
slr1909	NarL subfamily protein	-97, -83	ccga**CTACCATCATCCCTA**aata
slr1969	hybrid sensory kinase	-87, -73	atgc**CTAGGTTTTTGCCTA**accc

DNA repair

sll0876 (*ruvA*)	Holliday junction DNA helicase	-19, -5	ataa**CTATTTTCGTATCTA**aatc
sll1547	Holliday junction resolvase	-54, -40	aaag**CTAAAATTTCACCTA**agtt
sll1772 (*mutS*)	DNA mismatch repair	-16, -2	ttta**CTATATTTTTTCCTA**tgga

DNA and RNA metabolism

sll0757 (*purF*)	amidophosphoribosyl transferase	-63, -49	gaaa**CTAAATCTTAGACTA**tggg
sll1161	adenylate cyclase	-102, -88	atgc**CTATTAATTAACCTA**actg
sll1209 (*lig*)	DNA ligase	-70, -56	cttg**CTATAAGAGGATCTA**gagt
sll1507	cytidine deaminase	-37, -23	ttat**CTATCGGTGACTCTA**ctgc
slr0477 (*purN*)	phosphoribosylglycinamide formyltransferase	-16,-2	gccc**CTAATTTTTAACCTA**tgtc
slr0050	ribonuclease Z	-47, -33	gggg**CTAAGAACCATTCTA**ggcg
slr1123 (*gmk*)	guanylate kinase	-96, -82	ccag**CTATATTGCTATCTA**gccc
slr1369 (*cdsA*)	phosphatidate cytidylyl transferase	-94, -80	tgtt**CTAAAATAAAAACTA**agaa

Transport and binding proteins

sll0834 (*bicA*)	bicarbonate transporter reductase	-78, -64	agcc**CTAGAATATTCTCTA**tgga
sll1341 (*bfr*)	Bactoferritin	-56, -42	caac**CTAGGCACAAATCTA**ccta
sll1374 (*melB*)	melibiose carrier protein	-43, -29	tagc**CTACGCTGTGGGCTA**ggaa
sll1457 (*chrA*)	chromate transport protein	-54, -40	tacc**CTAAGGTAATCGCTA**acct
slr0513	periplasmic binding protein	-24, -10	ccca**CTATCCATTGCCCTA**gact
slr1454 (*cysW*)	sulfate transport system permease	-39, -25	attg**CTAATTATTAACCTA**ctgc
slr1316 (*fecC*)	iron (III) dicitrate transport system permease	-66, -52	tttc**CTAGTAACTTCACTA**aagt
slr1318 (*fecE*)	iron (III) dicitrate transport system permease	-45, -31	tcct**CTACAATAATCTCTA**atct
slr1471	inner membrane protein translocase	-19, -5	attg**CTAACAAGCTCTCTA**gata

Proteases and chaperones

sll0915 (*pqqE*)	Protease	-49, -35	tcat**CTAAAGTAGTTGCTA**taaa
sll1988 (*hslO*)	Hsp33-like chaperonin	-53, -39	gctt**CTATTGTGCCAACTA**gccc
slr0542 (*clpP*)	ATP-dependent protease	-38, -24	caat**CTAGGGAGAATACTA**cggt
slr1751 (p*rc*)	carboxyl terminal protease	-63, -49	gact**CTAAGCACCGCCCTA**gggt

Transposases

sll0665	Transposase	-95, -81	gatt**CTAGGCTTTCTACTA**tttt
sll1156	Transposase	-54, -40	catc**CTACGTCATTATCTA**tttg

sll7002	Transpoase	-92, -78	accaCTACAGCCCTAGCTAaaat
slr1683	Transposase	-16, -2	gacaCTATACCGCTTTCTAtttt
slr1715	Transposase	-86, -72	atgaCTATAGATTTGACTAtttt
slr2095	Transposase	-54, -40	catcCTACATCATTATCTAtttg
Metabolism			
sll0222 (*phoA*)	alkaline phosphatase	-79, -65	tcaaCTACTCAAAAAACTAgcga
sll0404 (*glcD*)	glycolate oxidase subunit	-37, -23	gtacCTATGCCAAATTCTAgtgg
sll0657 (*mraY*)	phospho-N-acetylmuramoyl-pentapeptide-transferase	-16, -2	tgaaCTAATTTTTTGACTAtggc
sll0990	formaldehyde dehydrogenase	-96, -82	tgctCTATCCACTGAGCTAcgca
sll1363 (*ilvC*)	ketol acid reductoisomerase	-27, -13	aattCTAGCTTTTGGTCTAtgct
sll1546 (*ppx*)	Exopolyphosphatase	-66, -52	aactCTACCAACCAGCCTAgggc
sll1900 (*act*)	acetyl transferase	-58, -44	ttctCTATTCTTAACTCTAggaa
slr0077 (*nifS*)	NifS	-47, -33	ctaaCTAAAAAATCAACTAaaat
slr0091	aldehyde dehydrogenase	-84, -70	acttCTAACTGTTTTTCTAaagt
slr0519	phosphoribosylformylglycinamidine synthase subunit	-54,-40	agatCTAGATTTGGGGCTAgtct
slr0519	phosphoribosylformylglycinamidine synthase subunit	-53,-39	aagaCTAGCCCCAAATCTAgatc
slr0661 (*proC*)	pyrroline-5-carboxylate reductase	-101, -87	aaccCTAGCAACTCCCCTAtctc
slr0812	panthothenate kinase	-55, -41	tggtCTAATGGGGAAGCTAgatt
slr0862 (*lmbP*)	LmbP	-100, -86	ctggCTAAACCCAAGGCTAgagg
slr1719 (*drag*)	quinone reductase	-37, -23	tctcCTAATCATTTTCCTAgcta
slr1945 (*yibO*)	phosphoglyceromutase	-100, -86	ataaCTAGTACTCGGTCTAgggc
slr1945 (*yibO*)	phosphoglyceromutase	-99, -85	tgccCTAGACCGAGTACTAgtt
Nitrogen metabolism			
slr0898 (*nirA*)	ferredoxin-nitrite	-39, -25	aaccCTATATTGATCTCTActgt
Stress proteins			
sll0897 (*dnaJ*)	DnaJ; heat shock protein	-60, -46	tttgCTACAGTAAACACTAcaga
ssr1169 (*ESI3*)	salt stress induced hydrophobic peptide	-25, -11	tatgCTATCCCATTGACTAattt
Other			
sll0002 (*ponA*)	penicillin binding protein A	-100, -86	tttcCTAATGTATCTCCTAccat
sll0202 (*gidA*)	mod enzyme	-65, -51	ttctCTAAAAGTTGAACTAaaga
sll0626 (*lim17*)	LIM17 protein	-95, -81	cctaCTATCTGTCAGCCTAgcaa
sll0698 (*dfr*)	drug sensory protein A	-63, -49	caacCTACTGGGCGTTCTAacag
sll1383 (*suhB*)	extragenic suppressor	-97, -83	tcatCTAAAGGCAAACCTAatcg
sll1621	membrane protein	-29, -15	ctggCTATCTAAACGACTAaatt

| sll7003 | plasmid stability protein | -102, -88 | aatc**CTAGAGCAGGCCCTA**ttac |
| ssl2296 (*dcoH*) | pterin-4-alpha-carbinolamine dehydratase | -29-15 | ttat**CTAGATTTTTAACTA**ttgg |

* Genome search of regulatory regions from -100 to +1 where +1 designates the translation start site
a. Location relative to translation start site
b. Search motif CTA (N_9) CTA; no mismatches accepted

Table 5.2 Annotated genes containing the CTA-N_9-CTA motif within the first 100 bp of their open reading frame

ID	Gene	Location[a]	CTA motif[b]
slr0017 (*murA*)	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	+14, +29	gttgCTACTCCTACCGCTAccga
slr0083 (*crhR*)	DEAD-box RNA helicase	+5, +19	atgaCTAATACTTTGACTAgtac
slr0228 (*ftsH*)	cell division protein	+25, +39	tgccCTACTTTGGTCCCTAcccc
slr1723	permease protein of sugar ABC transporter	+22, +36	ttacCTAGTTTTAGTCCTAttgg
slr1592	probable RNA pseudouridine synthase	+80, +94	acttCTACTCCCAACGCTAtctc
slr1991 (*cya1*)	adenylate cylcase	+16, +30	tgccCTACCCATTTACCTAgtag
slr2012	putative monovalent cation/H^+ antiporter subunit B	+16, +30	ttacCTAATTGCGGCCCTAttgt
slr2058 (*topA*)	DNA topoisomerase A	+32, +46	cccaCTAAAGCCCGCACTAtccg
sll0915 (*pqqE*)	periplasmic protease	+25, +39	ttctCTATCCCGTTGTCTAttgg
sll1869	probable dioxygenase, Rieske iron-sulfur component	+36, +50	ttgtCTATGCTGCGTACTAcccc

* Genome search of regulatory regions from +1 to +100 where +1 designates the translation start site

a. Location relative to translation start site

b. Search motif CTA (N_9) CTA; no mismatches tolerated

Synechocystis, a CTA-N$_9$-CTA motif was found in the hydrogenase accessory

gene, *hupE*. The conservation of LexA targets related to H$_2$ production in several

cyanobacteria suggests LexA-regulated expression of this process is important.

Additionally, a CTA-related sequence was identified within the upstream

sequences of the *hoxEFUYH* gene containing a putative LexA binding site

(Oliveira and Lindblad, 2005; Patterson-Fortin and Owttrim, 2008; Chapter 3).

The sheer number of genes identified containing the CTA motif within their

upstream sequences suggests further analysis into the degree of accepted

sequence variability and the nature of the N9 linker is essential for a clearer

consensus sequence to be identified. For example, motif analysis performed

using variability at the third position (e.g. A or T in the third position of the CTA

triplett) as suggested by the identified CTA repeat within a region of DNA

required for LexA interaction with the *hox* target, 807 putative LexA targets were

identified. Nevertheless, the initial identification of putative LexA targets offers an

initial glimpse into the *Synechocystis* LexA regulon and the relationship between

LexA and CrhR targets.

5.5 Evolution of the *Synechocystis* LexA protein

The striking sequence disparity from SOS-associated LexA proteins hints

at a complex evolutionary history for the *Synechocystis* LexA protein. It has been

suggested that *Synechocystis* LexA may have undergone a domain swap event.

The occurrence of a domain exchange may explain the relatedness of the amino-

terminal DNA binding domain and the divergence of the carboxyl-terminal

catalytic domain to the prototypical *E. coli* LexA protein. For example, replacement of the carboxyl-terminal proteolytic domain of the ancestral LexA protein would rationalize both the similarities and differences between *Synechocystis* LexA and other LexAs.

The existence of domain exchanges is clearly demonstrated in the analysis of the phage repressor, CI. For example, the CI proteins from Lambda and VT2-Sa phage repressor proteins share strong sequence homology within their carboxyl-terminal dimerization domains but the sequence homology is lost within their amino-terminal DNA binding domains (Fattah *et al.*, 2000). Similarly, the C-terminal domain of the shiga-toxin converting phage, 933W, is related to the C-terminal domain of phages H-19B (96.6% identity at the protein level) and φ80 yet its N-terminal domain is homologous with that of the phage HK022 (Plunkett *et al.*, 1999; Fattah *et al.*, 2000).

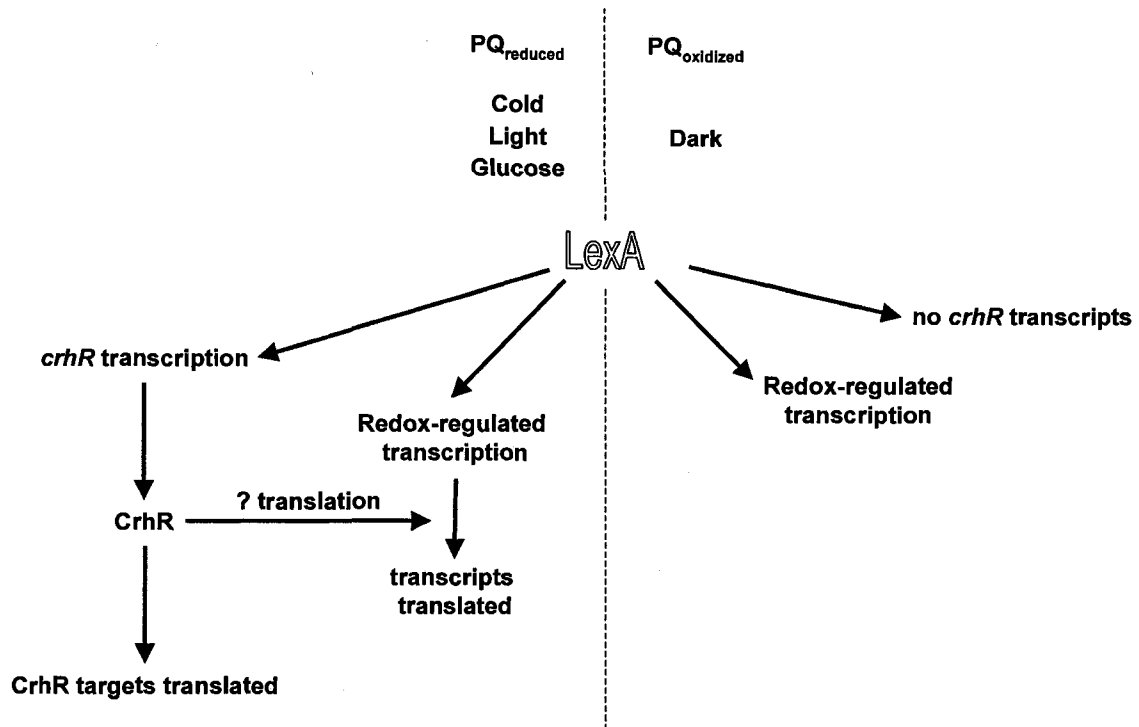## 5.6 Physiological role of the LexA and CrhR proteins in *Synechocystis*

Preliminary physiological roles for the LexA and CrhR proteins can be assigned based on the results presented in this thesis. LexA is an essential protein regulating expression of the *crhR* and *lexA* genes (Patterson-Fortin *et al.*, 2006; Patterson-Fortin and Owttrim, 2008; Chapter 4). LexA does not appear to be required for the response to and repair of DNA damage. Rather, knowledge of the gene products regulated by LexA identified in this thesis and by other research groups (Domain *et al.*, 2004; Gutekunst *et al.*, 2005; Oliveira and Lindblad, 2005) evoke an important role for LexA in responding to environmental

change. CrhR is essential for growth at low temperature as loss of the helicase activity inhibited growth at 20°C. This cold temperature requirement implies CrhR activity is needed to rearrange the stable RNA secondary structures that may form during cold shock.

crhR expression is responsive to both temperature and the redox poise of the electron transport chain (Kujat and Owttrim, 2000; Kujat-Choy, 2001). Low temperature is one of several environmental stresses shown to influence cellular photosynthetic capabilities and consequently redox poise (Jeanjean *et al.*, 1998; Allakhverdiev *et al.*, 2000; Pfannschmidt, 2003; Savitch *et al.*, 2001). At low temperatures, the rate of carbon fixation is decreased resulting in overreduction of the electron transport chain. This cold induced overreduction of the photosynthetic electron transport chain may be coupled to increased expression of *crhR*. Similarly, *lexA* transcript levels declined in the cold suggesting decreased expression of LexA is partially responsible for the observed induction in *crhR* transcript levels. A mechanism of cold induction requiring a functional electron transport chain has been shown for expression of the *Synechocystis* fatty acid desaturase genes (Los *et al.*, 1993; Kis *et al.*, 1998). Kis *et al.* (1998) concluded that cold induced changes in redox state were responsible for differential expression patterns rather than directly the result of the low growth temperature. Furthermore, the similarities in the mechanisms responsible for adjustments to both high light and low temperature further suggest cold induction is in part mediated by changes in redox poise of the electron transport chain (Huner *et al.*, 1996).

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LexA is an essential protein regulating gene expression in response to environmental cues such as light and cold. The protein products of the potential genes regulated by LexA appear to ensure the cell can successfully respond to a dynamic light and temperature environment. A model for LexA function in *Synechocystis* is depicted in Figure 5.4. LexA regulates expression of target genes whose protein products allow cellular adaptation to the predominating environment. Superimposed upon LexA regulation of gene expression is the potential for post-transcriptional regulation by the RNA helicase, CrhR. Biochemically, the CrhR RNA helicase is capable of both RNA helicase and annealing activities (Chamot et al., 2005) and may increase or decrease translation of target RNAs as previously described. For example, a possible involvement for CrhR in D1 protein turnover will ensure continued photosynthesis. The bidirectional hydrogenase, a LexA-activated gene (Gutekunst et al., 2005; Oliveira and Lindblad, 2005), is an electron valve during photosynthesis which may prevent over reduction of the electron transport chain (Appel et al., 2000). In both scenarios, LexA regulation ensures continued maximal photosynthetic capabilities in response to changing cellular redox conditions. The preliminary bioinformatic identification of regulon members described above also invokes an expanded role for LexA in the cell. Further characterization of the LexA regulon will extend upon our initial observations regarding its physiological role in the cell.

In summary, LexA's identification as a regulator of *crhR* expression has provided interesting insights into redox-responsive gene expression in the

Figure 5.5 Physiological roles of the LexA and CrhR proteins. Under PQ reducing conditions (i.e. cold, light, glucose), LexA regulates expression of redox-responsive genes including the *crhR* gene. Expression of CrhR may allow translation of its RNA targets through unwinding and annealing of RNA secondary structures. Additionally, CrhR may ensure translation of genes regulated transcriptionally by LexA leading to two levels of regulation. Oxidation of PQ leads to LexA repression of *crhR* transcript accumulation and consequently CrhR targets cannot be translated under these conditions due to inhibitory secondary structures (adapted from Kujat and Owttrim, 2000; Chamot *et al.*, 2005).

cyanobacterium *Synechocystis* sp. strain PCC 6803. These insights have demonstrated the evolutionary divergence of the *Synechocystis* LexA-orthologue from the canonical *E. coli* LexA protein, the ability of a gene to acquire and potentially swap functional domains to better suit its physiological role and environmental niche and the interplay among different environmental cues to regulate gene expression.

5.7 References

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Chapter 6: Appendix

Table A.1 Bacterial strains used in this study

Strain	Relevant genotype	Reference	Use
Synechocystis sp. strain PCC 6803	Wild type	University of Toronto Culture Collection	Study subject
Escherichia coli DH5α	*supE44ΔlacU169(φ80lac ZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Sambrook *et al.*, 1989	cloning
Escherichia coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^q lacZ φM15]*	Sambrook *et al.*, 1989	protein overexpression
Escherichia coli XL-1 Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB⁺ lacI^q Δ(lacZ)M15] hsdR17(r_K⁻ m_K⁺)*	Stratagene	site-directed mutagenesis
crhRΔ::Sp/Sm	*crhR* mutant	This study	CrhR functional analysis
lexA::Km	*lexA⁻/lexA⁺* heterodiploid	This study	LexA functional analysis
lexAΔ::Km	*lexA⁻/lexA⁺* heterodiploid	This study	LexA functional analysis

Table A2 Plasmids used in this study

Plasmid (parent vector)	Use	Reference or source
pBSL128	source of Km^r cassette for *lexA* inactivation	Alexeyev *et al.*, 1995
pBR*crhR*Δ::Sp (pBR322)	*crhR* deletion mutant	Kujat-Choy, 2001
pBluescript KS$^+$	cloning vector	Stratagene
cs0096-9 (pBSKS$^+$)	plasmid containing the ORF, 5' and 3' untranslated regions of the *crhR* gene, the sll0080 ORF and 3' sequence of slr0082	Kujat-Choy, 2001 Kujat and Owttrim, 2000
pCrhR (IV) (pBSKS$^+$)	*in vitro* transcription/translation	this study
P53, 54 (cs0096-9)	binding site mutagenesis; site-directed mutants of the LexA binding site within the *crhR* gene	this study
P60 to 68 (cs0096-9)	binding site mutagenesis; site-directed mutants of the LexA binding site within the *crhR* gene	this study
KC-05, -10, -15, -25, -30, -45, -50, -SL, -XM (pBSKS$^+$)	*crhR* promoter deletion series	Colvin, 2002
pflipped (cs0096-9)	binding site mutagenesis; 180° rotation of the *crhR* LexA binding site	this study
P5' (cs0096-9)	binding site mutagenesis; LexA binding sequence 5' to the *Spe*I site	this study
pWM3-2	plasmid containing the ORF, 5' and 3' untranslated regions of the *crhC* gene	Chamot *et al.*, 1999
JW-8	*crhC* promoter deletion plasmid	Ward, 1999
pRSETB	protein expression vector	Invitrogen
pLexA (pRSETB)	rLexA overexpression	this study
pNLexA (pRSETB)	NLexA overexpression	this study
psll0359 (pRSETB)	rAbrB-like protein overexpression	this study
pGem-T Easy	cloning vector	Promega
pGEMLexA	parent plasmid to generate *lexA* mutants	
pGemLexA::Km	*lexA* insertion mutant	this study
pGemLexAΔ::Km	*lexA* deletion mutant	this study

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Table A3 DNA targets used in this study

EMSA target	Primers	Template	Use
KC targets	GWO-13/GWO-45	KC deletion plasmids	identification of protein binding site/ EMSAs
lexA promoter	LPF-3/LPF-6	*Synechocystis* genomic DNA	EMSAs/DNaseI footprinting
LPF4:5	LPF-4/LPF-5	*Synechocystis* genomic DNA	non-specific competitor DNA
LPF55/GWO45	LPF-55/GWO-45	*Synechocystis* genomic DNA	DNaseI footprinting- *crhR* gene
LPF56:6	LPF-55/LPF-6	*Synechocystis* genomic DNA	DNaseI footprinting- *lexA* gene
crhR binding sites mutants (53, 54, 60-68)	GWO-13/GWO-45	*crhR* binding site mutant plasmids p53, 54, 60-68	characterization of the rLexA binding site in the *crhR* gene
f (flipped)	GWO-14/GWO-45	pfcrhR	characterization of the rLexA binding site in the *crhR* gene
5'	GWO-13/GWO-45	p5'crhR	characterization of the rLexA binding site in the *crhR* gene
3'	GWO-13/GWO-45	KCSL	characterization of the rLexA binding site in the *crhR* gene
GWO	GWO-25/GWO-45	*Synechocystis* genomic DNA	rLexA oligomeric state
hox	ShopxrF/ShopxrR	*Synechocystis* genomic DNA	*hox*/rAbrB-like EMSAs