Blue-light dependent expression of *Vibrio cholerae* photolyase

by

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ABSTRACT

The photolyase/cryptochrome (phrs) family is blue-light sensing flavoproteins. Although photolyases and cryptochromes are structurally similar proteins, their functions are different depending on organisms. *V. cholerae* possess one *photolyase* and two *cryptochrome* genes. Photolyase (VcPhr) repairs UV-damaged DNA using energy from the blue light while Cry1, belongs to DASH family, responsible for the single strand DNA repair. In this thesis we wished to identify blue-light dependent expression of *phrs* genes in *V. cholerae* using Real-Time PCR. To this end, the cells were exposed to blue light and the quantities of *cry1*, *cry2*, *phr* expressions were determined. Analysis of RT-PCR results indicated that only *phr* expression was significantly increased when cells were exposed to blue light compared with the control samples. To see this effect is not due to the free-radical formation upon exposure cells to the blue-light, cells were subjected to the hydrogen peroxide and methylene blue and *phr* gene expression level was monitored by Real-Time PCR. We observed that there is no induction of *phr* gene expression compared with control genes that are induced under the free radicals. Next we wish to identify the gene that is responsible for induction of the expression level of *phr*. We performed a genome wide analysis on *V. cholerae* to detect possible blue-light receptors and analysis end up with three candidates, which are Cry1, Cry2, and VcPhr proteins. To see *phr* induction is mediated by VcPhr, Cry1 or Cry2, we first over-expressed Cry1 protein in bacteria by transferring the plasmid that carry *cry1* gene, the result indicated a significant increase of *phr* gene compared with untransformed cells under the blue light condition. However Cry2 overexpression did not affect *phr* expression under the same conditions. Also to see the effect of photolyase on its own expression, similar experiments were performed on *phr* knockout *V. cholerae*. We did not observe a significant induction of *phr* gene by blue light when there is no functional VcPhr protein in the cell. All these results suggest that both VcPhr and Cry1 are involved in the induction of *phr* expression under blue light. The present study shows blue light dependency of repair activity in *V. cholerae* and it is possible that Cry1 and VcPhr take role in induction of *phr* expression and make cells ready to repair DNA damage, caused by UV light.

ÖZET

Fotoliaz/kriptokrom ailesi mavi ışığı algılayan, flavin içeren proteinlerdir. Fotoliaz ve kriptokrom yapısal olarak benzemelerine rağmen, farklı türlerde değişik görevlere sahiptirler. *V. cholerae* bakterisi, bir *fotoliaz (phr)* ve iki *kriptokrom (cry1, cry2)* genine sahiptir. Çalışmalar Cry1 proteininin DASH ailesinde yer aldığını ve tek zincirli DNA'nın tamirinde rol aldığını göstermiştir. Yaptığımız çalışmada *V. cholerae* bakterisi farklı dozlarda mavi ışığa (10, 25, 50, 100, 200 $\mu W/cm^2$) ve kırmızı ışığa maruz bırakıldığında, *cry1*, *cry2* ve *phr* genlerinin ifadesinde ışığa bağlı artışın olup olmadığı Gerçek Zamanlı PCR metoduyla incelendi. Deneyler sonucunda *fotoliaz (phr)* ifadesi mavi ışıkla doza bağlı olarak artış gösterirken kırmızı ışığın hiçbir etkisi olmadı. Bu etkinin mavi ışıkla oluşan oksijen radikallerinden kaynaklanmadığını göstermek için besiyerine metilen mavisi ve hidrojen peroksit eklenerek *phr* ifadesine etkileri belirlendi. Sonuçlar *phr* gen ifadesine radikal oluşumunun etki etmediğini gösterdi. Mavi ışığa bağlı *phr* ifadesindeki artışın fotoliaz veya Cry1 etkisiyle mi arttığını belirlemek için *cry1* genini taşıyan pMAL-c2X plazmidi *V. cholerae* bakterisine aktarılarak proteinin fazla ifadesi sağlandı. Yapılan çalışmada Cry1 proteininin miktarının artmasıyla mavi ışığın *phr* ifadesi üzerindeki etkisinin önemli bir miktarda arttığı görüldü, fakat Cry2 proteini artırıldığında bir değişim gözlenmedi. *fotoliaz* geninin etkisini görmek için *phr* geni susturulmuş hücreler ışığa maruz bırakılarak *phr* gen ifadesi belirlendi. Sonuçta mavi ışık etkisinin *phr* geni susturulmuş hücrelerde yok olduğu belirlendi. Çalışmam, *V. cholerae* bakterisinde, DNA tamirinin mavi ışığa bağlı olduğunu, Cry1 ve VcPhr proteinlerinin *fotoliaz geninin* ekspresyonunda rol aldığı, bu sayede bakterilerin kendilerini UV nedeniyle oluşan DNA hasarına karşı hazır hale getirdiklerini göstermiştir.

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NOMENCLATURE

VcPhr - CPD photolyase protein of *V.cholerae*

Cry1 - Cryptochrome 1 protein of *V.cholerae*

Cry2 - Cryptochrome 2 protein of *V.cholerae*

phr - the gene of *Vibrio* CPD photolyase

cry1 - the gene of *Vibrio Cryptochrome 1*

cry2 - the gene of *Vibrio Cryptochrome 2*

UV - ultraviolet light

ES - enzyme- substrate complex

EP - enzyme-product complex

P - product

FAD - flavin adenine dinucleotide

MTHF - 5,10-Methylenetetrahydrofolate

8-HDF - *8*-hydroxy-*5*-deazaflavin

ROS - reactive oxygen species

Cat - catalase

Mn-SOD - Mn superoxide dismutase

Fe-SOD - Fe superoxide dismutase

Cu-Zn SOD - Cu-Zn superoxide dismutase

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

ssDNA - single stranded DNA

L(-) - dark condition

MBP – *V.cholerae* including blank pMAL vector

MBPCRY1 - *V.cholerae* including pMAL vector with *cry1* gene

MBPCRY2 - *V.cholerae* including pMAL vector with *cry2* gene

Chapter I

INTRODUCTION

Light is one of the essential environmental factors, which is utilized by biological systems mainly in energy production and in enzymatic reactions as energy. The organisms possess photoreceptors, which activation is dependent on the wavelength and intensity of light, to sense and optimize perception of light and gain information about their environment.

Photolyase/cryptochrome family uses blue light as an energy source to repair UV induced lesions on DNA, to regulate growth and development in plants and to regulate circadian rhythms in animals. These proteins have been identified in diverse organisms and have light dependent and light independent functions (2).

The genomic and biochemical studies indicated that there are three photolyase like proteins in *Vibrio cholerae*: VcPhr, Cry1 and Cry2 proteins (50). VcPhr repairs cyclobutane pyrimidine dimers on DNA in a light dependent manner and Cry1 protein is involved in repair of ssDNA (41). The other functions of photolyase/cryptochrome family members have not been identified yet.

In this study, we found that blue light increases the expression of *phr* gene, but not *cry1* and *cry2* genes. This effect was wavelength dependent and specific to only blue light. Also, the level of induction was proportional to the intensity of blue light up to a point. We investigated the effect of reactive oxygen species (ROS) to the *phr* expression, in order to answer the possibility of that *phr* is induced by blue light produced ROS. The results revealed that oxygen radical formation did not affect *phr* expression. To determine whether Cry1 and

Cry2 are responsible for this influence, these proteins were overexpressed by using Pmal-c2 vector system. Cry1 protein overexpression leads to the increase in blue-light mediated *phr* induction. The studies with *phr* knockout cells showed that phr induces its own expression. To conclude, Cry1 and VcPhr participate in blue light mediated *phr* induction.

Chapter II provides pertinent background and literature review on the function and structure of photolyase and cryptochrome proteins, members of photolyase / cryptochrome family in *Vibrio cholerae* and reactive oxygen species.

Chapter III gives information about used materials and describes methods for determination of blue light, red light and reactive oxygen species effects on expressions of target genes, for overexpression of cryptochrome proteins and for determination of light effect in *phr* knockout cells.

The results are given and discussed in Chapter IV.

The thesis is concluded with a short summary of the performed study and future research work.

Chapter II

LITERATURE REVIEW

2.1. Blue Light Photoreceptors

Organisms need to gather information about the alteration in the conditions of environment, via employment of the data they maintain in their metabolic systems to survive. Though light is the source of energy and utilized in metabolic reactions, it also leads to deleterious consequences on organisms. Optimization of light perception and protection from its hazardous effects are significant points of survival, therefore diverse organisms possess photoreceptors involved in the sensing of and response to light.

To date, many types of photoreceptors have been discovered and grouped in bacteria, plants, animals and other organisms. They are basically classified with respect to their light-absorbing chromophores, which are activated by specific wavelength interval of light. The principle of photoreception is that light photons alter the conformation of chromophore, successively; signal transduction chain is triggered to generate a response (1).

Photoreceptors have had conserved role in all living organisms over time, however have also gained diverse functions in different organisms. Two photoreceptors from different families are able to be involved in the same process, which means one organism could possess dissimilar photoreceptors (1). The complexity of function and structure of photoreception systems and their evolution history still remain to be solved. Thanks to biochemical, genomic and phylogenetic investigations, we are able to classify the photoreceptor proteins into a limited number of families. Since I investigated the function and expressions of photolyase like proteins, I will focus on blue light photoreceptors at this point.

Most organisms possess photoreceptor proteins to sense blue light (350 – 450 nm) and respond via activation of signal transduction. Blue light photoreceptors contain flavin in the form of FAD (Flavin Adenine Nucleotide) or FMN (Flavin Mononucleotide) as chromophore. Action spectrum of these proteins is identical to the absorption spectrum of flavin, that functions as the cofactor of blue light photoreceptors. Second chromophore is involved in the structure of certain blue light photoreceptors in addition to flavin.

The functions of the blue-light photoreceptors are different in various organisms. Followings are the examples for the function of photoreceptors: repair of damaged DNA, seedling, de-etiolation, pigment production, flowering, entrainment of circadian clock, regulation of transcription, phototropism, chloroplast relocation, stomatal opening, progression of sexual cycle, perception of daily changes and phototaxis (Table 2.1) (1).

2.1.1. Photolyase / Cryptochrome Family

DNA photolyases and cryptochromes have structure resemblance and share the common ancestor albeit functional similarities. They both use blue-light energy to perform their function; however in some cases it is possible that they function as a light independent manner. Photolyases repair UV formed pyrimidine dimers via transfer of blue light photon energy to damaged DNA and blue light stimulated cryptochromes activate signaling cascades in diverse organisms (3). Photolyases also stimulate, light independently, nucleotide excision repair system which is responsible for removal of all types of DNA lesions in prokaryotes and eukaryotes (4,5,6). Cryptochromes synchronize circadian clock by acting as light-independent inhibitors in animals (7).

Table 2.1. General information of blue light photoreceptors (1)

Flavin-Containing Blue Light Photoreceptors					
Receptor	**chromophore extension**	**C-terminal activity**	**photolyase**	**DNA binding**	**Reference**
CPD Photolyase	FAD MTHF or HDF	No	Yes	Yes	[2,17]
(6-4) Photolyase	FAD MTHF or HDF	No	Yes	Yes	[2,17]
Plant Cry	FAD and MTHF	Yes	No	No	[2,18,19]
Animal Cry	FAD and MTHF	Yes	No	Yes	[2,18,19]
Cry-DASH	FAD 2nd chromophore may be distinct or absent	No	ssDNA repair activity	nonspecific	[20,21,22]
Phototropin	FMN				[23,24]
ZTL,LKP2,FKF1	FMN				[25]
VIVID	FMN or FAD				[26]
BLUF domain containing proteins	FAD				[27]

2.1.1.1. Photolyase

Far UV (200-300 nm) leads to the production of pyrimidine dimers in DNA, causing growth delay and death as a result of mutation. DNA photolyase reverses UV-induced harmful lesions by utilizing 350-450 nm light as an energy source or as a cosubstrate (8), that is called photoreactivation (9,10). There are two types of DNA photolyase: cyclobutane pyrimidine dimer (CPD) photolyase and pyrimidine-pyrimidone (6-4) photolyase, that are respectively specific to cyclobutane pyrimidine dimers (Pyr<>Pyr) and pyrimidine-pyrimidone (6-4) photoproduct (Pyr[6-4]Pyr) (Figure 2.1). 75% of UV-induced DNA lesions

are cyclobutane pyrimidine dimers (CPDs) (11). The enzyme, that repairs one, is not able to repair the other; however both have similar sequences, structures and reaction mechanisms (2).

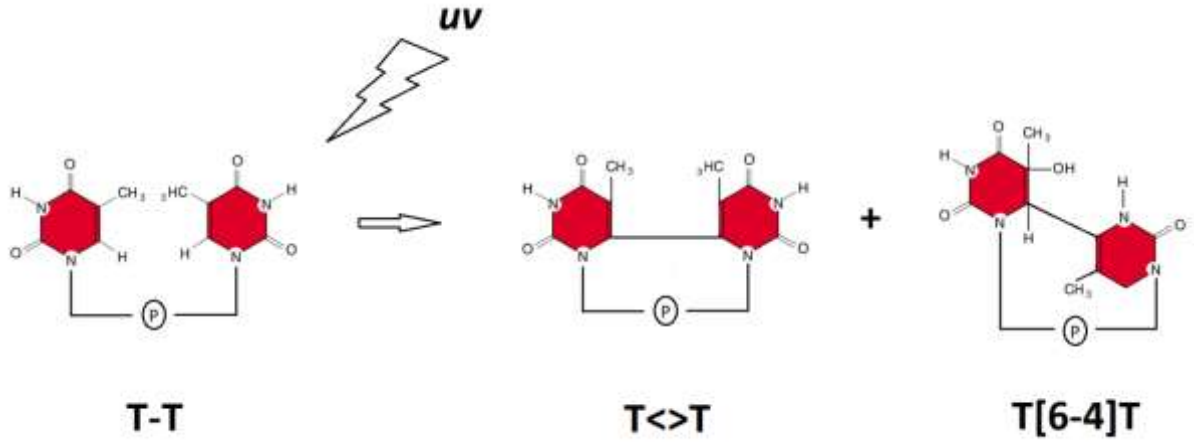


Figure 2.1. The UV induced lesions: cyclobutane pyrimidine dimers (Pyr<>Pyr) and pyrimidine-pyrimidone (6-4) photoproduct (Pyr[6-4]Pyr) (2)

Photolyases, monomeric proteins of 450-550 amino acids, have an amino terminal homology region (PHR region), where chromophores bind non-covalently. These cofactors are involved in binding to damaged DNA, catalysis and light absorption (1,2). The first chromophore is always FAD (Flavin Adenine Nucleotide), which is responsible for specifically binding to UV induced lesions and catalysis (12,13). The second chromophore is either MTHF (methenyltetrahydrofolate) or 8-HDF (8-hydroxy-7,8-didemethyl-5-deazariboflavin), that both increase DNA repair activity 10-100 fold under limiting light conditions. The photolyases are classified according to the type of second chromophore: folate class and deazaflavin class (2).

The homology of photolyases is ranging from %15 to %70 or more sequence identity Folate and deazaflavin classes possess highest degree of homology at 150 amino acids of C-

terminal (PHR domain) (4). Microbial photolyases have less homology to animal and plant photolyases (14,15). The phylogenetic analysis of photolyases exhibited there is also connection with other nucleotide binding proteins including class I aminoacyl-tRNA synthetases, electron-transport flavoproteins and photolyases (16).

To date, crystal structures of several photolyases have been determined. Owing to low sequence identity between some members and containing different types of second chromophores, most photolyases possess the same basic structure (28,29). *E.coli* photolyase, the member of folate class, has globular shape and includes N-terminal α/β-domain (residues 1-131) and C-terminal α-helical domain (residues 204-472). An interdomain loop (residues 132-203) wraps around the α/β-domain to connect two domains. MTHF domain is between two domains in a shallow cleft. FAD cofactor and α-helical domain interact with each other by 14 amino acids (Figure 2.2) (30).

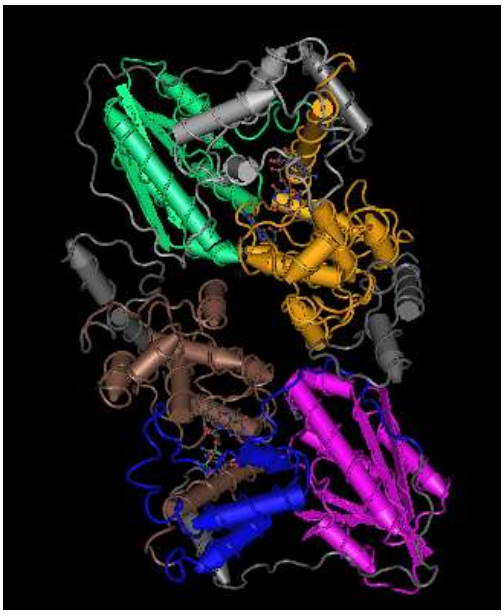


Figure 2.2. The structure of *E. coli* deoxyribopyrimidine photolyase (28)

The repair of UV lesions by photolyase can be divided into two steps: binding and catalysis. The enzyme works according to Michaelis-Menten kinetics and bisubstrate ordered sequential mechanism. It binds DNA in a light independent manner in order to form ES complex, ES changes to EP by catalysis, then P dissociates. ES→EP step is light dependent (2). Figure 2.3 describes the reaction mechanism of the enzyme: the enzyme binds to pyrimidine dimer in DNA, it flips the dimer out of the double helix in order to make stable interaction between dimer and active site cavity of enzyme. The second chromophore (MTHF or 8-HDF) absorbs a near-UV/blue light photon, then the excitation energy, formed by the effect of photon, is transferred to flavin by FRET (Fluorescence Resonance Energy Transfer), then to Pyr<>Pyr. The electron transfer results the reversal of dimer into two pyrimidines. The electron is transferred back to FADH° form in order to regenerate the $FADH^-$ form (2). I will give detailed information about the chromophores in photolyase/cryptochrome family in further sections.

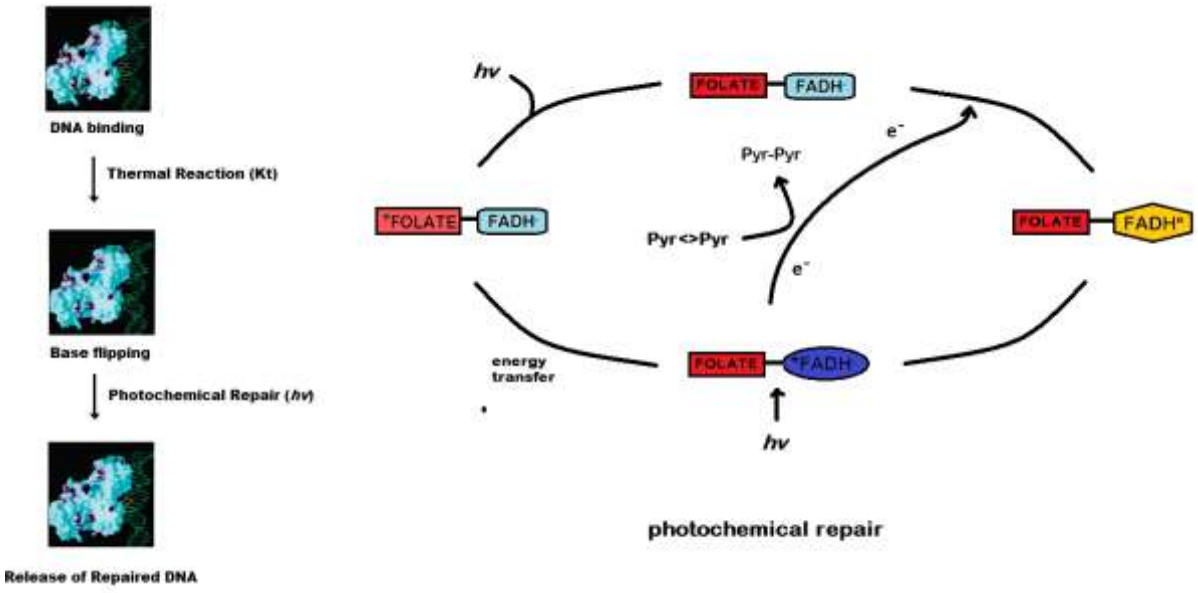


Figure 2.3. The reaction mechanism of photolyase (2)

The formation of pyrimidine dimers alters the structure of the duplex, which is bended by 30° toward the major groove and unwinded by 9° (2). The change in the structure is recognized by photolyase and ionic interactions which occur between the positively charged groove on the surface of the enzyme and first phosphate 5′ and the three phosphates 3′ of T<>T. Photolyase also interacts with the backbone of the complementary strand across from the dimer (31). These interactions lead to flipping out of the T<>T into the active site cavity to make stable complex, therefore the electron is transferred more efficiently during catalysis (28). Photolyase can recognize and bind to thymine dimers on single-stranded, double-stranded DNAs and also short oligonucleotides in order to repair efficiently (32). The overall reaction mechanism is also valid for (6-4) photolyase. The structure of substrate (6-4 photoproduct) and the second chromophore (8-HDF) are the differences (30).

In addition to activation of the enzyme, blue light also induces the expression of photolyases. The transcription of *CsPHR* (Cucumber CPD photolyase gene) is activated by light via cis acting element in a wavelength-dependent manner (33). Moreover, in *Xenopus* A6 cells, *(6-4) photolyase* expression is increased by blue-light, however it does not influence *CPD photolyase* expression (34). CPD photolyase in cultured cells of goldfish *Carassius auratus* is also transcriptionally activated by blue light (35). The question of by which mechanism blue light increase the expression of *photolyase* still has not been answered thoroughly. However, the study of *Trichoderma atrovi*s reveals that PHR1 induces its own expression (36). Moreover, although under stress conditions the genes of cellular defense proteins are induced, that is called SOS response, the amount of photolyase proteins in the cell is not altered (37). The transcription of *PHR1* gene of *S. cerevisiae* is activated by DNA damaging agents (38). Also, the findings about the regulation of *phrA* gene of *Rhodobacter*

sphaeroides by singlet oxygen and hydrogen peroxide in a σ^E-dependent manner (39) point the possibility that the radical formation via blue light is able to induce *photolyase* activation. However, it is observed that hydrogen peroxide and photosensitizer, phenol red, do not have any effect on *6-4 photolyase* transcription (34).

2.1.1.2. Cryptochrome

Cryptochromes were first isolated and characterized from Arabidopsis Thaliana (40). Later, the protein was identified in bacteria, animals and different species of plants (Figure 2.4). Although they are structurally similar to DNA photolyases, they have no repair activity except Cry-DASH proteins (41) and involve FAD and folate as cofactors (42). They contain Carboxy-Terminal Extension (CCT domain) (50-250 amino acids) in addition to PHR domain (Figure 2.4)(43). There is low similarity in this region between cryptochromes and also Cry-DASH does not contain C-terminal extension (1).

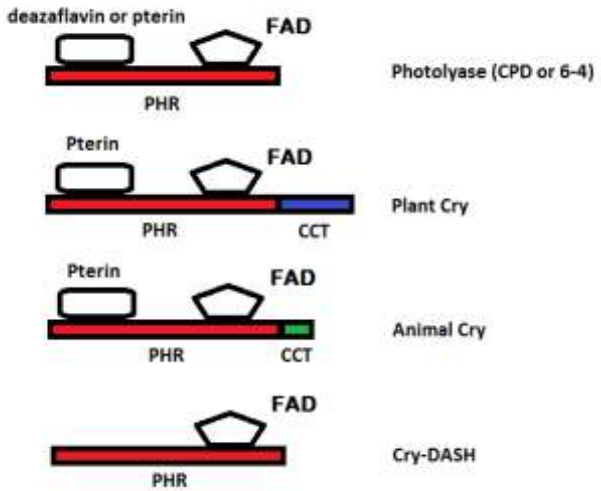


Figure 2.4. Schematic representation of functional domains in photolyase/cryptochrome family (1)

The photochemical mechanism of cryptochrome is unknown. AtCry1 and AtCry2 of *A.thaliana* mediate blue-light dependent inhibition of hypocotyls elongation, cotyledon expansion, chlorophyll, and anthocyanin production and transcription regulation (43). DmCRY sets the circadian clock in *D. melanogaster* via being activated by blue-light (44). Moreover, Cry1 and Cry2 act as transcription factor for the regulation circadian clock in a light-independent manner in mammals (45). In *Synechocystis and Arabidopsis*, CRY-DASH proteins bind DNA and are proposed to serve as transcriptional repressors (20,22). *Aspergillus nidulans* photolyase-like CryA regulates sexual development (46).

2.1.1.3. Photolyase / Cryptochrome Family Proteins in *V. cholerae*

Vibrio cholerae causes cholera disease by colonizing in the small intestine and producing an enterotoxin (51,52). During transit to human, this organism is exposed to UV, therefore it is expected that it has an efficient photorepair system to eliminate harmful consequences of this stress.

The sequencing project of *V. cholerae El Tor N16961* strain revealed there are three genes belonging to photolyase/cryptochrome family: two of them are cryptochromes (VcCry1 and VcCry2) and the other is CPD photolyase (VcPhr) (50). Later, it was reported that VcCry1 is a member of Cry-DASH family (20,53). Moreover, it was purified with excess amounts of RNA, which means that it might be an RNA-specific photolyase (50). However, previous experiments indicated that Cry1 had specificity for cyclobutane pyrimidine dimers in ssDNA instead of RNA and dsDNA (41). Although cryptochromes are accepted as blue light photoreceptors, there are no photochemical and biochemical data to prove this hypothesis. However, Cry1 was purified with flavin in two-electron reduced form, that means this protein

carries out its function by light-dependent mechanism as photolyase. VcCry1 and Cry2 contain MTHF as a second chromophore in addition to FAD (50). Owing to the genetic and biochemical information, the function of photolyase/cryptochrome family members in *V. cholerae* remains unknown.

Chapter III

MATERIALS & METHODS

3.1. Light Experiment

Single colony was picked using a pipetman with sterile tip. The tip was inoculated with colony into 2 ml LB Broth & streptomycin (100 µg/ml) and grown overnight at 37ºC. The tube was folded in aluminium foil not to be subjected to light. Note that, to enable dark conditions, the experiment was done at room under yellow light. 20 ml LB Broth & streptomycin (100 µg/ml) was inoculated with 200 µl of an overnight culture of cells at dark room. The flask was folded in aluminium foil and the cells were grown to A_{600}: 0.6-0.8 at dark at room temperature. The cells were harvested by centrifugation at 4000 x g for 5 minutes at 25 ºC and the supernatant was discarded. The cells were suspended in 20 ml 1X PBS Buffer. 3 ml of culture in 1X PBS Buffer were added into plates, which contain sterile cylindrical magnetic stirring bars (20mm x 8mm). One of plates was folded in aluminium foil to use as a dark control. The cells were exposed to different doses of blue light (10, 25, 50, 100, 200 $\mu W/cm^2$) for 15 minutes. During blue light exposure, the cells were mixed by stirring bars at 200 rpm on magnetic stirrer. The intensity of blue light was measured by UVmeter at 354 nm. The intensity at 254 nm was also determined to be sure that whether the cells were subjected to UV light. Also, thick glass was put between light source and cells to prevent UV exposure.

To determine radical formation effect, the cells were exposed to 100 µM hydrogen peroxide at dark. Also, 1 µM methylene blue was added to the culture under blue light to

produce singlet oxygen. In the wavelength specificity experiments, the cells were exposed to 200 μW/cm^2 red light for 15 minutes.

3.2. RNA Isolation

Following light exposure, 1.5 ml of culture in 1X PBS was transferred to Eppendorf tube for RNA isolation and centrifuged at 14800 x rpm for 1 min under dark conditions. The supernatant was discarded and 1 ml TRIZOL Reagent was added onto pellet. The pellet was dissolved in TRIZOL Reagent by pipetting and incubated at room temperature for 15 min. Then, 200 μl chloroform was added, mixed by inverting tubes 10 times and incubated 3 min at room temperature. The sample was centrifuged for 15 min at 12000 x rpm, 4ºC. The supernatant was transferred to fresh Eppendorf tube. After addition of 500 μl isopropanol, the sample was incubated for 10 min on ice, then centrifuged for 10 min at 12000 x rpm, 4ºC. The supernatant was discarded and the pellet was washed with 500 μl 70% ethanol by centrifugation for 5 min at 12000 x rpm, 4ºC. The pellet was dried on air for 10 min, then, dissolved in 50 μl DEPC-treated dH_2O. The sample was incubated at 65ºC for 10 min, then stored at -20ºC. RNA stability was detected on native % 1 agarose gel. The concentration was measured by NanoDrop Spectrophotometer.

3.3. cDNA Synthesis

RevertAid^{TM} First Strand cDNA Synthesis Kit was used to convert RNA to cDNA in order to use in Real-Time PCR. 1 μg of RNA sample was mixed with 1 μl random hexamer primer in nuclease-free tube on ice, and added DEPC-treated water to 12 μl (first mix). The mix was incubated at 65ºC for 5 min, then chilled on ice. Second mix [4 μl 5X reaction buffer, 1 μl Ribolock^{TM}RNAse Inhibitor (20 u/μl), 2 μl 10 mM dNTP mix, 1 μl RevertAid^{TM} M-MuLV Reverse Transcriptase (200 u/μl)] was prepared in the indicated order and added to

first mix. The sample was incubated for 5 min at 25ºC followed by 60 min at 42ºC. The reaction was terminated by heating at 70ºC for 5 min. The sample was stored at -20ºC.

3.4. Real-Time PCR

Roche Light Cycler 1.5 Instrument was used for quantification and analysis of expressions by monitoring fluorescence (530 nm) during amplification. Product characterization was done by melting curve analysis. The PCR reaction mixture was prepared as in Table 3.1. The primers, being used to amplify the region to determine the desired gene expressions, were given in Table 3.2. The Real-Time PCR details were given in Figure 3.1. During analysis, *gapdh* gene was used as a control.

Table 3.1. Real-Time PCR Reaction Mixture

Reagents	***volume***
SYBR Premix ExTag (2X)	*10* µl
PCR Forward-Reverse Primer Mix (4 pmol)	*5* µl
Template	*2* µl
dH_2O	*3* µl
Total	*20* µl

Table 3.2. Real-Time PCR primers

Primer	**Sequences**
Cry1	*5'-ACTGTTTCGCTTCTCTGGGA* -3'
	5'-AACCCGTTTGATTCAGTTGC -3'
Cry2	*5'-GCAGAGTAAAGGGGAGGTGTTG* -3'
	5'-GGAAACTCATGCCAGACGAT -3'
Phr	*5'-GGTGTGATGGACAGCCCTAT* -3'
	5'-GGTTACTTCTCTTTCCTGCTTATG -3'
Gapdh	*5'-GAAAACCTGTTGAAATGACCGTG* -3'
	5'-ACCGCTTTTTGCAAGTCAGT -3'
Cat	*5'-CCAGTTGCCTTGTTCGGTAT* -3'
	5'-GATCCAAAAATTGCAGCGTT-3'
Fe-SOD	*5'-CTTCCTTATGCGAAAGACGC*-3'
	5'-AAGTGTGGTTCCAAACCTGC -3'
Mn-SOD	*5'-TAGGCCACTGAGCGAGATTT* -3'
	5'-TGCTCAGTTGACCTTGTTGC -3'
Cu-Zn SOD	*5'-CACCCAAGTGATTGTCGTTG* -3'
	5'-TGACTGTGGTGATGACGGAT -3'

LightCycler Software 4.0

Programs

Program Name	Cycles	Analysis Mode
Denaturation	1	None
amplification	35	Quantification
melting	1	Melting Curves
cooling	1	None

Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:03:00	20	0	0	0	None

Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:00:02	20	0	0	0	None
61	00:00:10	20	0	0	0	None
72	00:00:20	20	0	0	0	Single

Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode
95	00:00:00	20	0	0	0	None
60	00:00:00	20	0	0	0	None
95	00:00:00	0.2	0	0	0	Continuous

Target (°C)	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Sec Target (°C)	Step Size (°C)	Step Delay (cycles)	Acquisition Mode
40	00:00:30	20	0	0	0	None

Figure 3.1. The details of Real-Time PCR Program

3.5. Transformation

PMAL-c2 vector was used to increase the amount of Cry1 and Cry2 proteins in *Vibrio cholerae*. PMAL-c2-Cry1 and PMAL-c2-Cry2 constructs were obtained from previous studies. The vectors were transformed by electroporation into the electrocompetent cells.

The electroporation was done by modifying the protocol by Nickoloff (60). The cells of host strain were incubated with shaking at 37°C, 200 rpm overnight in 2 ml LB Broth & streptomycin (100 µg/ml). 1 ml of overnight culture was inoculated into 50 ml LB Broth & streptomycin (100 µg/ml). The cells were grown to A_{600}: 0.6 with shaking 37°C, 200 rpm. The culture was chilled on ice for 20 min and transferred to 50 ml Falcon tube. Then cells were collected by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was discarded and the collected cells were washed with 40 ml of ice-cold Sucrose buffer. The cells were washed with 20 ml ice-cold Sucrose buffer two times. The washed cells were re-

suspended in 1 ml of cold Sucrose buffer and the concentrated suspension was divided into 100 µl aliquots. The aliquots were frozen by the use of liquid nitrogen and stored at -80°C. For electroporation, the frozen cells thawed on ice and 0.5 µg of pMAL-c2 vector were mixed with 100 µl of the competent cells. The mixture was placed on ice for 15 min. The mixture was transferred to a chilled BIORAD cuvette with 0.cm electrode gap. The samples were pulsed with a time constant of 25 ms (25 µF capacitance, 1000 Ω) at 1.5-1.75 kV (field strength, 7.5-8.75 kV/cm). The sample was diluted in 0.9 ml of pre-warmed SOC medium and incubated at 37°C for 60 min. Then the sample was plated on LB Broth Agar & streptomycin (100 µg/ml) & ampicillin (100 µg/ml). The plates were incubated at 37°C overnight.

Chapter IV

RESULTS

4.1. The increase of *phr* expression by the effect of blue-light

To determine the blue light effect on the expressions of photolyase/cryptochrome family in *V. cholerae,* cells were exposed to 100 μW/cm^2 of blue light for 15 min along with control which covered with aluminum foil. After the exposure of cells to the blue-light (300-420 nm), they were precipitated by centrifugation and total RNA was extracted and then converted into the cDNA. Then samples were subjected to the Real-Time analysis. The level of the *cry1, cry2, phr* and *gapdh,* (as a reference gene) expressions in light and dark conditions were determined as follow. First, the ratio of *gapdh* level was normalized to 1.0 with values that were taken from both dark and light conditions. Then the levels of *phr, cry1* and *cry2* expressions were normalized with respect to the normalized *gapdh*. The results revealed that *phr* expression was increased by the effect of blue light (Figure 4.1). However, there was not any significant change in the expression level of *cry1* and *cry2* genes. Next we investigated expression level of phr against to different doses of blue light (10, 25, 50, 100, 200 μW/cm^2). As can be seen in Figure 4.2, *phr* expression level was greatly increased at moderate amount of the blue-light and this increase saturated at 50 μW/cm^2.

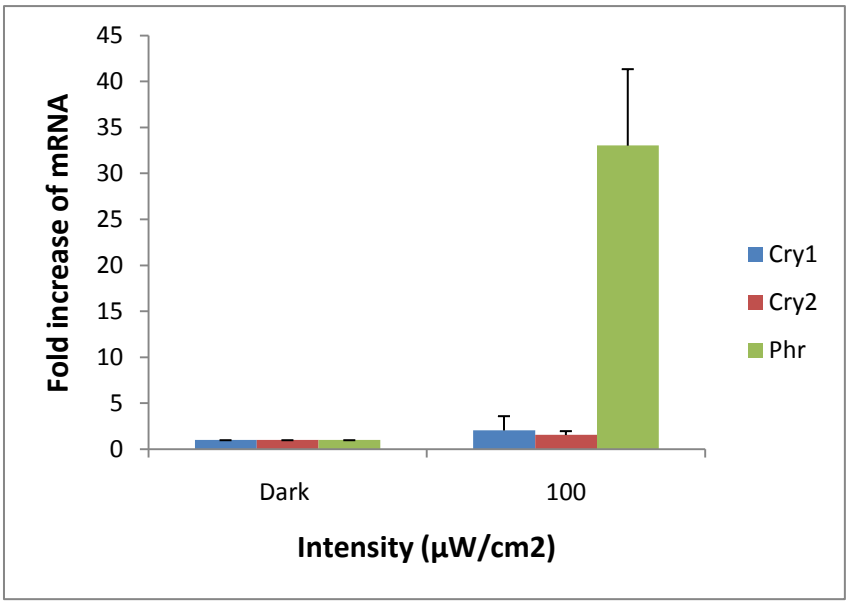


Figure 4.1. Real-Time PCR analysis of blue light effect on the expressions of *cry1*, *cry2* and *phr* genes. Values are expressed as means ± SD for each group.

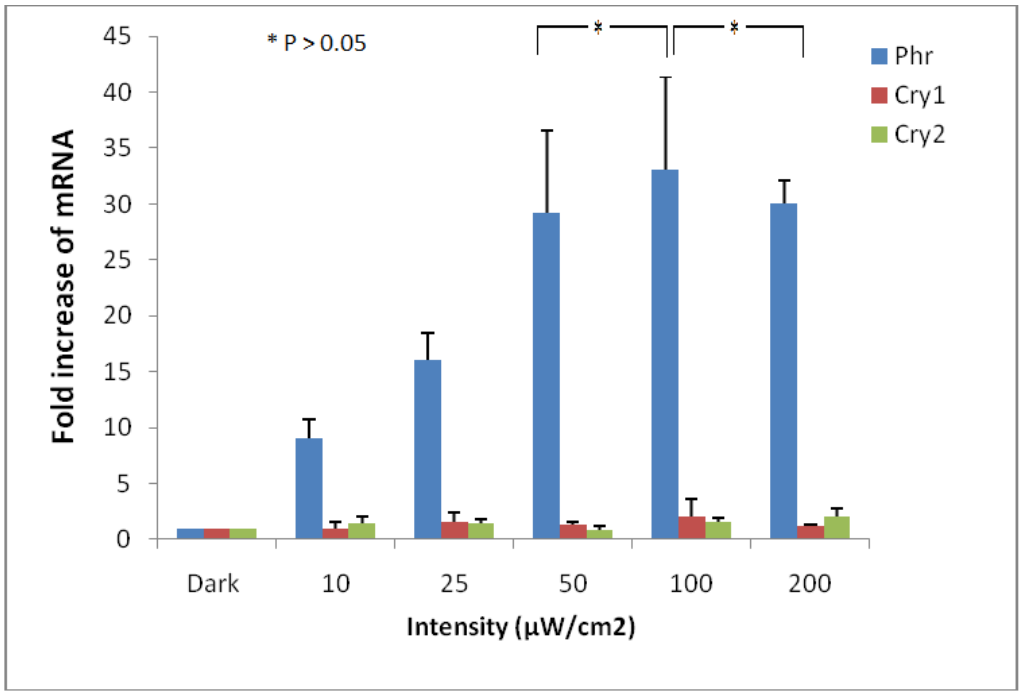


Figure 4.2. Real-Time PCR analysis of dose dependent blue light effect on the expressions of *cry1*, *cry2* and *phr* genes. Values are expressed as means ± SD for each group.

To confirm this observation is mediated specifically by blue-light, we performed same experiments under the red light (650 nm) conditions. The cells were exposed to 200 µW/cm² red light for 15 min along with the proper control. cDNAs were isolated and used in the Real-Time analysis. None of genes expression levels that we tested were altered under this condition (Figure 4.3). Specifically only phr gene expression was increased upon exposure cells to the blue-light, other genes *cry1* and *cry2* expression levels didn't change upon exposure cells either blue or red-light (Figure 4.3). Therefore, it can be concluded that the enhanced transcriptional expression of photolyase is achieved by blue light.

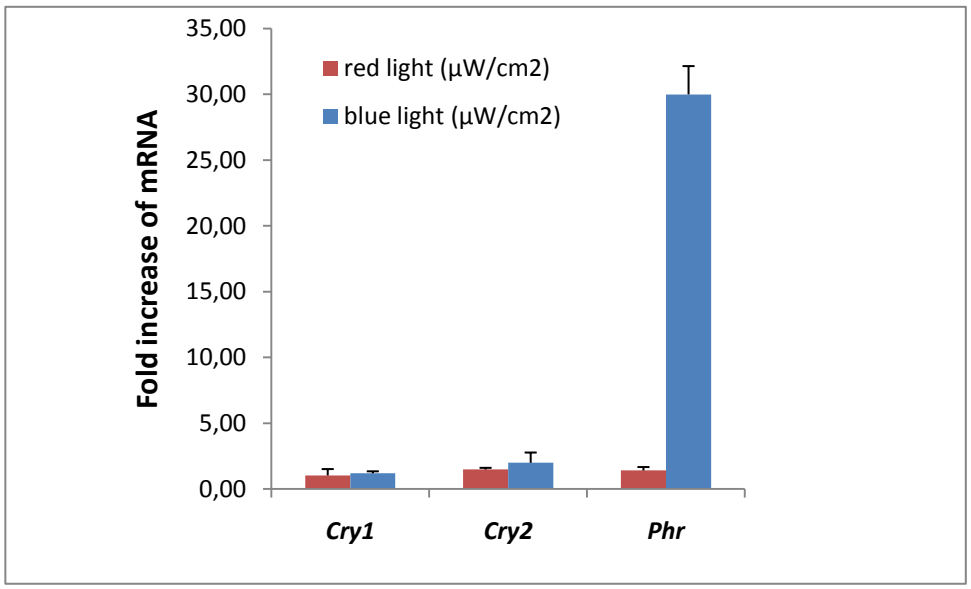


Figure 4.3. Real-Time PCR analysis of red light effect on the expressions of *cry1*, *cry2* and *phr* genes. Values are expressed as means ± SD for each group.

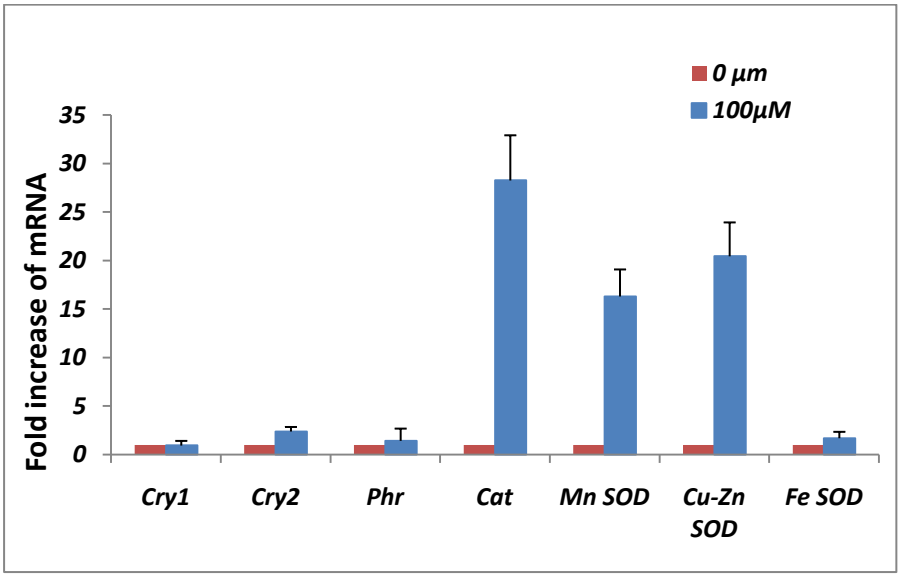
Previous studies have shown that blue light can indeed increase free radical formations and these free radicals effect transcription of *phr* expression in the cell (57). In next section we will perform experiments to see induction of *phr* expression a result of the free radical or solely from blue-light.

4.2. The effects of free radicals on *phr* induction

Molecular oxygen (O_2) and light energy, captured by organisms, are utilized in many key biological processes. However, the reactive byproducts, the outcome of the use of molecular oxygen in bioenergenetic or metabolic pathways, are toxic to cells and blue light constitutes one of the main sources of oxygen radical formation. Many organisms contain defense mechanisms to eliminate the hazardous consequences of these reactive oxygen species (ROS)(57). Therefore, blue light mediated radical formation might lead to *phr* induction to take precaution against DNA damage or to eliminate pyrimidine dimers on DNA. To determine the role of oxygen radicals in *phr* transcription, hydrogen peroxide and singlet oxygen were produced in cells and *phr* expression level was investigated.

The cells were treated with 100 μM hydrogen peroxide for 15 min at dark. *cry1, cry2, phr* and also *gapdh*, (as a reference gene) expressions were determined by Real-Time PCR. As seen in Fig 4.4A, the expression levels of the *phr* genes were not affected with treatment of H_2O_2. To prove the radical formation occurred via H_2O_2 in vivo we have also measured the level of radical scavenging genes which their levels are increased with exposure of the H_2O_2 (58,59). The expressions of *catalase (cat), Mn-superoxide dismutase (Mn-SOD), Cu-Zn superoxide dismutase (Cu-Zn SOD)* and *Fe-superoxide dismutase (Fe-SOD)* were significantly enhanced in H_2O_2 treated cells (Figure 4.4A). The induction of *cat, Mn-SOD* and *Cu-Zn SOD* genes showed that hydrogen peroxide exposure leads to formation of reactive oxygen species; however *phr* transcription was not activated, which means that *phr* activation is not influenced by radical formation in *V.cholerae.* In addition, catalase expression was determined by Real-Time PCR at different doses (50, 100, 200 μW/cm²) of blue light exposed cells to check the effect of blue light on free-radical formation in vivo. Our results indicated that blue light did not induce catalase expression, which show the doses that we used throughout the experiments is not sufficient to produce free radicals in vivo (Figure 4.4B).

A)



B)

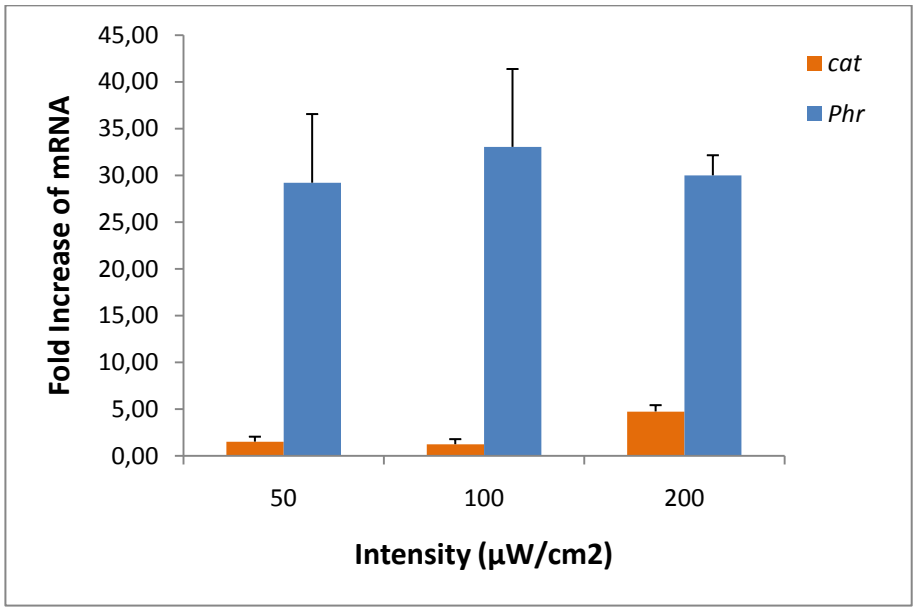


Figure 4.4. Real-Time PCR analysis of hydrogen peroxide effect on the expressions of *cry1*, *cry2* and *phr* genes (A) and determination of radical formation when cells were exposed to the doses of 50, 100, 200 µW/cm² blue light (B). Values are expressed as means ± SD for each group.

Hydrogen peroxide, superoxide dismutase and hydroxyl radicals can transmute to each other and formed via electron transfer, though singlet oxygen is produced by energy transfer to molecular oxygen (57). Therefore, we investigated the effect of the singlet oxygen separately. In order to determine whether blue light leads to the *phr* induction via production of singlet oxygen, the cells were treated with 1µM methylene blue under blue light (50 µW/cm²). Singlet oxygen did not bring about an increase in *phr* expression level (Figure 4.5). Thus, blue light generated transcriptional activation of *phr* gene does not result from radical formation.

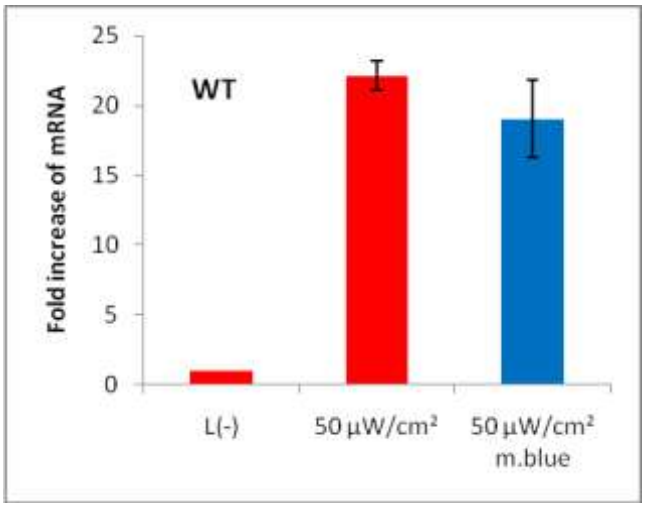


Figure 4.5. Real-Time PCR analysis of singlet oxygen effect on the expression of *phr* gene (L(-):dark). Values are expressed as means ± SD for each group

4.3. The role of Cry1 in blue-light mediated *phr* induction

Our results revealed that *phr* expression was induced dose dependently by blue light, not by radical formation or red light, indicating that at least one blue-light photoreceptor participates in the transcription of *phr* gene. Genome analysis of *Vibrio cholerae* suggested that there are only three blue-light sensing proteins: Cry1, Cry2 and VcPhr.

To determine whether cryptochromes are responsible for such induction of *phr* expression, bacterial expression plasmids that carry Cry1 (Cry-DASH) and Cry2 proteins were transferred and overexpressed in *V.cholerae.* Genes are in a plasmid (pMAL-c2) where they fused with maltose binding protein (MBP). The cells with blank pMAL-c2 vector were used as a control to eliminate the stress of the vector presence. Cells that contain pMAL-c2, pMAL-c2-Cry1 and pMAL-c2-Cry2 were exposed to 10µW/cm² blue light for 15 min. Real time analysis showed that there was significant increase in the induction of *phr* gene in the cells that carry pMAL-c2-Cry1; there was no significant increase in VcPhr mRNA level in the cells carry pMAL-c2-Cry2 (Figure 4.6) upon exposure cells to the blue-light.

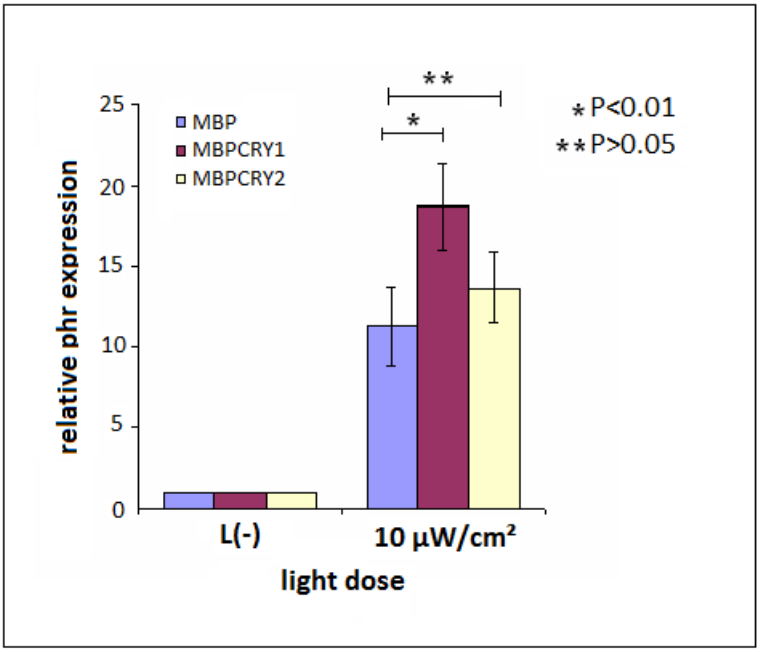


Figure 4.6. Real-Time PCR analysis *phr* induction on cells that carry plasmids that contains Cry1 and Cry2genes upon exposure to the blue light. (MBP: *V.cholerae* with pMAL vector; MBPCRY1: *V.cholerae* with pMAL-Cry1 vector; MBPCRY2: *V.cholerae* with pMAL-Cry2 vector) (L(-):dark) Values are expressed as means ± SD for each group

To make sure the both recombinant *cry1* and *cry2* gene were expressed in comparable level we carried out both Western Blot and RT-PCR. As can be seen in Figure 4.7-A,B, the mRNA level of both recombinant genes are significantly elevated, which are the indication of the recombinant genes. To confirm the presences of the recombinant protein we have carried out the Western Blot analysis on the cells using the MBP antibody. There were comparable amount of the both Cry1 and Cry2 proteins in the cells (Figure 4.7C)

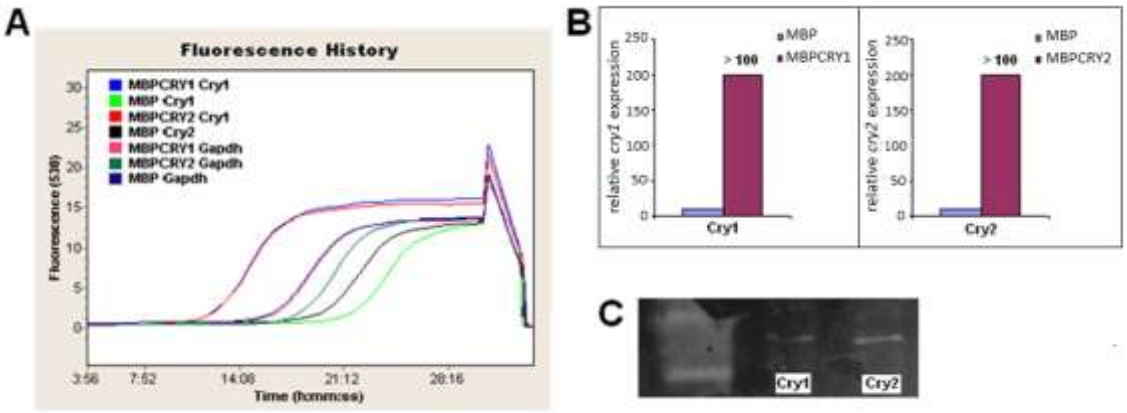


Figure 4.7. Real-Time PCR analysis of the effect of Cry1 and Cry2 overexpression on the blue light-dependent induction of *phr* gene. A-B) Real-Time PCR results of Cry1 and Cry2 expression and C) Western Blotting using the total proteins from the cells overexpressing the *crys* genes

4.4. The effect of photolyase to its own expression

To find out whether photolyase participates in this own induction by the effect of the blue light, we have performed a similar study on the *phr* mutant *V.cholerae*. The *phr* mutant cells were exposed to 50 μW/cm² blue light for 15 min. *phr* and also *gapdh*, (as a reference gene) expressions were determined by Real-Time PCR. The effect of blue light on *phr* expression was significantly alleviated in *phr* expression level of mutant cells and the

decrease was resulted from the high levels of phr expression of mutant cells at dark when it is compared with wild type cells. (Figure 4.7).

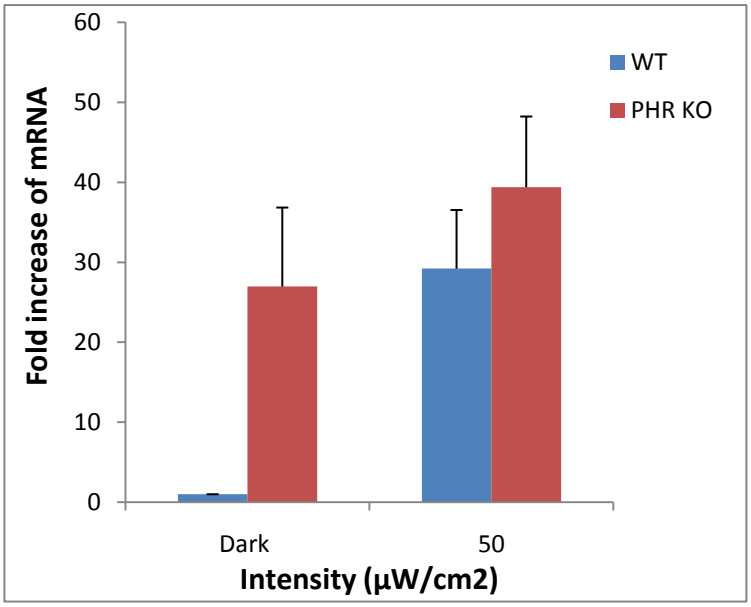


Figure 4.8. Determination of *phr* expression in *phr* mutant cells (PHR KO) by Real-Time PCR (L(-):dark). Values are expressed as means ± SD for each group

In order to investigate whether blue light lead to the *phr* induction via singlet oxygen, the *phr* knockout cells were also tested by treating with 1µM methylene blue under blue light (50 µW/cm²). The excess amounts of singlet oxygen in *phr* knockout cells did not increase *phr* expression in agreement with the results of wild type cells (Figure 4.9).

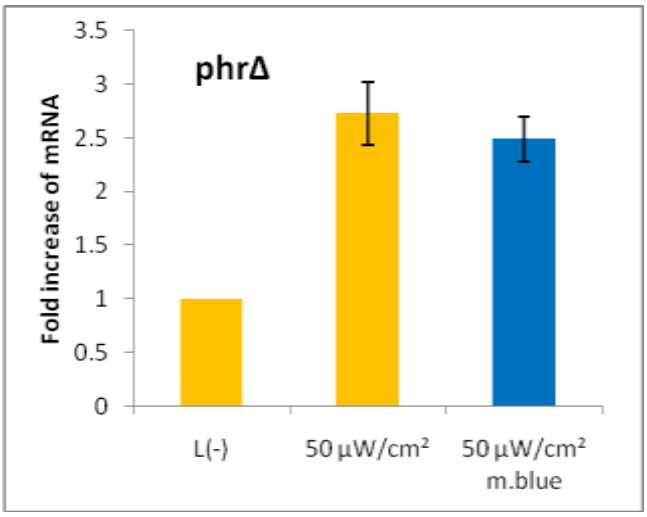


Figure 4.9. Real-Time PCR analysis of singlet oxygen effect on the expression of *phr* gene at *phr* knockout cells (phrΔ). (L(-):dark) Values are expressed as means ± SD for each group

Chapter V

DISCUSSION

Genomic and biochemical studies led to the discovery of three photolyase like proteins in *V.cholerae*: cryptochrome1 (Cry1), cryptochrome 2 (Cry2) and CPD photolyase (VcPhr) (50). It has been known that VcPhr repairs UV induced cyclobutane pyrimidine dimers on DNA by the use of blue light as an energy source (2). Subsequent studies found that Cry1 is a member of Cry-DASH family (20,53) and has blue-light dependent ssDNA repair activity (41). In addition to impact on activity, we found that blue light also induces photolyase expression in *V. cholerae.* This effect is dependent on the dose of blue-light; however intensity of blue light is significant up to a point, then the effect is saturated. We also investigated red light effect on *phr* expression to determine whether *phr* induction is wavelength specific or not. The results indicate that *photolyase* expression is induced only by blue-light.

The utilization of molecular oxygen (O_2) in biological processes leads to the production of reactive two oxygen species (ROS). ROS was originated by two different ways: electron transfer (type I) and energy transfer (type II) (54,55). Superoxide, hydrogen peroxide and hydroxyl radicals are created by transfer of electron to O_2. These reactive oxygen species are toxic to cells and can be converted to each other by metabolic reactions. Singlet oxygen, the most reactive oxygen species, is produced via the energy transfer to molecular oxygen. Superoxide and hydrogen peroxide damage proteins, through oxidation (54,55), whereas

hydroxyl radical brings about damage on DNA, that might be mutagenic and lethal. Singlet oxygen undergoes chemical reaction with myriads of cellular components, including membranes, proteins and DNA (54,56). Organisms mounted rapid response mechanisms against the lethal consequences of ROS to survive. Cells activate the transcription of genes, that are involved in conversion of oxygen radicals to nonhazardous species (57). Divergent oxygen radicals stimulate different response mechanisms in the organisms, though hydrogen peroxide, superoxide dismutase and hydroxyl radicals can transmute to each other. Therefore, singlet oxygen and other reactive species should be evaluated separately. The findings about the regulation of *phrA* gene of *Rhodobacter sphaeroides* by singlet oxygen and hydrogen peroxide in a σ^E-dependent manner (39) pointed the possibility that the radical formation via blue light is able to induce *photolyase* activation in *V. cholerae*. However, our results indicate that hydrogen peroxide and singlet oxygen did not lead to *phr* induction, indicating that increase in *phr* transcription results from blue light, not from blue light produced radical formation.

Our results indicate that the blue light effect on photolyase expression is specific to only blue light and results from radical formation. Therefore, we reached the solution that at least one blue light photoreceptors are involved in blue-light mediated *phr* induction. The sequencing project, genomic and biochemical analysis determined that *V. cholerae* possesses three blue-light sensing proteins: Cry1, Cry2 and Phr.

Cry-DASH proteins function as transcriptional regulators in particular organisms (20,22). Cry1 is the member of Cry-DASH protein family and was purified with FAD in the two electron reduced form, indicating that this protein mediate photo-induced reactions and has light dependent functions (40). Our study revealed that the excess amounts of Cry1 significantly increased the blue-light mediated *phr* induction. The finding indicates the role of

Cry1 protein in the regulation of *phr* expression; however Cry2 seems not participate. The increase in blue light mediated *phr* expression by the overproduction of Cry1 could be only supporting evidence. Blue light effect on Cry1 and Cry2 mutant cells will be required to be sure about the roles of these proteins in blue-light mediated photolyase expression. How cryptochrome might contribute to blue-light dependent *phr* expression has not been found out yet. One possibility is that it might directly bind to the promoter region of *phr*; however, binding to this site could not be detected by Gel Shift Assay (data not shown). Considering these results, we could conclude that Cry1 participates in the mechanism by interacting with other proteins, which are responsible for *phr* expression. Also, Cry1 might increase the RNA stability in a light dependent manner.

The study of *Trichoderma atrovi*s revealed that PHR1 induces its own expression (36). Therefore, photolyase might be involved in its own induction by blue-light in *V. cholerae*. The exogenous *phr* expression from vector did not let us to analyze the overexpression effect on phr expression by Real Time PCR. So, we tested the effect of blue-light in *phr* mutant cells. We reached the solution that blue light dependent *phr* expression is nearly eliminated in *phr* mutant cells. At dark, the *phr* expression level of mutant cells is higher than wild type cells. This data reveals that the difference in phr expression when cells are exposed to blue light is resulted from the suppression of its own expression by photolyase at dark. Photolyase takes role as a repressor of its own expression. It is likely that VcPhr and Cry1 proteins participate together in blue light mediated *phr* expression. Whether VcPhr binds to its promoter region remains to be determined. The gel shift assay exhibited no specific affinity to its promoter region (data not shown). However, both Cry1 and photolyase proteins exhibit high nonspecific binding affinities to DNA, it is possible that binding to promoter region could not be detected by in vitro assays. ChIP (chromatin immunoprecipitation), in

vivo technique, could be an alternative to determine the binding of Cry1 and VcPhr to the promoter region of *phr* gene.

Chapter VI

CONCLUSION

Photolyase and cryptochromes are structurally similar proteins; however, they differ in function in the cell depending on the organisms. Light dependent and independent functions of photolyase like proteins have been reported.

Vibrio cholerae possesses VcPhr, Cry1 and Cry2 proteins. VcPhr repairs UV induced lesions on DNA and Cry1 has repair activity of ssDNA proteins. Both utilizes blue light photons as an energy source in their activity. This thesis study determines the effect of blue-light on the expressions of photolyase in V. cholerae and indicates that Cry1 and VcPhr participate together in blue-light effect on photolyase expression.

In this study, we observed that photolyase gene was induced when the cells were exposed to blue light. To determine whether this effect is specific to blue-light, red light effect on *phr* expression was analyzed; however no influence was reported. To see the effect of radical formation on *phr* expression, hydrogen peroxide and singlet oxygen effect were determined. The results indicated that blue-light dependent expression did not result from radical formation.

Vibrio cholerae genome sequencing project revealed that there are only three blue light sensing proteins: VcPhr, Cry1 and Cry2. Therefore, only three candidates that might play role in blue light mediated *phr* induction. We overexpressed Cry1 and Cry2 in cells by using vector system and observed that Cry1 overexpression increased the blue light effect on *phr* transcription, supporting that Cry1 participates in blue-light regulated *phr* expression. To

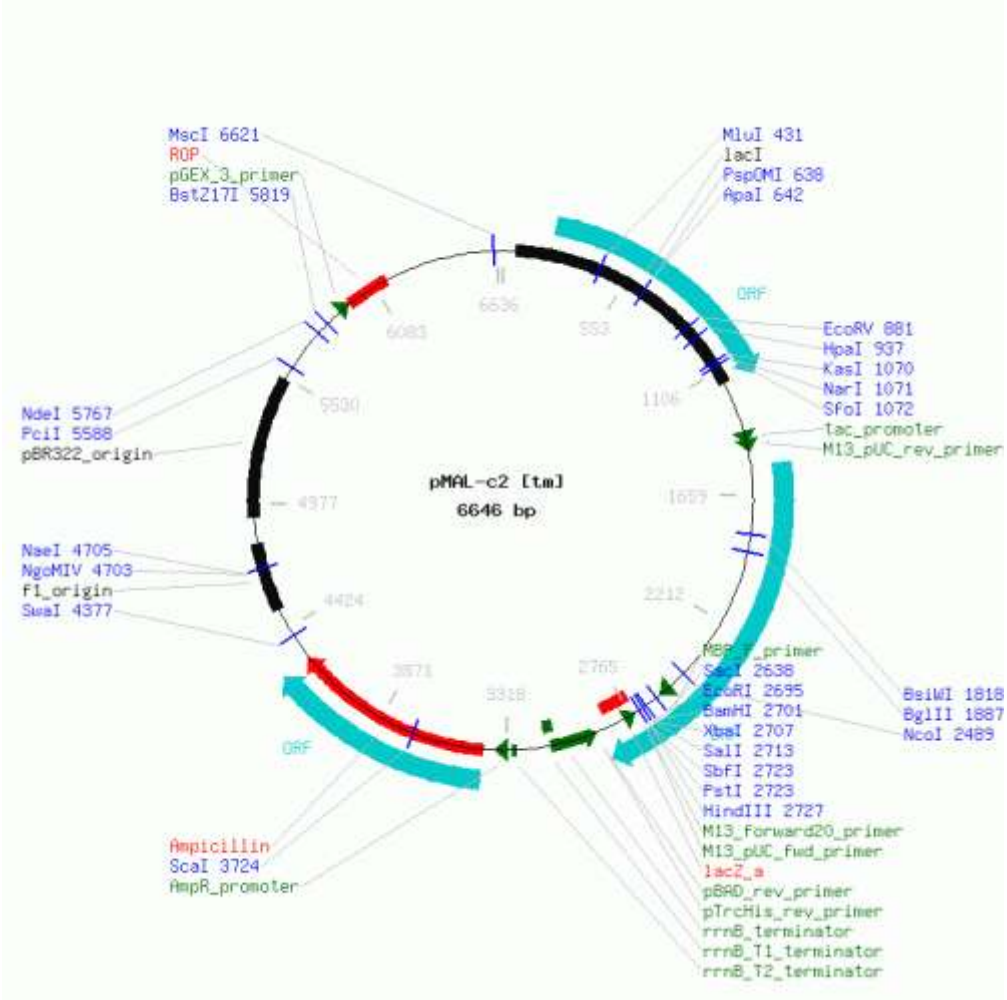
determine the role of VcPhr on its own induction, we investigated blue light effect in *phr* knockout. The blue light effect nearly was eliminated owing to the high levels of phr expression of mutant cells at dark. Therefore, photolyase takes role as a blue light inducible suppressor of its own expression

This study indicates the point that blue light induces *phr* expression; this effect does not result from blue-light produced radical formation. Cry1 and VcPhr are involved in blue-light mediated *phr* transcription.

APPENDIX

Appendix A.

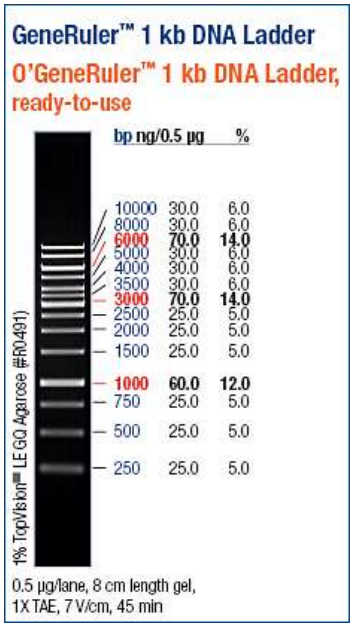
Vector map of pMAL-c2



The vector includes maltose-binding protein (MBP) fusions, where the protein of interest can be cleaved from MBP with the specific protease Factor Xa. Because the *mal*E gene on this vector is deleted for the signal sequence, the fusion protein produced remains in the cytoplasm It was purchased from New England Biolabs®

Appendix B.

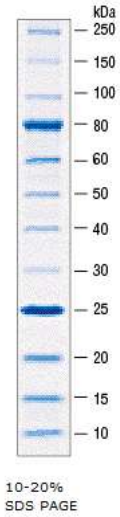
DNA Molecular Weight Marker



The ladder is a mixture of chromatography-purified individual DNA fragments.

Appendix C.

Protein Molecular Weight Marker



Protein Ladder is a mixture of 12 recombinant, highly purified proteins, which resolve into clearly identifiable sharp bands from 10-250 kDa when analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250

Appendix D.

Lab Equipments

Real-Time PCR	: Roche LightCycler 1.5
Autoclaves	**:** CL-40S/SDP (60L) ALP autoclave
Centrifuges	**:** 4K15, Sigma Laboratory
	: Microfuge 14-15, Sigma Laboratory
Deep freezes and refrigerators	**:** Heto Polar Bear 4410 ultra freezer, JOUAN Nordic A/S, catalog# 003431.
	: 2021 D deep freezer, Arcelik.
	: 1061 M refrigerator, Arcelik.
Electrophoresis equipments	**:** E-C Mini Cell Primo EC320, E-C Apparatus.
	: Mini-PROTEAN 3 Cell and Single-Row AnyGel Stand, Catalog# 165-3321,Bio-Rad.
Gel documentation system	**:** UVIpro GAS7000, UVItec Limited.
Ice Machine	**:** AF 10, Scotsman.
Shaker	**:** Innova 4300 incubator shaker
Magnetic stirrer	**:** Heidolph MR 3001
Pipettes	**:** Pipetteman P10, P 100, P1000, Eppendorf
pH meter	**:** Inolab pH level 1, order# 1A10-1113,
Power supply	**:** PowerPac Basic (300V,400mA,75W) Biorad
Pure water systems	**:** DV25 PureLab Option ELGA
Spectrophotometer	**:** W-1700 PharmaSpec, Shimadzu Corporation.
Illuminator	**:** Sankyo Denki Blacklight Blue (352 nm)

Light intensity measurer : UVX radiometer (364 nm, 254 nm)

Vortexing machine **:** Reax Top, Heidolph2.2.

Appendix E.

Bacterial Strains

***E. coli* DH5α host strain**

F- φ80lacZΔ (lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1/F' proAB+ laclqZ 15 Tn10

***Vibrio cholerae* O1 biovar eltor str. N16961 strain**

General: Gram negative, γ-Proteobacterium, (str^R),

Taxonomy: (Taxon ID:243277) Kingdom: Bacteria; Intermediate Rank 1:Proteobacteria; Intermediate Rank 2: Gammaproteobacteria; Intermediate Rank 3:Vibrionales; Intermediate Rank 4: Vibrionaceae; Intermediate Rank 5:Vibrio.

Genome: The annotated genome sequence and the gene family alignments are available at http://www.tigr.org/tbd/mdb. The information about the genome is provided at http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gvc. The sequences have been deposited in GenBank with accession number AE003852 (chromosome 1) and AE003853 (chromosome 2)

V. cholerae 01 eltor was obtained from Dr. Fitnat Yıldız (University of California, Santa Cruz, CA).

Appendix F.

Enzymes

RevertAid™ M-MuLV Reverse Transcriptase

The enzyme was obtained in RevertAid™ First cDNA Synthesis Kit from Fermentas (catalog # K1621)

3.1.3.2. Ribolock™RNAse Inhibitor

The enzyme was obtained in RevertAid™ First cDNA Synthesis Kit from Fermentas (catalog # K1621)

Appendix G.

Media and Solutions

LB (Luria-Bertani) Media

20 g LB Broth, Lennox Powder (Fischer Scientific) were dissolved in distilled water up to 1 lt and sterilized for 15 min. under 1.5 atm at 121°C.

LB Agar Medium

20 g LB Broth, Lennox Powder (Fischer Scientific) and 15 g agar were dissolved in distilled water up to 1 lt and sterilized by autoclaving.

SOC Medium

2 g Bacto-tryptone, 0.5 g Yeast Extract were dissolved in distilled water and 200 µl 5M NaCl, 250 µl 1M KCl, 1 ml 1M $MgCl_2$, 4 ml 0.5 M $MgSO_4$ and distilled water was added up to 99 ml. Then, 1 ml 40% glucose was added after sterilization by autoclave.

Streptomycin Stock

100 mg / ml of tetracycline was dissolved in distilled water and stored at 4°C

Ampicillin Stock

100 mg / ml of tetracycline was dissolved in distilled water and stored at 4°C

Kanamycin Stock

50 mg / ml of kanamycin was dissolved in distilled water and stored at 4°C

TRIZOL Reagent

The Reagent was obtained from Invitrogen (catalog # 15596-026)

10X PBS Buffer

80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 were dissolved in 800 ml of distilled water, then the pH was adjusted to 7.4 with HCl. Distilled water was added up to 1 lt.

Sucrose Buffer

272 mM sucrose was dissolved in distilled water and %15 glycerol was added, then sterilized by autoclaving.

5X TBE Buffer

4.6 g disodium EDTA, 54 g Trizma Base and 27 g Boric Acid were dissolved in distilled water up to 1 lt.

TE Buffer

10 ml 1M Tris-Cl (pH 7.5) and 2 ml 500mM EDTA pH 8.0 were dissolved in distilled water up to 1 lt.

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