Molecular characterization of a protein kinase involved in extraordinary radioresistance of *Deinococcus radiodurans*

By

Yogendra Singh Rajpurohit

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Recommendations of the Doctoral committee

As members of the Viva Voce Board, we certify that we have read the dissertation prepared by **Yogendra Singh Rajpurohit** entitled "**Molecular characterization of a protein kinase involved in extraordinary radioresistance of *Deinococcus radiodurans***" and recommend that it may be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Chairman-	Dr. S. K. Apte	Date:	10 / 09 / 2012
Member 1-	Dr. A. K. Sharma	Date:	10 / 09 / 2012
Member 2-	Dr. (Mrs) Malini Krishna	Date:	10 / 09 / 2012
External Examiner	Dr. J. Gowrishankar	Date:	10 / 09 / 2012

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Research Guide- Dr. H. S. Misra 10/9/2012 Date: 10 / 09 / 2012

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DECLARATION

I, hereby declare that the investigations presented in the thesis have been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution/University.

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DEDICATED TO MY

PARENTS AND

FAMILY

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Yogendra Singh Rajpurohit,
MBD, BARC

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ABBREVIATIONS

Pyrroloquinoline-quinine	PQQ
Double strand breaks	DSBs
D.radiodurans	DEIRA
Ionizing radiation	IR
Single strand breaks	SSBs
Escherichia coli	*E. coli*
Single-strand binding	SSB
Colony forming units per milliliter	cfu/ml
Far-ultraviolet radiation	FUV
Near-ultraviolet radiation	NUV
UV damage endonuclease	UVDE
Methylmethanesulphonic acid	MMS
Mitomycin C	MMC
Extended synthesis-dependent strand annealing	ESDSA
DNA damage response A	DdrA
Post irradiation recovery	PIR
Two component systems	TCS
2-Dimensional electrophoresis	2-DE
Pulsed field gel electrophoresis	PFGE
Counts per minute	CPM
DNA binding proteins	DBPs
Polyacryamide gel electrophoresis	PAGE
Isoelectric focusing	IEF
Cell free extract	CFE
Ataxia telangiectasia mutated	ATM
Ataxia telangiectasia and Rad3-related protein	ATR
Mre11/Rad50/ Nbs1	MRN
Protein kinase A	PkA

SYNOPSIS OF THE THESIS TO BE SUBMITTED TO THE HOMI BHABHA NATIONAL INSTITUTE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN LIFE SCIENCES

Name of the Candidate	Yogendra Singh Rajpurohit
Name of the Guide	Dr. H. S. Misra Molecular Biology Division Bhabha Atomic Research Centre & Homi Bhabha National Institute, Mumbai - 400094, INDIA
Registration Number	LIFE01200704002
Date of the registration	3th September, 2007
Title of the thesis	Molecular characterization of a protein kinase involved in extraordinary radioresistance of *Deinococcus radiodurans*.
Place of the Research Work	Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400 085, India.
Signature of the Candidate	
Signature of the Guide	

Thesis title: Molecular characterization of a protein kinase involved in extraordinary radioresistance of *Deinococcus radiodurans*

Deinococcus radiodurans R1 a radioresistant bacterium, exhibits extreme tolerance to the lethal and mutagenic effects of DNA damaging agents including radiations and desiccation (Makarova *et al.,* 2001). *D. radiodurans* can tolerate nearly 200 DNA double strand breaks (DSB) and 3000 single strand breaks per genome (Battista, 2000). A proficient DSB repair and a strong oxidative stress tolerance are amongst the mechanisms that have been attributed to its extreme phenotypes (Blasius *et al.,* 2008; Slade and Radman, 2011). It exhibits an efficient biphasic DSB repair (Daly *et al.,* 1994; Daly and Minton, 1996), which gets accomplished by two distinct mechanisms like extended synthesis dependent strand annealing (ESDSA) and a slow process of maturation by homologous recombination (Zahradka *et al.,* 2006, Blasius *et al.,* 2009). The stability and protection of shattered genome from nucleolytic degradation, recession of protected ends by deprotecting proteins and synthesis as well as assembling of various DNA repair proteins in macromolecular complexes are some of the essential pre-requisites for efficient DSB repair of this bacterium. Although, the molecular mechanisms supporting these pre-requisites are not known, the γ radiation induced changes the expression of a large number of genes and recycling to proteomes, and the involvement of two DNA protecting proteins such as PprA (Narumi *et al.*, 2004) and DdrA (Harris *et al.*, 2004) in radiation resistance, have been shown in this bacterium. Surprisingly, this bacterium does not display the typical SOS response mechanism that is known for controlling the SOS gene expression in response to DNA damage, as known in other bacteria. But it contains a large number of uncharacterised stress response components like eukaryotic type Serine / Threonine protein kinases (eSTPK), histidine kinases,

tyrosine kinase and response regulators. In bacteria, the histidine kinases are common stress sensor proteins, which are involved in sensing the environmental signal and for transducing it to cellular levels through the cascades of protein phosphorylation/ dephosphorylation The signal transduction using two-component system, a simplest form of signaling mechanisms has been known in bacteria (Parkinson, 1993). It is composed of a sensor protein kinase and a response regulator where the activation of protein kinases and phosphorylation of response regulator bring about a systematic change in regulation of gene expression. While the existence of DNA damage response in form of SOS response is known in bacteria, the DNA damage linking to signal transduction have not been reported in prokaryotes.. The Ser / Thr type protein kinases (STPKs) are less abundant in prokaryotes. *D. radiodurans* genome also encodes PQQ synthase (*pqqE*) an enzyme that regulate PQQ synthesis in bacteria. Recently, the roles of pyrroloquinoline-quinone (PQQ) as an antioxidant *in vitro* and in the regulation of oxidative stress tolerance in both bacteria and mammalian systems have been demonstrated (He *et al.,* 2003; Misra *et al.,* 2004). PQQ is also shown to stimulate the autokinase activity of a periplasmic protein kinase (YfgL) that has a role in UVC resistance in *Escherichia coli* (Khairnar *et al*., 2007), and various other signaling protein kinases in eukarytoes (reviewed in Rucker *et al.,* 2009). Unlike eukaryotic system, the involvement of protein kinases in regulation of DNA damage response has not been studied in greater details in bacteria. However, the role of a quinoprotein kinase in higher UVC resistance in *E. coli* (Khairnar *et al.,* 2007) and phopshorylation of single stranded DNA binding protein of *Bacillus subtilis* (Mijokovic *et al.,* 2006) have been demonstrated. Existence of PQQ synthase supporting the synthesis of PQQ as inducer of STPKs, and a large number of STPKs and other sensor kinases in *D. radiodurans* makes this bacterium a good model system for

studying the roles of STPKs in bacterial response to γ radiation and other DNA damaging agents at cellular and molecular levels.

The present study aimed to investigate the functional significance of pyrroloquinoline- quinone (PQQ) as an inducer of protein kinase, and possible roles of hypothetical quinoproteins including a putative quinoprotein kinase, in the radiation resistance and DSB repair of *D. radiodurans.* This has been accomplished through following objectives.

1. Studies on the role(s) of pyrroloquinoline-quinone, a protein kinase activity inducer in radiation resistance and DSB repair of *D. radiodurans*.

2. Studies on hypothetical proteins of *D. radiodurans* containing PQQ binding motifs, for their possible involvements in radiation resistance.

3. Functional characterization of a PQQ binding protein kinase DR2518, having a well defined eukaryotic type Ser/Thr protein kinase (eSTPK) motif and its role in radiation resistance and DSB repair of *D. radiodurans*.

The work carried out to address the above objectives, will be presented in this thesis as following chapters.

Chapter 1 General Introduction and review of literature

Chapter 2 Materials and experimental procedures

Chapter 3 Results

3.1. Studies on the role(s) of pyrroloquinoline-quinone (PQQ) and five PQQ binding motifs containing proteins in radiation resistance of *D. radiodurans*.

3.2. Functional characterization of DR2518 protein kinase having PQQ binding motifs and an eSTPK domain.

3.3. Identification of DR2518 kinase substrates and effect of protein phosphorylation on the known functions of PprA, a representative substrate

Chapter 4. Discussion

Chapter 5. Summary and **conclusions**

Chapter 1. This chapter describes the general information on *Deinococcus radiodurans*, identification of bacterium, classification, microbiological features and it's response to various DNA damaging agents. Different DNA repair pathways and various models proposed in support of efficient DSB repair in this microbe will be described. The literature related to different classes of protein kinases in bacteria, involvement of protein phosphorylation in bacterial responses to abiotic stresses and their regulatory effect on various cellular processes would be described.

Chapter 2 This chapter describes the details of materials used with their sources and common experimental methods used in this study. Different techniques and approaches used in this study will be described under the category of (i) biochemical techniques including protein purification, estimation and *in vitro* protein phosphorylation, and activity characterization at protein levels, (ii) microbiological techniques such as bacterial culture growth, treatments of *D. radiodurans* cells with different DNA damaging agents, and *in vivo* labeling of total phosphoproteins by [$^{32}PO_4$] etc., and (iii) recombinant DNA technology and molecular biology techniques including cloning, generation of deletion mutants and their confirmation, pulsed field gel electrophoresis etc. Detailed protocols of method used in transcriptome analysis of *D. radiodurans* and its derivatives grown under different conditions will be covered in this chapter.

Chapter 3 This chapter **s**tarts with preamble describing the hypotheses and logical anticipation from this study. The results obtained to test the hypotheses along with other new findings during

the course of this investigation will be summarized. Results obtained from this study have been presented in four sections. Each starts with a brief introduction, experimental procedures and results obtained from this specific study, followed by a brief discussion on the findings specific to results included in this section.

3.1 Studies on the role(s) of pyrroloquinoline-quinone (PQQ) and five proteins containing PQQ binding motifs, in radiation resistance of *D. radiodurans*

Disruption mutant of *pqqE* (*pqqE:nptII*) encoding PQQ synthase was generated and the possibility of this gene regulating the synthesis of PQQ was ascertained. Cell free extract from mutant showed the absence of a peak with retention time of 3.29 min on C18 column as against of 3.27 min observed for standard PQQ. This suggested that *pqqE* possibly regulates the synthesis of PQQ in this bacterium. The transgenic *E. coli* expressing *pqqE* gene from *D. radiodurans* showed functional complementation of a PQQ mediated phenotype i.e. mineral phosphate solubilization and improved oxidative stress tolerance (Khairnar *et al.*, 2003). This indicated that unlike wild type, the *pqqE:nptII* mutant did not make PQQ and hence checked for its response to various DNA damaging agents. The *pqqE* mutant showed nearly 3-log cycle decrease in survival to γ radiation at 10 kGy dose and differential sensitivity to other DNA damaging agents like hydrogen peroxide, mitomycin C. This mutant did not show any effect of UVC radiation. These cells exhibited delayed kinetics in DSB assembly during PIR. Mutant cells took nearly 24h to achieve the wild type levels of DSB repair seen in 4h PIR. These results suggested that PQQ plays an important role in radiation resistance of *D. radiodurans*. The molecular mechanisms underlying PQQ function was further investigated.

The PQQ binding motif search analysis (http://smart.embl-heidelberg.de/) showed that the *dr0503, dr0766, dr1769, dr2518* and *drc0015* genes encode uncharacterized proteins having multiple PQQ binding motifs. Amongst these, the DR2518 contains a well-characterized eukaryotic type Ser/Thr kinase (STK) domain while DR1769 contains a relatively less defined signature of kinase domain. To ascertain the involvement of these genes in radiation resistance of *D. radiodurans*, the deletion mutants of *dr0503, dr0766, dr1769, dr2518* and *drc0015* genes were made and effect of γ radiation on their cell survival was monitored. The levels of γ-radiation tolerance in Δ*dr1769* mutant decreased to less than 1.0 log cycle at 10kGy; while Δ*dr2518* mutant exhibited nearly 3-log cycle decreased resistance at 6kGy γ-radiation dose as compared to wild type. Other mutants showed no effect of γ radiation treatment and their tolerance to different DNA damaging agents, continued to be similar to wild type. Since, Δ*dr2518* cells showed significantly higher sensitivity to γ radiation, the *dr2518* was selected for further studies. The Δ*dr2518* cells showed decreased resistance to UV-C by 3-log cycle at 1200 Jm^{-2} UV (254nm) dose, to desiccation by 2.5 log cycle upon 14 days incubation at 5% humidity. The Δ*dr2518* mutant also showed impairment of DSB repair and disappearance of a few phosphoproteins. In order to ascertain if DR2518 also functions through PQQ, the Δ*dr2518pqqE:cat* double mutant was isolated and found to have γ-radiation resistance similar to Δ*dr2518* single mutant indicating that both PQQ and DR2518 function through common pathway(s) in conferring the γ radiation resistance in *D. radiodurans*. To ascertain that the loss of γ radiation resistance in Δ*dr2518* mutant was not due to the polar effect of this deletion but due to the loss of DR2518 enzyme *per se*, the wild type DR2518 was expressed into Δ*dr2518* mutant and γ radiation resistance was checked. The Δ*dr2518* cells expressing DR2518 showed

near to complete recovery of wild type resistance indicating that the γ radiation sensitivity in Δ*dr2518* cells was due to absence of DR2518 protein. These results suggested that amongst the five putative PQQ binding proteins of *D. radiodurans*, the DR2518 contributes maximum to the γ radiation resistance in this bacterium. Further, the DR2518 seems to be the most preferred protein through which PQQ possibly functions in radiation resistance and DSB repair of *D. radiodurans.*

3.2 Functional characterization of DR2518 protein having PQQ binding motifs and an eSTPK domain

Bioinformatics analysis revealed that the N-terminal of DR2518 contains a Hank type kinase domain normally present in eSTPKs, while C-terminal is having a sensory PQQ interacting domain. The kinase domain showed 40% identity with PknB at amino acid levels and a high degree of conservation at secondary structure levels. The DR2518 had all the conserved motifs like P-loop, Helix-C, DFG motif, and catalytic loop as known in other eSTPKs. The activation loop, which receives the phosphate during autokinase activity, has varying number of phosphate receiving residues in different members of this sub-family. To find out the subclass of DR2518 sensory C-terminal domain, the multiple sequence alignments was carried out with conserved amino acid sequences of each class of β-propeller family protein using Clustal X program. The alignment results showed that C-terminal sensor domain of DR2518 had seven WD repeats of approximately 40 amino acids that start with glycine (G) and end with tryptophan (W) in each repeat. Topology analysis (http://www.ch.embnet.org/software/TMPRED_form.html) predicted that DR2518 is an N-in, C-out membrane protein with three transmembrane domains hypothetically placing the catalytic domain in cytoplasm and the sensory C-terminal domain

possibly in periplasmic space. The SMART software (http://smart.embl-heidelberg.de/) predicted the presence of seven tandem β propeller repeat motifs at C-terminal sensory domain.

***In vivo* studies**

The effect of γ radiation on both transcription of *dr2518* gene and phosphorylation of DR2518 protein was studied. The results showed nearly 15.355 ± 0.179 fold increase in levels of *dr2518* transcript within 30 min PIR and that was maintained closely to this level till 3h PIR and then decreased to nearly unirradiated level in 24h. The phosphorylation status of DR2518 in *D. radiodurans* cells and the effect of γ-radiation on its *in vivo* phosphorylation were also determined by coimmunoprecipitation with antibodies raised against purified recombinant DR2518. Cell free extracts of radiolabeled wild type and *pqqE:nptII* mutant cells were prepared and incubated with DR2518 antibodies. Purified DR2518 showed immunoprecipitation with antibodies against this protein. The [^{32}P]-phosphoprotein signal was very low in immunoprecipitate of cell free extract from unirrradiated wild type cells, which increased to its maximum in 3h PIR and then reduces to unirradiated level in 5h PIR. This increase in phosphoprotein signal intensity in response to γ radiation was not noticed in *pqqE:nptII* mutant cells. The cells showed no change in phospho-immunoprecipitate in first 5h PIR. These findings suggested that although the DR2518 undergoes phosphorylation *in vivo* and its both transcription and phosphorylation are induced by γ radiation in wild type, the PQQ plays a critical but yet uncharacterized regulatory role in γ radiation induced autophosphorylation of DR2518 *in vivo*. Furthermore, the absence of DR2518 autophosphorylation stimulation in PQQ negative cells when exposed with γ radiation might indicate that DR2518 activity regulation by DSBs possibly requires PQQ.

***In vitro* studies**

In order to study the biochemical properties of DR2518 and the regulatory roles of C-terminal putative sensory domain, the full length (DR2518) as well as kinase domain (DR2518KD) of *dr2518* were expressed in *E. coli* and recombinant proteins were purified using nickel affinity chromatography. The phospho nature of DR2518 polypeptide and the autokinase activity of this protein were ascertained using polyclonal antibodies against phospho-Ser/Thr epitopes, by *in vitro* phosphorylation using [^{32}P] γ-ATP. Recombinant DR2518 showed cross reactivity with phospho-Thr epitopes antibodies and autophosphorylation with [^{32}P] γ-ATP. Recombinant DR2518 showed autokinase activity, which was stimulated with PQQ by 2.679 ± 0.324 fold *in solution*. This indicated the functional interaction of PQQ with DR2518. Circular Dichroism (CD) spectroscopy was employed to probe the change in secondary structure of DR2518 upon interaction with PQQ. The CD spectra of DR2518 incubated with increasing concentration of PQQ were different from DR2518 protein alone. Since PQQ alone did not show circular dichroism, a small but significant change in spectral characteristics as a function of PQQ concentration indicated physical interaction of PQQ with DR2518. Recombinant DR2518KD the protein missing C-terminal PQQ binding domain, although showed kinase activity, its stimulation by PQQ was not observed indicating the regulatory role of PQQ and its interaction through C-terminal domain of this protein. These results indicated that DR2518 is a quino-phosphoprotein kinase exhibiting the PQQ stimulated autokinase activity *in vitro*.

Since the expression of *dr2518* gene was induced at 6.5kGy γ-radiation, which produces high-density DNA strand breaks, the effect of double-stranded DNA (dsDNA) on autokinase activity of DR2518 was examined by *in vitro* phosphorylation with [^{32}P] γ-ATP. The autokinase activity of DR2518 increased by 2.134 ± 0.321 fold in presence of 1kb linear dsDNA fragment

irrespective of *in vitro* γ-radiation exposure. This indicated that DR2518 is a dsDNA inducible kinase. The stimulation of DR2518 activity by nearly 2 fold with linear plasmid DNA but not with circular DNA suggested that DR2518 activity stimulation by DNA requires DNA ends. Interestingly, the autokinase activity of DR2518KD when checked in presence of PQQ and 1kb linear dsDNA fragment, it showed activity, which did not change significantly in presence of PQQ and 1kb linear dsDNA. These results provided evidence that the C-terminal domain of DR2518 kinase plays a regulatory role in PQQ and linear dsDNA stimulation of its kinase activity at least, *in vitro*.

In order to understand the roles of amino acids located in putative activation loop and catalytic site of kinase domain, in kinase activity regulation and radiation resistance in *D. radiodurans,* the site directed mutagenesis were carried out and recombinant derivatives were produced. Activity characterization confirmed that the Lys42 in N lobe of kinase domain is essential for its kinase activity while T169 and S171 amino acids were functionally important as phosphoaccetor site as the K42A mutant was completely inactive while T169A and S171A showed partial loss of autokinase activity. These results suggested that DR2518 has a well-defined kinase domain similar to other eSTPKs and thus a kinase negative mutant of this protein was isolated. Further, the functional significance of DR2518 kinase activity *per se,* in radiation resistance of this bacterium, was monitored by functional complementation studies. The mutated K42A, T169A and S171A proteins were expressed *in trans* into Δ*dr2518* cells and the survival of these derivatives to γ radiation was monitored. Results showed that the γ-radiation resistance of transgenic Δ*dr2518* cells expressing wild type DR2518 was fully recovered, while K42A protein did not complement for loss of mutant's function. The T169A and S171A proteins supported only 40% and 60% to γ radiation resistance in Δ*dr2518* at 6kGy dose. These results suggested

that protein kinase activity of DR2518 *per se* was required for its role in radiation resistance of *D. radiodurans*.

3.3. Identification of DR2518 kinase substrates and effect of protein phosphorylation on the known functions of PprA, a representative substrate

DR2518 has been characterized as an autokinase. The possibilities of DR2518 had transkinase activity and can phosphorylate the other proteins of *D. radiodurans* were hypothesized. Using bioinformatic approaches and based on the known phospho-motifs found in protein substrates of eSTPKs, an approximately 30 proteins of *D. radiodurans* were found to have the conserved "XααααTX(X/V)$(X)I" and/or "X-X-T-Q/D/E-X/V-$-X-$" phospho-motifs, where α is an acidic residues, $ a large hydrophobic residue and X any amino acid. The notable ones include PprA, ParB1 and ParB of megaplasmid (MIB) and RecA. These proteins were checked for phosphorylation by DR2518 kinase *in solution*. Interestingly, DR2518 could phosphorylate though to different extent, the PprA, RecA, ParB1 and MIB, while phosphorylation of BSA and deinococcal-SSB (Dr-SSB) was not detected with this kinase. These results suggest that DR2518 kinase could phosphorylate these perspective substrates by its transkinase activity *in vitro*. Effect of PQQ and linear dsDNA on regulation of transkinase activity of DR2518 was checked with PprA as a representative substrate. Results showed that both PQQ and DNA fragment could increases DR2518 phosphorylation of PprA by ~3 fold. It further suggested the regulatory role of PQQ and dsDNA for its kinase activity. The effect of phosphorylation on function of PprA was further studied. Earlier, PprA was shown to protect dsDNA from exonuclease III degradation and stimulates the T4 DNA ligase (Narumi *et al.*, 2004) and *E. coli* catalase (Kota and Misra, 2006) activities *in vitro*. Therefore, the effect of PprA phosphorylation on its DNA binding activity and

T4 DNA ligase activity stimulation functions was checked *in vitro*. The dissociation constant (Kd) of phosphorylated PprA (0.348 ± 0.081 nM) was nearly 4 fold less than Kd of unphosphorylated PprA (1.766 ± 0.70). Similarly, the equilibrium dissociation constant (Ki) of unphosphorylated PprA (16.3 ± 0.672) was also nearly 4 fold lower than Ki of phosphorylated PprA (63.82 ± 0.612). This indicated that phosphorylated PprA has higher affinity to dsDNA than unphosphorylated form and the phosphorylation of PprA increases its affinity toward dsDNA. Although, the net increase in T4 DNA ligase activity by both these forms of PprA was not distinguishable, the phosphorylated PprA showed significantly higher amount of ligated products, which presumably generated through intermolecular DNA ends joining. These results suggested that phosphorylation of PprA resulted in increased DNA binding and stimulated faster rate of intermolecular ligation by T4 DNA ligase than unphosphorylated PprA. The possibility of deinococcal DNA ligase's (such as *ligA* and *ligB*) activity getting modulated by the state of PprA phosphorylation / dephosphorylation would be worth investigating independently.

Chapter 4. Discussion

Earlier studies from this laboratory have shown that *E. coli* cells expressing deinococcal-*pqqE*, a gene, which regulates the synthesis of PQQ, become more resistant to oxidative stress (Khairnar *et al.,* 2003). PQQ was subsequently shown as an antioxidant *in vitro* (Misra *et al.,* 2004) and as an inducer of a periplasmic membrane protein kinase (YfgL) in *Escherichia coli* (Khairnar *et al.,* 2007). The role of YfgL was demonstrated in radiation resistance and recombination repair of UV induced DNA damage in *E.coli* (Khairnar *et al.,* 2008). Role (s) of PQQ as an antioxidant in protection of mitochondrial functions (He *et al.,* 2003) and an inducer of eukaryotic protein kinases having roles in cellular differentiation and cancerous growth have been demonstrated

(Rucker *et al.,* 2009). Although, SOS response has been characterized as the sole DNA damage response mechanism present in most of the bacteria, the involvement of eukaryotic type Ser/Thr protein kinases in regulation of γ radiation response has not been demonstrated; perhaps because the *E. coli*, which has been extensively studied for DNA damage response mechanisms, is not known for having classical STPKs. Bacteria belonging to *Deinococcaceae* family are highly resistant to various DNA damaging agents. *D. radiodurans* R1 contains a gene for PQQ synthesis and a large number of protein kinases that include several putative eSTPKs and a large number of response regulators.

Here, we show that PQQ has a role in radiation resistance and DSB repair in *D. radiodurans*. PQQ is known as an antioxidant and its contribution in γ radiation stress could have accounted to its antioxidant roles. However, the severe effect of *pqqE* deletion on the DSB assembly during PIR, and PQQ stimulation of *E. coli* resistance to UVC that contributes very little to oxidative stress (Khairnar *et al.,* 2007), together argued for a possibility of this compound functioning in DNA strand break repair through modulation of enzymatic functions. The PQQ binding motif as defined in dehydrogenases, search showed five ORFs including DR2518, encoding polypeptides with multiple PQQ binding signatures in *D. radiodurans.* DR2518 that showed a role in higher DNA damage tolerance in *D. radiodurans* was finally characterized as a eSTPK. The kinase activity of this protein was modulated by PQQ both *in vitro* and *in vivo*. Although, linear dsDNA could stimulate its kinase activity *in vitro*, the absence of kinase activity stimulation in PQQ minus cells exposed to extensive DNA damage by lethal dose of γ radiation indicated that perhaps DNA stimulation to this protein requires its interaction with PQQ. How exactly PQQ coordinates the γ radiation induced stimulation of DR2518 activity is not understood. However, the hypothetical architecture of transmembrane domain organization indicated that DR2518

follow N-in and C-out rules. Possibly this would place the N-terminal of this protein having kinase domain, in cytoplasm and C-terminal sensory domain with PQQ binding motifs, in periplasmic space of bacterial membrane. Since PQQ is reported in periplasmic space on other bacteria, the possibility of PQQ interacting with C-terminal in periplasm and bringing about the interaction of intracellular domain for its higher kinase activity could be speculated. The possibility of intracellular domains interaction leading to the generation of DNA binding pocket cannot be ruled out in this protein. Nevertheless, this study has characterized DR2518 as a first eSTPK having roles in radiation resistance and DSB repair in any prokaryote. Recently some of the independent findings have emphasized the importance of protein phosphorylation in DNA repair and γ radiation resistance. For examples, the presence of phosphoprotein and protein kinase in multiprotein complex characterized from *D. radiodurans* (Kota & Misra, 2008), the role of DR2418, a response regulator in regulation of RecA and PprA (Wang *et al.,* 2008) and tyrosine phosphorylation of bacterial SSB protein (Mijakovic *et al.,* 2006) have been demonstrated.

The possibilities of DR2518 working through phosphorylation of some important DNA repair proteins by modulating their functions in response to γ radiation could be hypothesized. Since, DR2518 showed high levels phopshorylation of PprA, the effect of phosphorylation on PprA activity was evaluated. As expected and known in other proteins like SSB of *Bacillus subtilis* (Mijakovic *et al.,* 2006), the PprA showed both qualitative as well as quantitative improvement in its functions upon phosphorylation. In spite of having functional interaction for their activity, it may be noted that *pqqE* and *dr2518* mutants showed differential response to UVC radiation. Unlike *pqqE* mutant, *dr2518* mutant was highly sensitive to UVC radiation. Since, there are five different proteins having PQQ binding possibility, it might have greater roles beyond DR2518

kinase stimulation. Absence of both PQQ and linear dsDNA stimulation of kinase domain (DR2518KD) activity indicated the role of C-terminal sensory domain in regulation of DR2518 function. Interestingly, it was observed that levels of both *pqqE* and *dr2518* transcript increase in response to γ radiation was much higher than the levels of DNA ends and γ radiation stimulated *in vivo* phosphorylation of DR2518. Differences in the increase of *dr2518* transcription by γ radiation and DR2518 autokinase activity by DNA ends are intriguing. It could account either to the activation of this gene expression by other components of γ radiation effect or due to completely different mechanisms involved in regulation of these two processes *per se*. Similar levels of DR2518 phosphorylation stimulation by DNA ends *in vitro* and γ radiation *in vivo*, may further indicate the possibility of a threshold in the levels of autophosphorylation of this protein, which may be regulated by requirement of its transkinase activity for phosphorylation of other protein substrates *in vivo*. This study however, comprehensively presented the investigation on the roles of PQQ and PQQ interacting STPK type protein kinases in radiation resistance and DSB repair of *D. radiodurans* and has first time reported a lucid story describing the role of eSTPK in radiation resistance and DSB repair in any prokaryote.

Chapter 5. Summary and conclusion

D. radiodurans is characterized for its extraordinary resistance to γ radiation. This bacterium shows the induced transcription of numerous genes including *dr2518* and *pqqE*, and a high proteome turnover in response to γ radiation. Earlier, PQQ roles as antioxidant *in solution* and as an inducer of STPKs in eukaryotes have been reported. The roles of pyrroloquinoline quinone and the possible mechanisms of its functions in radiation resistance of *D. radiodurans* have now been reported. *D. radiodurans* genome encodes enzyme PQQ synthase (*pqqE*), which expresses

in this bacterium and *in trans* expression of it in *E. coli* did help the later to produce PQQ. The role of this enzyme in making PQQ was ascertained by molecular genetic studies followed by HPLC analysis. Loss of γ resistance and delayed DSB repair in absence of PQQ has indicated this compound role in both these phenotypes of this bacterium. *Deinococcus radiodurans's* genome contains five genes encoding hypothetical proteins with multiple PQQ binding domains. Deletion mutant of five deinococcal-genes encoding proteins having multiple PQQ binding motifs, showed differential responses to γ radiation. DR2518 deletion resulted to hypersensitivity to γ radiation and this was characterized as an eSTPK, having roles in radiation resistance and DSB repair of this bacterium. The autophosphorylation of this protein was stimulated by both PQQ and DNA ends *in solution*. The level of phosphorylation of this protein was induced in response to γ irradiation in wild type cells but not in mutant lacking PQQ suggesting the requirement of PQQ for activation of this kinase *in vivo*. *In vitro* studies on full length and N-terminal protein kinase domain of DR2518 further suggested that its C-terminal seems to be involved in PQQ and dsDNA stimulation of its kinase activity. The possibility of C-terminal domain acting as sensory domain for γ radiation effect may be speculated. Requirement of protein kinase activity of DR2518 in radiation resistance phenotype is ascertained as the kinase negative mutant had failed to complement the γ radiation resistance loss of Δ*dr2518* mutant. Subsequently it has been shown that many important DNA repair related proteins (PprA, RecA etc.) being phosphorylated by DR2518 kinase. It was found that DR2518 mediated phosphorylation of PprA as a candidate substrate could change PprA activity *in vitro*. Since, the SOS type DNA damage response mechanism has been nearly ruled out in this bacterium and its genome contains a large number of eSTPKs, the possibility of this bacterium constituting DR2518 mediated hypothetical DNA damage response mechanism might be suggested. The

phopshorylation mediated higher turnover of existing proteins in absence of their *de novo* synthesis, may be required to cope up the greater demand of their functions under PIR and DR2518 or other possible kinases contribute to this cannot be ruled out. These results suggested that kinase activity of DR2518 plays an important role in radiation resistance through a strong possibility of regulating the function of DNA repair proteins as seen for example, with PprA.

The present study has brought forth the detailed characterization of PQQ roles in radiation resistance and DSB repair, and provided evidence to suggest that PQQ functions as an inducer of a γ radiation responsive eSTPK protein kinase DR2518, in *D. radiodurans.* DR2518 comprises of N-terminal protein kinase domain and C-terminal sensory domain. DR2518 Derivative missing C-terminal domain showed protein kinase activity but no response from PQQ and dsDNA, indicating the role of C terminal domain as a regulatory domain of this kinase. Molecular studies have clearly indicated that DR2518 activity is inducible with γ radiation *in vivo* and dsDNA and PQQ *in vitro* and protein kinase activity of this protein is must for its role in γ radiation resistance in *D. radiodurans*. Results showing (i) requirement of protein kinase activity in radiation resistance, (ii) enhanced transcription as well as *in vivo* phosphorylation of DR2518 in response to γ radiation, and (iii) the stimulation of *in vitro* kinase activity by PQQ and DNA ends but requirement of PQQ for its *in vivo* increase in autophosphorylation, together suggested that DR2518 play an important roles in γ radiation resistance. Mechanistically, the possibility of this protein either directly modulating the functions through phosphorylation, and /or regulating the expression of proteins crucial for the extraordinary radiation resistance and DNA damage tolerance in *D. radiodurans,* could be suggested.

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CHAPTER 1

INTRODUCTION

Deinococcus radiodurans R1, a radioresistant bacterium, exhibits extreme tolerance to the lethal and mutagenic effects of DNA damaging agents including ionizing and non-ionizing radiations. Genome of *D. radiodurans* contains a large number of uncharacterised stress response components including eukaryotic type Ser / Thr protein kinases, sensor kinase and at least one tyrosine kinase. These features along with its extraordinary tolerance to DSBs offer *Deinococcus radiodurans* R1, a good model system for understanding the DNA damage response mechanisms at cellular and molecular levels. Earlier studies from this laboratory have shown that *E. coli* cells expressing deinococcal-*pqqE;* a gene regulates the synthesis of PQQ become more resistant to oxidative stress (Khairnar *et al.,* 2003). Recently PQQ has been shown to be an inducer of eukaryotic protein kinases having roles in cellular differentiation and cancerous growth (Rucker *et al.*, 2009) and an inducer of a periplasmic membrane protein kinase (YfgL) in *Escherichia coli*. This study for first time has characterized the gamma radiation and PQQ inducible membrane Ser/Thr type protein kinase regulating the DSB repair in *D. radiodurans.*

In this chapter, we have first discussed the general information about the involvement of protein phosphorylation in regulation of bacterial responses to abiotic stresses and their regulatory effect on various cellular processes. Different classes of protein kinases in bacteria and functional significance of protein phosphorylation / dephosphorylation in signal transduction mechanisms associated with DNA damage response mechanisms are also presented. The brief introduction of system and suitability of system is also summarized. At the end, aims and objectives of the present thesis are listed.

1.1 Protein phosphorylation- A general introduction

Protein phosphorylation is ubiquitously recognized as an important regulatory and signaling mechanism which involved in rapid and reversible modification of physio-chemical properties of a protein, triggering a number of possible consequences: change of enzyme activity, oligomerization state, interaction with other proteins, subcellular localization or half-life (Kobir *et al.,* 2011). Ser/Thr/Tyr kinases (STPKs) family is among the largest known protein superfamily (Cheek *et al.,* 2005). Members of this family have been extensively studied in *Eukarya* and are involved in regulating various cellular activities mediated by signaling cascades.

DNA damage is a relatively common event in the life of a cell and may lead to mutation, cancer, and cellular or organismic death. Damage to DNA induces several cellular responses that enable the cell either to eliminate or cope with the damage or to activate a programmed cell death process, presumably to eliminate cells with potentially catastrophic mutations. These DNA damage response reactions include: (*a*) removal of DNA damage and restoration of the continuity of the DNA duplex; (*b*) activation of a DNA damage checkpoint, which arrests cell cycle progression so as to allow for repair and prevention of the transmission of damaged or incompletely replicated chromosomes; (*c*) transcriptional response, which causes changes in the transcription profile that may be beneficial to the cell ; and (*d*) apoptosis, which eliminates heavily damaged or seriously deregulated cells (Sancar *et al.,* 2004) (Fig.1).

DNA lesions trigger the activation of various protein kinases; Phosphoinositide-3-kinase-related protein kinase (PIKK) family members ATM, ATR and DNA-Pkcs are important candidate protein kinases involved in DNA lesions induced signaling cascades. Such cascades convey information to effectors, coordinate incoming information from other signaling pathways, amplify signals, and allow for a variety of response patterns. The DNA damage checkpoints

employ damage sensor proteins, such as ATM, ATR, the Rad17-RFC complex, and the 9-1-1 complex, to detect DNA damage and to initiate signal transduction cascades that employ Chk1 and Chk2 Ser/Thr kinases and Cdc25 phosphatases. The signal transducers activate p53 and inactivate cyclin-dependent kinases to inhibit cell cycle progression from G1 to S (the G1/S checkpoint), DNA replication (the intra-S checkpoint), or G2 to mitosis (the G2/M checkpoint) (Sancar *et al.*, 2004) (Fig.2).

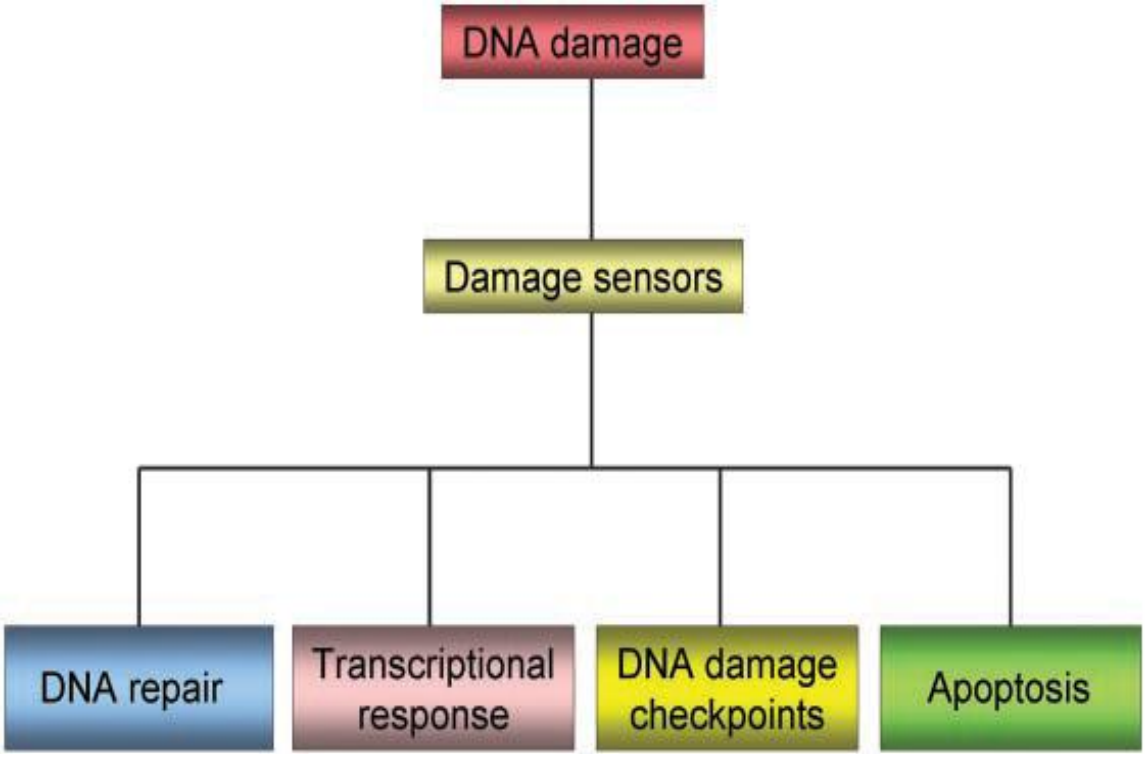


Fig.1 DNA damage response reactions in mammalian cells (Sancar *et al.*, 2004)

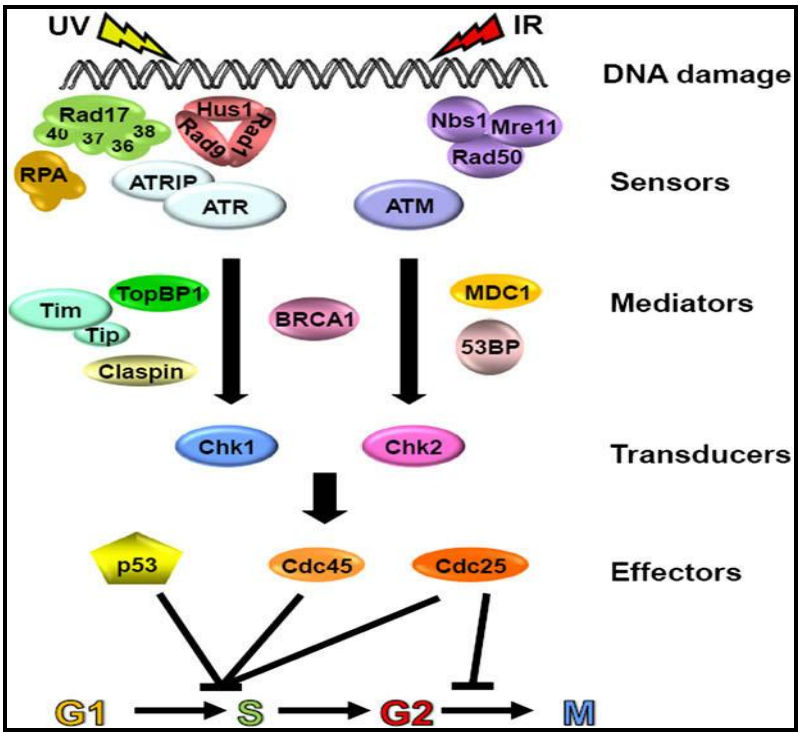


Fig.2 DNA damage checkpoint pathways in eukaryotes. (Sancar *et al.*, 2004)

In contrary to eukaryotes, prokaryotes are considered as simple bags of metabolites; however they can execute various cellular activities with highest precision. Bacteria in their natural habitat often cope with the extreme environment like, lack of nutrients, constant biochemical warfare against other microbes. In addition to that they also respond very quickly in favorable condition by burst of rapid growth. Therefore it is not surprising that bacteria also use extensive cellular signaling network to coordinate cellular functions. In bacteria, majority of environmental signal transduce through two-component signal-transduction pathway; a Histidine kinase and cognate response regulator. Phosphoproteome analysis of model organism *E.coli* and *B. subtilis* species showed several phosphoproteins with single or multiple phsophorylation events on their Ser/Thr/Tyr residues. Also phosphoproteome of these two organisms showed evolutionary

conservation in their Ser/Thr/Tyr phosphorylation (Macek *et al.,* 2007 and 2008). Ser/Thr/Tyr protein kinases (STYPKs) were also characterized in several bacterial species in the past two decades, including in many pathogenic bacteria. These 'eukaryotic-like STYPKs' play essential roles in growth (Sassetti *et al.,* 2003 and Fernandez *et al.,* 2006), virulence (Cozzone, 2005), persistence and reactivation (Shah *et al.,* 2008), However, the involvement of Ser/Thr/Tyr protein kinases in DNA damage induced signal transduction mechanism has not been established in bacteria.

1.2 Protein phosphorylation in bacteria

Protein phosphorylation is the covalent attachment of a phosphate group to a protein and is a widely employed post translational modification found in all three domains of life, where it plays key roles in regulation and signal transduction. In bacteria protein phosphorylation is categorized in two major class; (1) Two-component histidine and aspartate phosphorylation system and (2) Serine-Threonine and Tyrosine phsphorylation.

1.2.1 Histidine and Aspartate phosphorylatioin

In bacteria, the common phosphorylation takes place on histidine and aspartate residues in what are called two-component systems. This is simplest way of transducing signal; however it can fulfill purpose similar to those in eukaryotes (Mijakovic, 2010). In a typical two-component system, the first component is an input-sensing histidine-kinase (usually transmembrane) that autophosphorylates in response to a specific stimulus. This kinase then transfers the acquired phosphate to an aspartate residue of the second component, which is the response regulator. It actively participates in transferring the phosphate residue, a step that triggers the cellular

response, most commonly by directly regulating gene transcription by binding to regulatory DNA sequence (Fig.1). Two-component systems sometimes are integrated into more complex signaling cascades, known as phosphorelay systems (Hoch, 2000). Characteristically, such two-component systems serve as the sensory organs of bacteria, sensing various environmental signals such as nutrient availability, pH, salinity, temperature, and the presence of other bacteria. They also regulate pathogenicity-related functions, including toxin production, cell adhesion, quorum sensing, capsule synthesis, motility, and drug resistance (reviewed in Stock *et al.,* 1995; Parkinson and Kofoid, 1992; Alex and Simon, 1994). Histidine phosphorylation is also prominent in bacterial phosphoenolpyruvate-dependent phosphotransferase systems (PTS). Bacteria depend on these systems to import carbon sources and to regulate metabolic activities related to carbohydrate consumption (Deutscher *et al.,* 2006). Also some bacterial pathogens depend on PTS-related control mechanisms to regulate virulence gene expression. Recently two-component system also characterized for its involvement in DNA double strand break repair in radioresistance bacterium *D. radiodurans* (Shruti *et al.,* 2011; Wang *et al.,* 2008). Majority of two-component systems are positively auto-regulated at the transcription level "all or nothing" response, whereas those without a positive feedback loop exhibit graded and mixed mode responses. The cross-talk between two-component systems is very low, however there are increasing evidence of cross-talk between two-component systems and serine-threonine kinases (Nariya *et al.,* 2006 and Rajagopal *et al.,* 2006). So far high-throughput analyses is not possible for histidine and aspartate phosphorylation, because phospho-histidine and phospho-aspartate residues are unstable under acidic conditions however, technological advancement may achieved such analysis in future.

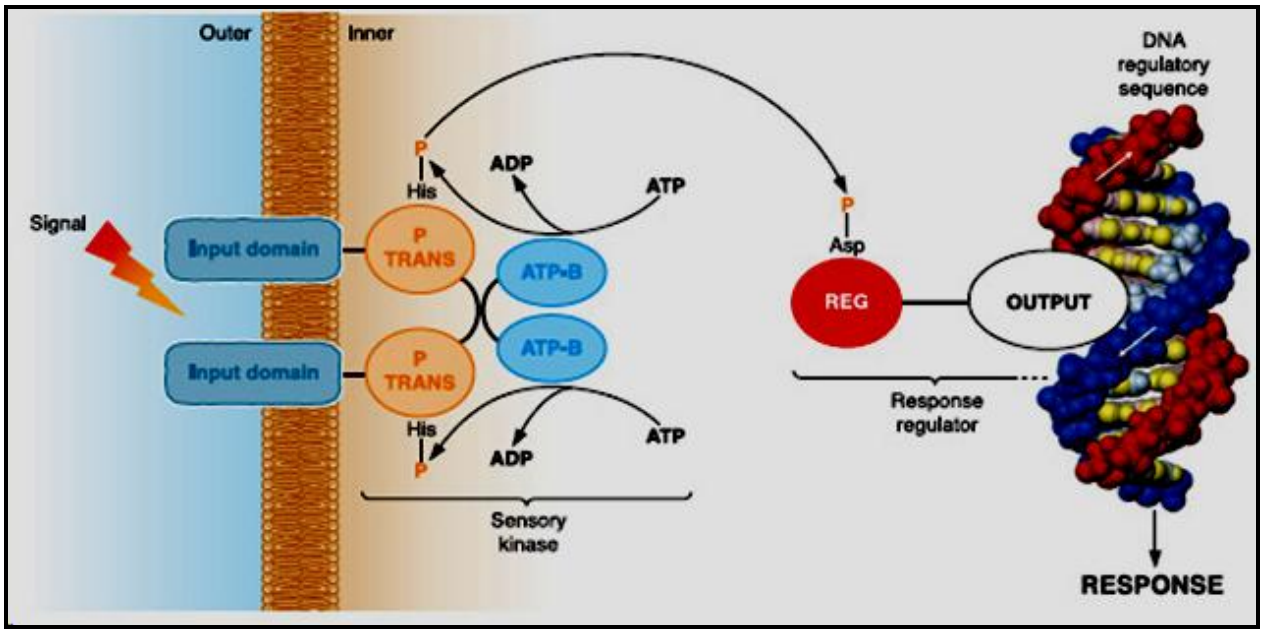


Fig.3 A typical two-component system (Mijakovic, 2010)

1.2.2 Serine-Threonine and Tyrosine phsphorylation

Protein phosphorylation was first described in *Eukarya* with the demonstration of enzymatic protein phosphorylation in 1954 (Burnett and Kennedy, 1954). Although there were a few reports of protein phosphorylation in bacteria in the sixties, the general opinion emerged that protein phosphorylation on serine, threonine and tyrosine was not present in bacteria (Rubin and Rosen, 1975). The first evidence for the presence of this type of phosphorylation in bacteria came in 1978 with the identification of serine and threonine phosphorylated proteins in *Salmonella typhimurium* (Wang and Koshland, 1978). The first detailed studies on eukaryotic type bacterial kinases published in 1990s with the characterization of the eukaryotic-like serine/threonine protein kinase, Pkn1, from *Myxococcus xanthus* (Muñoz-Dorado *et al*., 1991). Thereafter, it was generally accepted that serine/threonine phosphorylation takes place in bacteria. Another important type of protein phosphorylaion reported in bacteria is tyrosine

phosphorylation. The acceptance of tyrosine phosphorylation in bacteria has been longer underway. Although phosphotyrosine was demonstrated as early as 1986 in *Escherichia coli* (Cortay *et al*., 1986) and further established in *Acinetobacter calcoaceticus* in 1990 (Dadssi and Cozzone, 1990). Unlike serine/threonine phosphorylation, tyrosine phosphorylation is mediated mainly by a bacteria specific kinase showing no resemblance to eukaryotic-like tyrosine kinases (Cozzone *et al*., 2004). The first endogenous substrate of a tyrosine kinase was identified in 2003 and since then the number of characterized kinases and substrates have increased steadily.

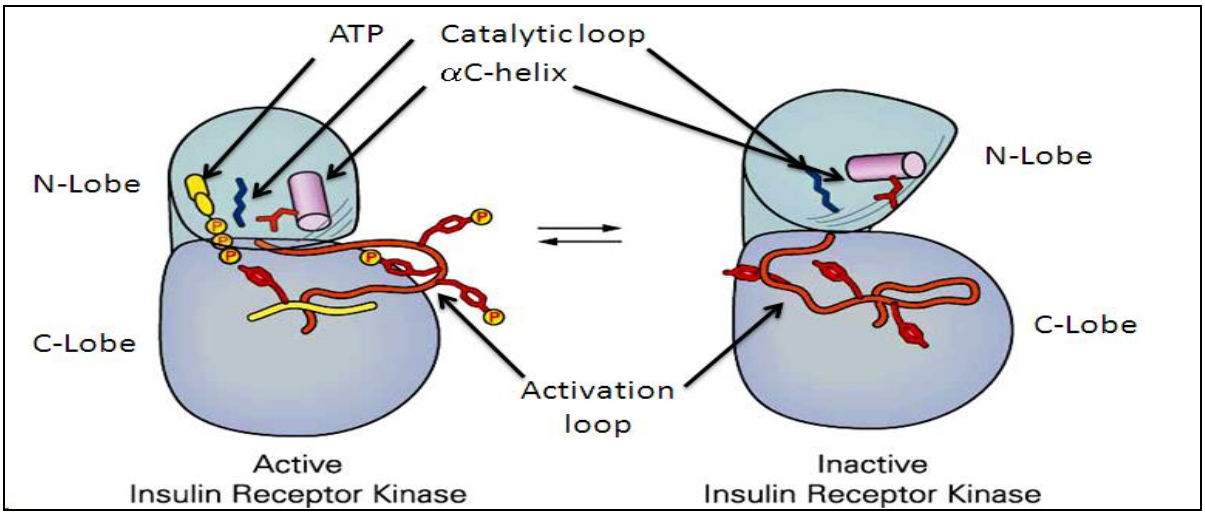
Based on sequence homology between kinase domains, bacterial eukaryotic Ser/Thr kinases are grouped together in the eukaryotic protein kinase superfamily (Hanks *et al.,* 1995). These domains are typically organized in to 12 subdomains that fold in a characteristic two-lobed catalytic core structure, with the catalytic active site lying in a deep cleft formed between the two lobes (Fig. 4A). The smaller, N-terminal lobe is involved primarily in binding and orienting the phosphop-donar ATP molecule, whereas the larger, C-terminal lobe binds the protein substrate and initiates the transfer of the phosphate group. While subdomains vary in size, however, the kinase catalytic domain can be defined by the presence of specific conserved motifs, showed remarkable conservation and is maintained across kingdom. Kinases are molecular switches that exist in either an off 'inactive state' or an on 'active state' (Huse *et al.,* 2002). Transition between these two states is tightly regulated by different mechanisms, including the binding of allosteric effectors molecule and their subcellular localization. Both the αC helicx in the N-terminal lobe and the activation loop in the C-terminal lobe undergo extensive conformational changes essential for this transition which is required for the kinase activity. In kinase domain of eukaryotic STKs contains activation segment designated by the conserved DFG and APE motifs. This segment includes the magnesium binding loop, the activation loop and P+1 loop (Fig. 4B).

The activation loop is involves in determining substrate specificity and is containing variable number of phosphoacceptor serine/threonine residues. Many kinases are activated by phosphorylation on at least one Ser/Thr (Tyr for tyrosine kinases) residue in the activation loop, by either autophosphorylation or transphosphorylation by another kinase. This modification promotes several interactions that stabilize the loop in a conformation that allows substrate binding and catalysis (Nolen *et al.,* 2004). This activation involve the interaction among phosphoacceptor site in activation loop, conserved arginine/aspartate (RD) residues and αC helicx, resulted interaction bringing the two lobes together in active conformation. The activation loop is also a site of protein-protein interaction for activity modulator in many kinaes. The P+1 loop is a critical point of contact between the kinase and its substrate and is a major determinant of the distinct substrate specificity between Ser/Thr and Tyr kinases. Another important conserved glycine-rich region known as P loop, which covers both the β- and γ-phosphate and plays an important role in both phosphoryl transfer and ATP/ADP exchange during the catalytic cycle. All of these conformational changes bring the ATP γ-phosphate from, the kinase catalytic Asp residue and the substrate phosphor-acceptor residue together, in such a close proximity that allowing the transfer of γ-phosphate from ATP to the phosphor-acceptor Ser or Thr residue (Tyr in tyrosine kinase) in the substrate protein.

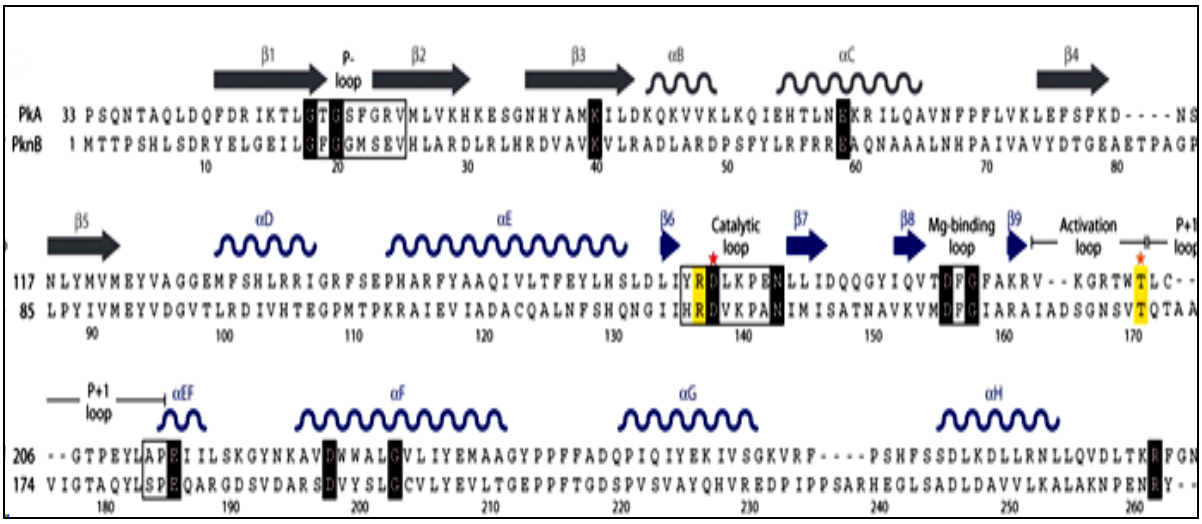
1.2.2.1 Structural and functional relationship of bacterial eSTPKs

The PknB eSTPK of *M. tuberculosis* is the first bacterial kinase whose crystal structure has been solved. The cocrystal of the PknB catalytic domain in complex with an ATP analog suggest that PknB is structurally very similar to the mouse cyclic AMP (cAMP) dependent protein kinase (PKA) in the activated state (Ortiz-lombardia *et al.,* 2003). PknB catalytic domain showed

typical two-lobed structure, with the nucleotide analog tightly bound in catalytic cleft between two lobes. All regulatory regions in active state of PknB resemble with the activated state of eukaryotic Ser/Thr kinases, however there is some sort of mismatch like αC-helix is oriented away from the active site, in a position characteristic of the inactive state. Despite this orientation, the essential interaction between the conserved Lys40 residue (Lys72 in PKA) in the β3 strand and the conserved Glu59 residue (Glu91 in PKA) in the αC helix is still maintained, as well as the interaction with α- and β-phosphate of nucleotide (Huse and Kuriyan, 2002). Different ortholog of PknB when mapped on the surface of the PknB structure showed extensive conservation in ATP binding sites and substrate binding sites which is similar to PKA. This suggests a common activation mechanism shared by homologous eukaryotic and prokaryotic Ser/Thr kinases. Eukaryotic protein kinases are activated by dimerization through conserved dimer interface. Dimerization is important phenomenon as it is required for activation of eSTPKs (Fig.5). PknB crystallized back-to-back dimer fashion, which is similar to the eukaryotic double-stranded RNA-activated protein kinase PKR. Site directed mutation in conserved dimer interface residues reduced autophosphorylation and altered substrate specificity of PknD from Mycobacterium (Greenstein *et al.,* 2007). This specific back-to-back dimerization suggests that possible activation can occur through transphosphorylation by another monomeric or dimeric activated kinase. Majority of eSTPKs catalytic domain is associated with an additional domain(s) primarily represented by PASTA (penicillin-binding protein and *Ser/Thr* kinase-associated), proline rich repeats and β propeller repeats. These extracellular domains can be enzymatic but they mainly mediate ligand binding or protein-protein interactions and work as a sensory domain.



[A]



[B]

Fig. 4A Catalytically active and inactive conformation of eukaryotic protein kinase

(Huse and Kuriyan, 2002)

4B Primary sequence alignment between the PKA (residues 33 to 283) and PknB (residues 1 to 266), **catalytic domains.** Conserved motifs are shown in boxes, and the invariant residues are depicted in black (Pereira *et al.*, 2011).

Beta propeller repeats presents in many eukaryotic proteins with a wide variety of functions and in bacteria it appear to present mainly in pathogenic bacteria. Ligand mediated dimerization of eSTPKs suggested by some researcher as PASTA repeats of *B. subtilis* PrkC involved in binding with peptidoglycan (Shah *et al.,* 2008). However, this is not only the activation mechanism for bacterial eSTPKs as it has been demonstrated that PknD kinase become activated in absence of ligand-binding domain (Greenstein *et al.,* 2007). Recently in PknB new front-to-front dimer interface has been identified where dimerization interface occur through interaction between the αC helix and the ordered activation loop of one kinase domain and the αC helix of the second kinase domain, in this association one monomer function as an activator and the other function as a substrate (Mieczkowski *et al.,* 2008). This dimerization provides a new mechanism by which an allosterically activated kinase could phosphorylate and thereby activate other kinases that are not associated with a receptor domain.

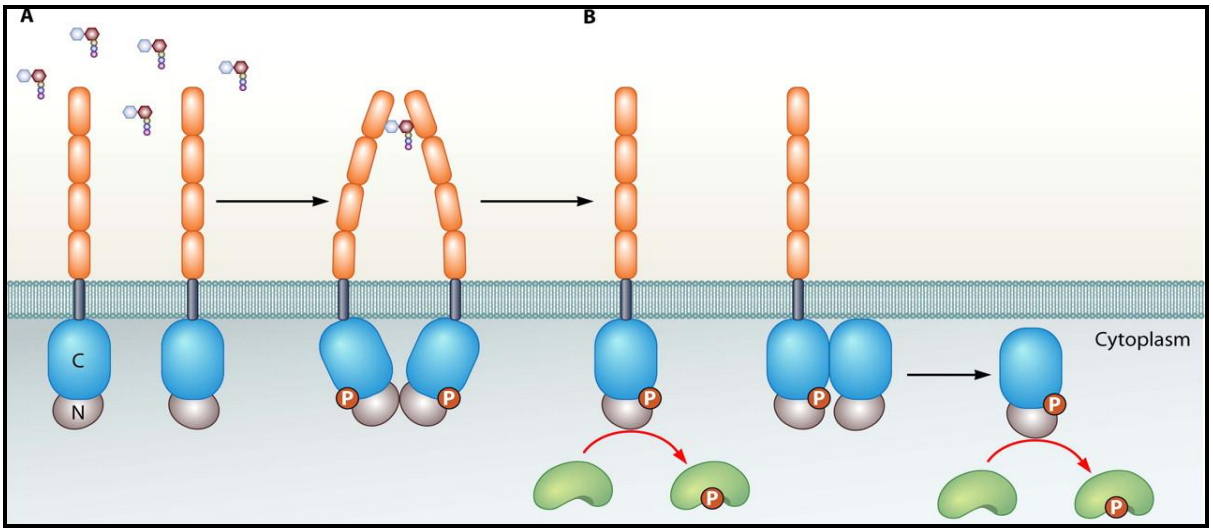


Fig. 5 Ligand mediated Activation model for eSTPKs (Pereira *et al.,* 2011)

1.2.2.2 Targets and physiological roles of eSTPKs in bacteria

Bacterial eSTPKs are involved in various cellular processes including cellular metabolism, virulence, cell division, cell wall synthesis etc. The involvement of these eSTPKs in various cellular functions occurs through complex signaling pathways. These pathways are also showing functional redundancy and/or substrate promiscuity. Although large number of substrates of eSTPKs has been identified, but majority of these substrates were identified by phosphopreoteomic approaches and / or in vitro kinase assay and site-directed mutagenesis but lack *in vivo* confirmation. Also only limited information exists concerning the effect of phosphorylation on the activity of substrates. Here following heading will discuss involvement of eSTPKs in known functions.

(i) Developmental processes

Development and differentiation are often defined in the context of multicellular eucaryotic organisms. However, there are some examples in prokaryotes where prokaryotic organism showed both development and differentiation during their life cycle. *Myxococcus xanthus* a gram-negative bacterium, has a complex life cycle. In response to starvation, most cells in a population participate in the formation of multicellular aggregates (i.e., fruiting bodies) in which cells differentiate into spores. *M. Xanthus.* FruA is a key transcription factor that regulates the expression of a large number of genes involved in both fruiting body formation and sporulation. Another transcription factor, MrpC, controls *fruA* expression and is itself under a dual regulatory mechanism involving both a TCS (Two component system) and an eSTPK (Inouye *et al.*, 2008). Transcription of *mrpC* is controlled by the MrpA/MrpB TCS, encoded on the same locus. In response to starvation signals, the sensor His kinase MrpA is activated and phosphorylates the

response regulator MrpB, which in turn positively regulates *mrpC* expression, leading to aggregation. The eSTPK Pkn14 also regulates MrpC by phosphorylating it on a Thr residue(s), reducing its affinity for both its own promoter and the *fruA* promoter (Nariya *et al.*, 2005, 2006). The transmembrane eSTPK Pkn8 phosphorylates Pkn14, and it has been suggested that Pkn8 and Pkn14 form an eSTPK cascade that inhibits the activity of MrpC during vegetative growth. Similar observations were reported for other organisms, such as cyanobacteria (Zang *et al.*, 2005) and streptomycetes (Nadvornik *et al.*, 2005). *Bacillus subtilis* is another example where eSTPKs play important role in forming heat and desiccation-resistant spores under conditions of nutrient deprivation. The transmembrane eSTPK PrkC is necessary for spore germination in favorable condition (Shah *et al.*, 2008). Peptidoglycan fragments bind the PASTA repeats in the extracellular domain of PrkC and lead to kinase activation and subsequent germination. The essential translational GTPase elongation factor EF-G is phosphorylated in this PrkC-dependent germination pathway. Ser/Thr phosphorylation of another elongation factor, EF-Tu, has been reported for *E. coli* (Schumacher *et al.*, 2009), *Thermus thermophilus* (Lippmann *et at.*, 1993), *Streptomyces coelicolor* (Holub *et al.*, 2007), *B. subtilis* (Absalon *et al.*, 2009). For *Streptomyces collinus*, a ribosomeassociated kinase was reported to phosphorylate several ribosomal proteins on Ser and Thr residues, which led to a significant reduction in protein synthesis (Mikulik *et al.*, 1997, 2001), also Ser/Thr phosphorylation of a number of other ribosomal and ribosome-associated proteins has been observed in phylogenetically diverse bacteria by use of phosphoproteomic methodologies (Bendt *et al.*, 2003; Macek *et al.*, 2008; Prisic *et al.*, 2010). Regulation of translational initiation and elongation by phosphorylation in response to diverse stimuli is well established for eukaryotes (Browne *et al.*, 2002).

(ii) Secondary metabolism

eSTPKs are involved in secondary metabolites production. The best studied example is regulation of production of actinorhodin by *Streptomyces coelicolor* which is gram-positive filamentous soil bacteria. The production of actinorhodin is under the control of regulator AfsR, which itself is target of memebarne assiciated eSTPK AfsK (Matsumoto *et al.*, 1994). Afsk can phosphorylate AfsR *in vitro* on a Ser and /or Thr residue(s). AfsK activity is necessary for production of actinorhodin *in vivo* as the production of actinorhodin is reduced in *ΔafsK* mutant. Activity of AfsK is further regulated by actinorhodin responsive protein KbpA, whose gene located upstream of afsK gene. Regulation of actinorhodin is showed redundancy as two other eSTPKS, AfsL and PkaG can phosphorylate AfsR *in vitro*.

(iii) Cell division and cell wall synthesis

Involvement of eSTPK substrates in cell division or cell shape have been reported in many bacteria species like *S. pyogenes, S. agalactiae, S. pneumoniae, S. mutans, E. faecalis* and *M. tuberculosis.* In *M. tuberculosis* the presence of eSTPK genes such as those for *M. tuberculosis* PknA and PknB in the operon containing the genes encoding penicillin-binding protein A (PBPA) and RodA suggests a role for these eSTPKs in cell shape. Both *pknA* and *pknB* are expressed preferentially during exponential growth phase, and their overexpression results in long, broad, and in some cases branched cells, whereas partial depletion results in long cells (Kang *et al.*, 2005). PknB phosphorylated PBPA on Thr362 and Thr437 when the proteins were coexpressed in *E. coli* (Dasgupta *et al.*, 2006). Moreover, a PBPA T437A mutant did not complement a *pbpA* deletion in a heterologous system and led to an increased number of nucleoids per cell and to mislocation of the protein. Another *in vivo* substrate of PknB is Wag31,

a homolog of the essential cell shape/cell division protein DivIVA, and overexpression of a mutated form of Wag31 in which the Threonine residue identified as the phosphoacceptor was replaced by a Glutamate residue resulted in morphological defects (Kang *et al.,* 2005). Another cell division protein FtsZ, a homolog of eukaryotic tubulin and a major component of the division septum, phosphorylated by PknA of M. *tuberculosis* on a Thr residue(s) *in vitro* (Thakur *et al.,* 2008). The coexpression of both proteins (PknA and FtsZ) in *E. coli* resulted in significantly reduced FtsZ GTPase and polymerization activities. Similarly the *S. pneumoniae* eSTPK StkP colocalized with FtsZ at midcell (Giefing *et al.,* 2010) and interacted with and phosphorylated FtsZ *in vitro*. In the actinobacterium *Corynebacterium glutamicum*, overexpression of the PknA and PknB kinases also resulted in growth and morphological defects (Fiuza *et al.,* 2008). In addition, PknA inhibits the activity of the muropeptide ligase MurC by phosphorylating it on multiple Thr residues *in vivo*. The involvement of eSTPKs in cell wall synthesis and division has been reported for other Gram-positive pathogens. For example, a *Staphylococcus aureus* Δ*pknB* strain grew slower and exhibited severe cell division defects, including multiple and incomplete septa, bulging, and irregular cell size (Beltramini *et al.,* 2009).

(iv) In virulence and central metabolism

The involvement of eSTPKs in virulence has been reported for several pathogens, such as *M. tuberculosis, Y. pseudotuberculosis S. pneumoniae, and Salmonella enterica* serovar *Typhi*. An *M. tuberculosis* PknG and PknH eSTPKs regulate infection in host cells as Δ*pknG* strain exhibited delayed mortality in SCID mice and reduced viability in a BALB/c mouse infection model, with significant reductions in the bacillary loads in the lungs, spleen, and liver (Cowley *et al.,* 2004). Similarily, an *M. tuberculosis* Δ*pknH* mutation increased the bacillary load in a

BALB/c mouse infection model (Papavinasasundaram *et. al.,* 2005). PknH phosphorylates the transcription activator EmbR. Phosphorylation of EmbR activates the expression of the *embCAB* operon, which encodes arabinosyltransferases involved in the metabolism of arabinan, an essential component of the mycobacterial cell wall, and resistance to ethambutol. Since EmbC catalyzes the conversion of lipomannan into lipoarabinomannan, a cell wall lipoglycan that prevents the host proinflammatory response (Briken *et al.,* 2004). PknH also phosphorylates DosR on two Thr residues, and DosR together with DosS controls the DosRS regulon responsible for the nitric oxide and hypoxia response, an important evasive response to host macrophages (Chao *et. al.,* 2010). Similarly the *Yersinia pseudotuberculosis* eSTPK YpkA is absolutely essential for virulence (Galyov *et al.,* 1993). The eSTPK PrkC is required for *M. pneumoniae* adhesive growth and cytotoxicity (Schmidl *et al.,* 2010)

M. tuberculosis PknG exemplifies the involvement of eSTPKs in central metabolism. A Δ*pknG* mutant showed higher intracellular levels of glutamate, glutamine, and derivatives, suggesting that PknG controls glutamate synthesis in response to nutritional stress signals (Cowley *et. al.,* 2004). In *C. glutamicum* deletion of the *pknG* homolog impaired glutamine utilization. OdhI, an FHA-containing protein that inhibits the 2-oxoglutarate dehydrogenase (ODH) complex of the tricarboxylic acid cycle, was inhibited by phosphorylation and identified as an *in vivo* PknG substrate (Niebisch and Kabus, 2006). Similarly, *M. tuberculosis* PknG phosphorylates GarA, an OdhI homolog, on Thr21 *in vivo* (O'Hare *et al.,* 2008).

1.3 *Deinococcus radiodurans* (DEIRA), a model organism

Member of Deinococcaceae family have been isolated from a variety of sources including different environments, rich in organic nutrients, such as soil (Brooks and Murray, 1981), animal

feces and sewage (Ito *et al*., 1983), processed meats (Davis, 1963) and sometimes even from harsh environments such as dried foods (Lewis, 1973), room dust, medical instruments and textiles (Christensen and Kristensen, 1981). The *Deinococcus* lineage comprises mesophilic, thermophilic, and psychophilic representative, which can be found in a variety of habitats, such as animal gut, hot springs, deserts, alpine environments, and antartica (Slade and Radman, 2011). Deinococci are non-motile and non-spore forming organisms and the majority of the species are found to grow best in rich media at a temperature range between 30^{o}C and 37^{o}C, with a doubling time ranging between 1.5 and 3 hours. Certain species such as *D. geothermalis* and *D. murrayi* are true thermophiles growing happily at 45^{o}C-55^{o}C. All *Deinococcus* species, except *D. grandis* (rod shaped) are spherical cells and exist either as single, as diads or tetrads with an average cell diameter of 1µm (range, 0.5 to 3.5µm) in liquid culture.

D. radiodurans was first isolated from canned ground meat that had been irradiated at 4000Gray (Gy), a dose 250 times higher than the lethal dose for *E. coli* (Anderson *et al*., 1956). Earlier it was classified as a species belonging to genus *Micrococcus* for its morphological resemblance; however, research in the subsequent years has reclassified in *Deinococcaceae* family, and renamed *Deinococcus radiodurans* (Hensel *et al*., 1986; Weisburg *et al.*, 1989). In the genus *Deinococcus,* the Greek adjective 'deinos' means strange or unusual; an apt description for an organism, which has the unique ability to survive the genetic damage and distinguishes it from rest of the life forms. Nearly 43 different species of *Deinococcus* have been discovered till date from diverse surroundings they are, *D. radiodurans, D. radiopugnans ,D. radiophilus, D. proteolyticus, D. grandis, D. geothermalis, D. murrayi, D. indicus, D. frigens, D. sexicola, D. marmoris, D. hohokamensis, D. navajonensis, D. hopiensis, D. apachensis, D. maricopensis, D. pimensis, D. yavapaiensis, D. papgonensis, D. sonorensis, D. deserti, D. ficus, D. mumbaiensis,*

D. peraridilitoris, D. radiomollis, D. claudionis, D. altitudinis, D. alpinitundrae, D. aquaticus, D. caeni, D. aquatilis, D. aquiradiocola, D. xinjiangensis, D. gobiensis, D. aerius, D. piscis, D. aetherius, D. aerolatus, D. aerophilus, D. wulumuquiensis, D. xibeiensis, D. guangriensis and *D. depolymerans.*

Among them, *D.radiodurans,* has gained a considerable attention because of its unique radioresistance, better understood genetics and amenability to genetic modifications. The genome sequence of this bacterium has been decoded and released to public domain (White *et al*., 1999).

1.3.1 Genome composition

The Genome of *D. radiodurans* is 3.28-Mb, which consist of a 2.64-Mb chromosome (chromosome I), a 0.41-Mb chromosome (chromosome II), a 0.18-Mb megaplasmid and a 0.045-Mb plasmid (Lin *et al.*, 1999). Total numbers of ORFs are 3192 and GC content of 66.6%, with 52 insertion sequences (ISs) and 247 small noncoding repeats (SNRs) randomly scattered in the genome. Depending on the growth phase, *D. radiodurans* may have nearly 4-10 genome copies per cell (Hansen, 1978; Harsojo *et al*., 1981). *D. radiodurans* R1 has a D_{37} dose of approximately 6,500 Gy. This sublethal dose can produce potentially catastrophic deletions and genome rearrangements may occur at low frequencies, however the process of double-strand-break repair in *D. radiodurans* appears to be error-free. It was suggested that the robust complement of repair enzymes, strong oxidative stress management and passive features of deinococcal physiology aid in the recovery of *D. radiodurans* from this substantial DNA damages (Cox and Battista, 2005; Slade and Radman, 2011).

1.3.2 Classification

Classification of *Deinococcus radiodurans* was difficult as it could be grouped neither in the members of archaebacteria (due to peptidoglycan content in its cell wall) nor in eubacteria as it contained a few genes not typical to this particular group. It was removed from the genus Micrococcus after the discovery of its unique cell wall containing L-ornithine and lacking teichoic acid in the peptidoglycan and later was grouped with its related species in a phylum of their own as it had a unique cell wall and exhibited radiation resistance phenomenon. Even though the phyla *Thermus* are radiation sensitive thermophiles and that of *Deinococcus* are radiation resistant mesophiles they were found related to each other based on the 16S rRNA sequence suggesting evolved from a common ancestor (Hensel *et al*., 1986; Weisburg *et al.,* 1989). These two phyla even share a great deal of homology in their megaplasmids and most of the genes found identical in these two bacteria are common to majority of the bacteria and it was suggested that the members of phyla *Thermus-Deinococcus* are the closest living relatives of the archeabacteria. The classification study of *D. radiodurans* is based primarily on the analysis of 16S rRNA sequences. *D. radiodurans* is classified to be in the domain and kingdom of bacteria due to peptidoglycan component in the cell wall, with all of the members of class *Deinococci* being radiation resistant and the majority of them are mesophiles.

Deinococcus radiodurans classified as - Kingdom: Bacteria Phylum: Deinococcus-Thermus Class: Deinococci Order: Deinococcales Family: Deinococcaceae Genus: *Deinococcus* Species: *radiodurans*

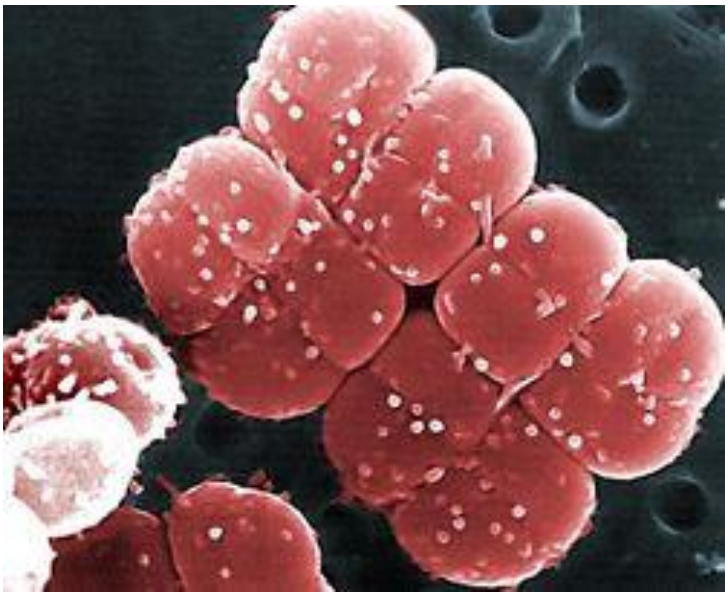


Fig.6 Electron micrograph showing tetards of *D. radiodurans*

1.3.3 Microbiological features

D. radiodurans is a Gram positive, pigmented, nonsporeforming, nonmotile, spherical bacterium ranging from 1.5 to 3.5 μm in diameter (Murray, 1986; 1992) and forms pairs and tetrads when grown in a liquid culture, when grown on solid media it forms convex, smooth, and pink to red colored colonies. Optimal growth temperature for this organism is 32^{o}C, but growth is seen even at 37^{o}C and gets reduced at the higher temperatures. *D. radiodurans* cultures are typically grown in a TGY medium (0.5% tryptone, 0.3% yeast extract and 0.1% glucose) with aeration and exhibit a doubling time of approx. 80 minutes and attain a stationary phase density of about 1 x 10^{9} colony forming units per milliliter (cfu/ml). For the transfer of genetic information it is not known whether it uses conjugation or transduction (Moseley and Setlow, 1968; Smith *et al*., 1989). However, it is easily transformable during exponential phase of growth. The efficiency of transformation is higher with double-stranded DNA (dsDNA) than with single stranded DNA (ssDNA) (Moseley and Setlow, 1968) and with plasmid DNA than with linearized DNA with

circular and more efficiently with linear forms of DNA throughout exponential growth phase (Tigari and Moseley, 1980).

1.3.4 Cell structure

The cell envelope is reminiscent of the cell walls of Gram-negative organisms (Brooks *et al*., 1981; Work and Griffiths, 1968) and is unusual in terms of its structure and composition. It stains as Gram positive because of the inability of its thick peptidoglycan layer to decolorize. It consists of the plasma and outer membranes, divided by a 14 to 20 nm peptidoglycan layer and a compartmentalized layer. Out of the six layers identified by electron microscopy, the innermost layer is the plasma membrane with the next layer a peptidoglycan-containing perforated cell wall. The third layer is divided into numerous fine compartments (the compartmentalized layer); the fourth layer is the outer membrane, and the fifth one, a distinct electrolucent zone. Regularly packed hexagonal protein subunits compose the sixth layer of cell envelope (Baumeister *et al*., 1986; Peters *et al.*, 1987). Only the cytoplasmic membrane and the peptidoglycan layer participate in the septum formation during cell division. Whereas the other layers act as a sheath, surrounding groups of cells and form on the surface of daughter cells as they separate (Sleytr *et al*., 1976; Thornley *et al*., 1965; Work and Griffiths, 1968). The plasma and outer membranes share nearly the same lipid composition (Thompson and Murray, 1981).

A few strains of *Deinococcus* also exhibit a dense carbohydrate coat (Baumeister *et al*., 1986;; Lancy and Murray, 1978; Sleytr *et al*., 1976; Thompson and Murray, 1981). The fatty acid composition of *D. radiodurans* (Ferreira *et al*., 1997) includes a mixture of 15-, 16-, 17-, and 18-carbon saturated and monounsaturated acids, while polyunsaturated, cyclopropyl and branched-chain fatty acids are absent. *D. radiodurans* lacks the conventional phospholipids (Thompson *et*

al., 1980), 43% of membrane lipid is composed of unique phosphoglycolipids containing a series of alkylamines as structural components (Anderson and Hansen**,** 1985) that seem to be derived from a novel phosphatidylglycerolalkylamine and formed when it is glycosylated with galactose or glucosamine. A variety of proteins and carotenoids present in DEIRA are suggested to play a role in resistance to its oxidative stress (Slade and Radman, 2011). It is reported that deinoxanthin, a major product of the carotenoid synthesis pathway in DEIRA scavenges H_2O_2 and singlet oxygen, exerting a protective effect on DNA (Tian *et al.*, 2009). *Deinococcus* cells lacking phytoene desaturase CrtI (DR0861) are colourless and show sensitivity to desiccation, oxidative stress and γ -irradiation (Zhang *et al.*, 2007). However, the role of pigmentation in radioresistance appears to be less significant as the unpigmented *D. deserti* bacteria and even some colourless mutants of *Deinococcus* show nearly the same level of radioresistance as that of *D. radiodurans* (Slade and Radman, 2011).

1.3.5 Radiation resistance in *Deinococcus radiodurans*

D. radidurans is 30-fold and 1000-fold more resistance to ionizing radiation than *E.coli* and humans repectively, and can repair approximately 200 DSbs or 190 cross-links per genome copy without a loss of viability (Battista, 1997). Various factors contributing in ionization radiation resistance are depicted in Fig.7**.** Earlier it was assumed that the genome of *Deinococcus* genome suffers less damage during irradiation, however later it was found not to be the case as the rate of DSB formation was identical in case of *Deinococcus* as well as *E.coli* irradiated under the same conditions (Gerard *et al*., 2001). Extreme radiation resistance of *D. radiodurans* is manifestation of desiccation resistance (Battista, 1995) therefore, hypothesized that *D. radiodurans* is an

organism where its DNA repair ability appears to be the expression of its adaptation to dehydration.

D. radiodurans also exhibits very high resistance to the lethal and mutagenic effects of other DNA damaging agents such as UVC (254 nm), nitrous acid, mitomycin C, hydrogen peroxide and bulky chemical monoadducts such as 4-nitroquinoline-N-oxide. Even after exposure to lethal doses of these DNA damaging agents, *Deinococcus* shows a very low level of mutagenesis which normally occurs during replication. Unique restoration of the genome with great fidelity suggest that the error prone SOS repair pathway might be absent in this organism (Narumi, 2001). In case of other damaging agents, *Deinococcus* is found equally sensitive as *E. coli to* the lethal effects of ethyl methanesulphonate (EMS), but is not mutable by it, and shows sensitivity towards the mutagenic but not the lethal effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Sweet and Moseley, 1976; Rebeyrotte, 1983; Caimi and Eisenstark, 1986). MNNG is known to induce O^6-methylguanine, which causes transitions not well recognized by *D. radiodurans* (Minton, 1994). The high genome copy number in case of *Deinococcus* was suggested to aid in the recombinational repair of DNA double strand breaks (Hansen, 1978; Harsojo *et al*., 1981), which restricts the dispersion of DNA fragments in the condensed genome (Levin-Zaidman *et al*., 2003) and leads to an efficient recombination through their homologous interactions (Minton and Daly, 1995). It was also suggested that the removal of damaged oligonucleotides and presence of NUDIX hydrolases in this organism might help in the degradation of oxidized nucleotides adding further to its radioresistance (Vukovic-Nagy *et al*., 1974; Xu *et al.,* 2001). *D. radiodurans* is distinctly rich in Nudix pyrophosphohydrolases proteins, some of which may act to remove the residual intracellular mutagenic precursors (White *et al*., 1999). Final steps are done by superoxide dismutases and catalases such as SodA

and KatA (Markillie *et al*., 1999), which help in the elimination of activated oxygen species with long half-lives. This initial phase of cellular cleansing also involves import of amino acids, nucleotides, nucleosides, sugars, and phosphate into the cell while precursors for DNA synthesis are made by way of ribonucleoside diphosphate Reductase followed by genomic restoration and synchronization of different repair pathways (Makarova *et al.*, 2001). The initial products are a mixture of damaged and undamaged nucleotides and nucleosides (Battista, 1997; Vukovic-Nagy *et al*., 1974) found in the cytoplasm and also in the surrounding growth medium, suggesting their export once they are formed and prevents their reincorporation during further DNA synthesis (Battista, 1997). Carotenoids, deinoxanthin, present in *D. radiodurans* has a stronger scavenging ability on H_2O_2 and $^1O_2$ (Lemee *et al.*, 1997) as well as it is reported that its high intracellular Mn^{2+}/Fe^{2+} content helps to prevent oxidative damage to the proteins (Daly *et al*., 2004; 2007).

Deinococcus radiodurans is an excellent example of an organism in which all systems for DNA repair, DNA damage export, desiccation and starvation recovery, and genetic redundancy are present together (White *et al*., 1999). Transcriptome analysis of *D. radiodurans* has revealed an exciting group of genes up-regulated in response to stresses such as desiccation or ionizing radiation (Tanaka *et al*., 2004). There is correlation between the genes up-regulated during the first hour after a sublethal dose of ionizing radiation to that of highly expressed genes in *D. radiodurans* cultures recovering from desiccation (Tanaka *et al*., 2004) e.g. number of well defined DNA repair genes including *recA* (DR2340), *uvrA* (DR1771), *uvrB* (DR2275), *gyrA* (DR1913) and *gyrB* (DR0906). Inactivation of the five most highly induced genes, *ddrA* (DR0423), *ddrB* (DR0070), *ddrC* (DR0003), *ddrD* (DR0326), and another gene called *pprA* (DRA0346) indicated their role in radioresistanc (Tanaka *et al.*, 2004). However, sometimes this

is not the case for example, expression of DRB0100, a homolog of eukaryotic DNA ligase III, showed high induction after exposure to radiation and desiccation but mutant lacking this gene had the same radioresistance as the wild type R1 strain (S. Sommer, unpublished results; Blasius *et al*., 2008). The gene *recA*, essential for genomic restoration after irradiation, was found to get substantially up-regulated during the early phase of DNA damage and down-regulated before the start of exponential growth (late phase). The Induced genes in early phase of recovery included those involved in DNA replication, repair, and recombination, cell wall metabolism, cellular transport, and many encoding uncharacterized proteins suggesting efficient coordination of these genes in a complex network during the DNA repair and metabolic functions (Liu *et al*., 2003). Recent D. radiodurans proteome study also suggests that gamma radiation primerly induce the DNA repair and oxidative stress related proteins (Basu and Apte, 2012).

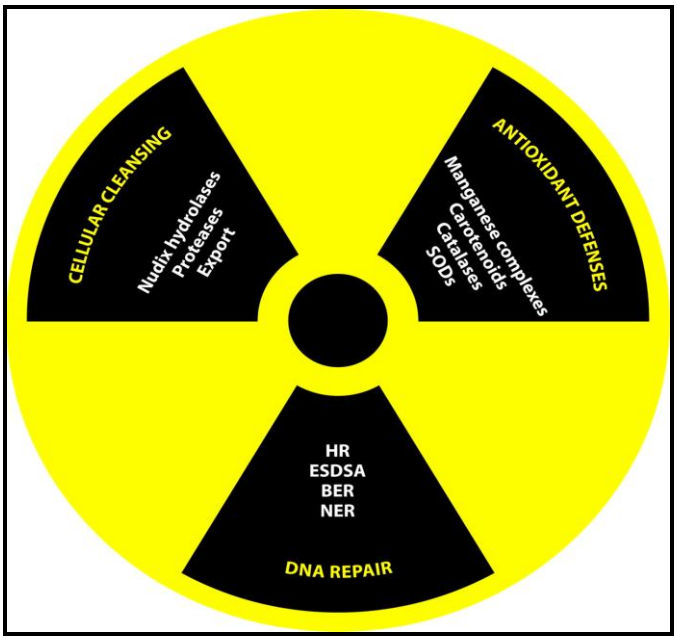


Fig. 7 Factor contributing for ionizing radiation resistance in *D. radiodurans* (Slade *et al.*, 2011)

1.3.5.1 Hypothesis proposed for radiation resistance in DEIRA

Various theories have been proposed by a group of researchers to unravel the extreme radiation resistance of this bacterium. Although *Deinococcus* has nearly identical DNA repair system as that of *E.coli*, it appears that the interaction of the different proteins involved in these repair pathways might be different. It has been suggested that *D. radiodurans* combines a variety of physiological tools that are strongly synchronized and several different mechanisms along with some undiscovered factors contribute to its radioresistance (Cox and Battista, 2005).

1.3.5.1.1 Desiccation adaptation hypothesis

According to the "desiccation adaptation hypothesis," the extreme radiation resistance is an incidental consequence of the adaptation to dehydration, because dry environments are more prevalent on earth than those that generate a high flux of ionizing radiation, which is a common physiological stress (Mattimore and Battista, 1996.). Without an exception, all 41 examined ionizing-radiation-sensitive strains of *D. radiodurans* were also found to be proportionally sensitive to desiccation, indicating that radiation resistance and desiccation resistance are functionally related phenomena (Mattimore and Battista, 1996). Further showed that ionizing-radiation-resistant bacteria from various habitats are also highly resistant to desiccation, MMC, and H_2O_2 (Shukla *et al.,* 2007). However, desiccation tolerance mechanisms do not always result in radiation resistance, as desiccation-tolerant bacteria from the *Actinobacteria*, *Arthrobacter*, and *Rhodococcus* families are not radiation resistant, therefore an alternatively hypothesis "radiation adaptation hypothesis" of the extreme radiation resistance has been proposed, which states that radiation resistance may have evolved as an adaptation to permafrost or semifrozen conditions where background radiation-induced DNA damage is accumulated (Richmond *et*

al.,1999) or to high natural ionizing radiation levels in deeply buried manganese-rich marine sediments) (Sghaier *et al.,* 2007).

1.3.5.1.2 Protection of the damaged genome

D. radiodurans can repair approximately 200 DSBs or 190 cross-links per genome copy without a loss of viability, when exposed to a high dose of γ radiation, (Dean *et al.,* 1966; Sweet and Moseley, 1976; Daly *et al.,* 1994). Exposure to such a huge amount of radiation results in the degradation of the chromosomal DNA by its own cellular exonucleases which may chew back DNA from starting at the ends of DSBs (Battista *et al*.,1999; Lipton *et al* ., 2002) and could be lethal for most of the bacteria. In *D. radiodurans,* it has been suggested that there are some novel proteins playing a role to prevent complete chromosomal digestion thus protecting the genome for its further repair. DdrA (DR0423) a novel protein in *Deinococcus,* is distantly but specifically related to the Rad52 family of eukaryotic proteins. DdrA protects 3'ssDNA overhangs from degradation by *E. coli* exonuclease I, presumably ensuring long-lived recombination substrates and the recycling of RecA (Jolivet *et al.,* 2006). Its absence results in excessive DNA degradation following radiation exposure. Hence, DdrA is proposed to preserve genome integrity by protecting DNA fragments generated by ionizing radiation from nuclease degradation, particularly when nutrients are scarce and when DNA repair processes are hindered (Harris *et al.,* 2004). PprA ("pleiotropic protein promoting DNA repair", DRA0346) is another novel DNA repair protein which is unique to the *Deinococcaceae* (Griffiths and Gupta, 2007). It binds DNA ends with a greater affinity than internal DNA regions thus protect DNA ends from extensive degradation. PprA also stimulates DNA end-joining reactions catalyzed by ATP- and NAD-dependent DNA ligases while inhibiting *E.coli* exonuclease III activity and contribute in

radiation resistance of this organism as pprA mutant is slow growing and highly sensitive to ionizing radiation, MMC and UV-A radiation (Narumi *et al.,* 2004)

1.3.5.1.3 Physical scaffolds for DNA repair

DNA repair proteins in *D. radiodurans* are enzymatically very similar to those in other radiation sensitive bacteria, their remarkable efficiency in assembling DNA fragments may be partially imparted by structural organization. It has been observed that *D. radiodurans* nucloeoid is in more condensed form compare than that of radiation-sensitive species. Genome condensation is thought to preserve DNA linear continuity even in the presence of numerous breaks by (i) restricting the diffusion of DNA fragments, (ii) protecting the fragments from free radicals generated in the cytoplasm by water radiolysis, and (iii) limiting accessibility to degradation enzymes. Apart from restricting the diffusion of broken fragments, genome condensation may also prevent the occurrence of DSBs by stabilizing proximal SSBs and preventing the separation of DNA ends (Englander *et al*., 2004; Levin-Zaidman *et al*., 2003; Minsky, 2006). Transmission electron microscopy images of *Deinococcus* have suggested that nucleoid of this species exists as a "ring-like" body, called toroid and contributes to the radioresistance of the species. DNA toroids correspond to a particularly stable mode of DNA packaging, within which DNA molecules are densely packed and possibly consisting of SMC proteins for packing. It is observed that genomes of all extremely radioresistant species are relatively highly condensed than the radiosensitive species which may help to limit diffusion of fragments generated post-irradiation even in cells incapable of repairing strand breaks, however *D. geothermalis*, a radiation resistance bacterium lack a specific nucleoid organization (Zimmerman and Battista, 2005). Some researcher believes that membrane association of genome might, support the DNA

structure to ensure the correct sequential restitution of the genome (Burrell *et al.*, 1971). Also pre-alignment of juxtaposition of homologous regions of multiple genome copies, would ensure the quick access of the recombinational repair machinery for searching of homology for rapid and error free repair (Minton and Daly, 1995**).** However, molecular studies have shown high levels of recombination between homologous DSB fragments irrespective of their genomic origin, which argues against the existence of structures linking chromosomes (Daly and Minton, 1994 and 1997).

1.3.5.1.4 Recombinational Processes in *D. radiodurans* DNA Repair

Even after exposure to lethal doses of γ radiation, *Deinococcus* remarkably reassembles its genome from the resulting thousands of fragments with high fidelity which was proposed to occur through two homologous recombination processes, ESDSA (Extended synthesis dependent strand annealing) and homologous recombination by crossovers, both of which require RecA recombinase (Zahradka *et al*., 2006; Slade *et al.*, 2009). They suggested that DNA polymerase I (PolA, DR1707) is the key component for the high fidelity of RecA dependent DSB fragment reassembly. At the earliest stages of recovery this enzyme supports a very efficient DNA replication as compared to normal replication process (Zahradka *et al*., 2006).The *polA* paralogs were suggested be involved in the genomic network of resistance to γ radiation and tolerance of desiccation and help in ESDSA (Sghaier *et al*., 2007). DSB repair is initiated by the RecFOR pathway, as *D. radiodurans* lacks RecBC (Misra *et al.*, 2006). The ends of DSBs are presumably processed by UvrD and RecJ into 3'single-stranded DNA substrates, onto which the RecFOR complex loads RecA (Bentchikou *et al.*, 2010). RecA is essential, as for DNA synthesis priming as RadA cannot replace RecA-mediated priming. Following RecA-RadA-catalyzed priming,

DNA Pol III initiates DNA repair synthesis, whereas the elongation step is performed by either (i) Pol III alone /or with Pol I protecting the DNA fragments' integrity following BER. The unwinding of the dsDNA template during D-loop migration may be mediated by UvrD, RecD, RecQ, RuvAB, and/or other helicases, such as Rad54 (DR1258/DR1259). DNA repair synthesis generates long newly synthesized single strands, which progressively dissociate from the migrating D loops, aided by DNA helicases, and can readily anneal with complementary strands (Fig. 6, steps 6 and 7). Whether single strand annealing is a spontaneous process promoted by the proximity of the annealing strands or requires a specialized protein, such as Rad52 in eukaryotes, has yet to be examined. The 3' flaps generated after the annealing of single strands could be incised by SbcCD (Kamble 2010; Hu *et al.,* 2010) (Fig. 6, step 8). The long linear products of ESDSA require RecA-mediated crossovers within overlapping homologies to mature into circular chromosomes (Zahradka *et al*., 2006) (Fig. 6, step 9).

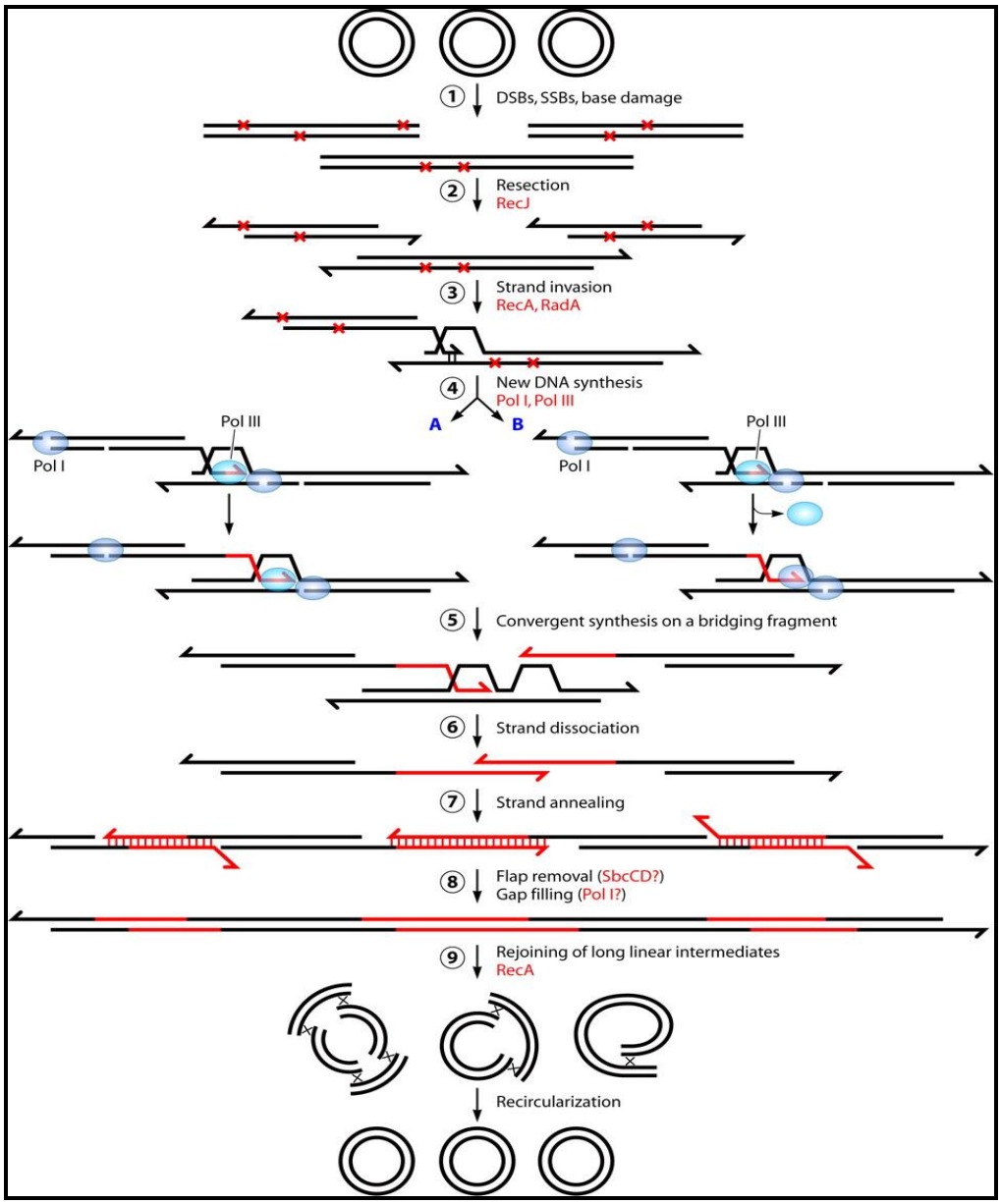


Fig.8 Two step mechanism of DNA repair in *D. radiodurans* shattered by ionizing radiation (Slade *et al.*, 2011)

1.3.5.1.5 Protection of biomolecules from oxidation

Restoration of deinococcal cellular life is results of reconstitution of shattered genome after receiving the lethal doses of DNA damaging agents. It was believed that robust capacity of

DNA repair is key for radiation resistance of *D. radiodurans*, however the facts that yeast and human cells can survive hundreds of endogenous DSBs (Burgoyne *et al.,* 2007), they succumb to ionizing radiation doses that generate the same number of DSBs but which also cause protein damage. Together, these observations suggested that cellular macromolecules other than DNA determine survival following exposure to ionizing radiation. In recent years it has been found that in irradiated bacteria the level of protein oxidation is inversely correlated with survival and that protein oxidation. This suggests that proteins are the primary target of radiation toxicity and protein oxidation is the primarly cause of cell death (Daly, 2007; Krisko and Radman, 2010). In *D. radiodurans* protein protection from oxidative stress is collective effect of (i) cell cleaning through the elimination of damaged (oxidized) macromolecules by either exporting oxidized macromolecule or degradation of oxidized proteins by proteases, (ii) the selective protection of some proteins against oxidative damage, (iii) the suppression of endogenous ROS production, and (iv) Strong antioxidant defense systems. Endogenous ROS production is suppressed by the reduction in the number of respiratory chain enzymes and enzymes with iron-sulfur clusters as the major sources of endogenous ROS (Ghosal *et al.*, 2005). Direct scavenging of ROS by nonenzymatic Mn^{+2}-complex (complexes between Mn^{2+}, orthophosphate, peptides, and possibly nucleosides), by carotenoids (deinoxanthin) and by enzymatic (e.g. catalases, superoxide dismutases, and peroxidases) scavengers that synergistically scavenge all ROS (Daly *et al.*, 2004; Tian *et al.,* 2009; Lemee *et al.,* 1997). The genome of *D. radiodurans* contains the *pqqE* gene involved in the synthesis of PQQ synthase enzyme. Pyrroloquinoline -quinone (PQQ) (4, 5-dihydro-4, 5-dioxo-1H-pyrrolo [2,3-f]quinoline-2,7,9-tricarboxylic acid) has been known as a redox cofactor for periplasmic as well as cytosolic dehydrogenases contributing in the mineral phosphate solubilization (MPS) phenotype in bacteria (Goldstein, 1994) and also PQQ producing

bacteria shown to be more resistant to hydrogen peroxide and gamma radiation effect (Shrivastava *et al.,* 2010). PQQ has been reported to act as an antioxidant *in vitro* (Misra *et al.,* 2004.), in animal system (He *et al.,* 2003) and bacterial system (Khairnar *et al.,* 2003) *in vivo,* and as a member of B group vitamins (Kasahara and Kato, 2003). Recently the possible role of PQQ as an inducer for proteins kinases involved in distinctly different metabolic and physiological processes has been suggested (Khairnar *et al.,* 2007). In bacteria *in vivo* synthesis of PQQ require 6 to 7 gene (PqqA-G), however *pqqE* gene product play crucial role in catalysis of cyclization of tyrosine and glutamate amino acids of precursor polypeptide chain. The catalysis by *PqqE* is a radical driven C-C bond formation required to link the glutamate and tyrosine moieties at atoms C5a and C9a of PQQ (Wecksler *et al.,* 2009) (Fig. 9). The exact mechanism is unknown but the analogy to radical SAM proteins implies the following mechanism: the reduced 4Fe-4S cluster transfers an electron to the sulfur of SAM. The C5'-S+ bond of SAM is cleaved, producing methionine and a highly oxidizing 5'-deoxyadenosyl radical (Fig.10). The radical abstracts a hydrogen atom from tyrosine in PqqA, creating a tyrosine radical at position C9a. The radical reacts with atom C5 of glutamate leading to cyclization. PQQ has been detected in a wide variety of foods and other sources (Stites *et al.* 2000). Quantitative analyses of PQQ by LC/MS/MS showed that free PQQ was present in almost all food samples, in the range of 0.19-7.02 ng per gm fresh weights (for solid foods) and per milliliter in liquid foods (Noji *et al.* 2007).

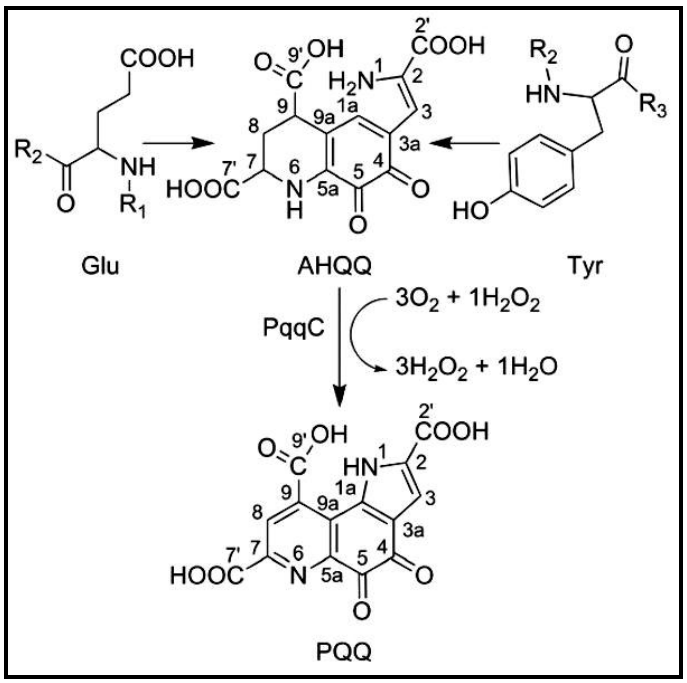


Fig. 9 Formation of PQQ from the fusion of glutamate and tyrosine via the intermediacy of AHQQ (Wecksler *et al.,* 2009)

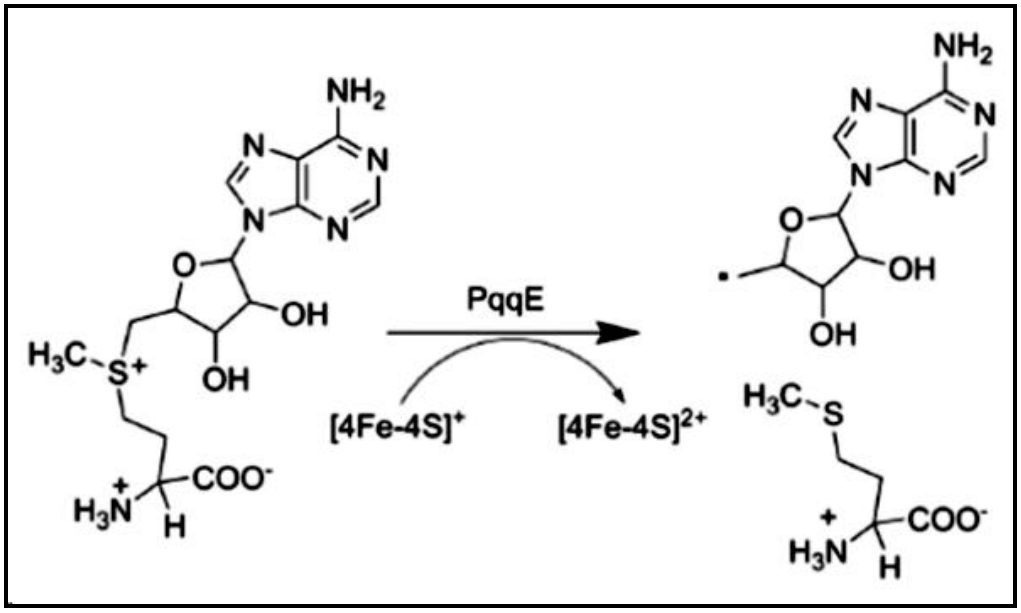


Fig. 10 PqqE catalyzes the reductive cleavage of SAM to form methionine and a 5′-deoxyadenosyl radical. (Wecksler *et al.,* 2009)

1.4 Aims and Objectives

In *D. radiodurans,* ionizing radiation induced DNA repair require temporal regulation of DNA degradation and DNA synthesis. DNA degradation process, immediate after gamma radiation exposer, ought to be highly regulated as the uncontrolled exonucleolytic activity might lead to loss of genetic materials and can hinder in error free reassembling of gamma shattered genome fragments. It has been suggested that competition for DNA free ends between DNA exonucleases and Polymerases (Pol I and Pol III) might limit the DNA degradation. Apart from the meticulous temporal coordination between DNA degradation and DNA synthesis, the dose-dependent extension of the lag period before the onset of DNA replication might suggest the existence of a checkpoint regulation of growth arrest and DNA metabolism during post irradiation receiver of this bacterium (Cox and Battista, 2005). Intestresingly, the SOS response known as a DNA damage response mechanism in bacteria (Walker, 1996; Shimoni *et al.,* 2009) is absent in *D. radiodurans*. Therefore, the possible existence of an alternate DNA damage response mechanism in *D. radiodurans* could be hypothesized. The possibility of a checkpoint control mechanism which could presumably detect and respond to DSB in a manner similar to ATM (Ataxia Telangiectasia, Mutated) and ATR (ATM and Rad3 related) initiated DNA damage checkpoint in eukaryotes (Sancar *et al.,* 2004) may be envisaged. Regulation of these important phenomenons through post translational modification especially by protein phosphorylation has been discussesed in both eukaryotes (Sancar *et al.,* 2004) and prokaryotes (Mijakovic *et al.,* 2006). However, in prokaryotes, DNA damage induced signal transduction through protein phosphorylation is yet to be established. With our very limited knowledge of protein phosphorylation in response to DNA damage, the *D. radiodurans* a bacterium with extraordinary radiation resistance and DNA strand break repair ability, and the presence of large

number of uncharacterised stress response components including eukaryotic type Ser / Thr protein kinases, sensor kinase, offer excellent model system for deciphering role of protein phosphorylation in DNA damage response of *D. radiodurans*. The work porposed in present study has been accomplished in following objectives.

1. Studies on the role(s) of pyrroloquinoline –quinone (PQQ) and five PQQ binding motifs containing proteins in radiation resistance of *D. radiodurans*.
2. Functional characterization of DR2518 protein kinase having PQQ binding motifs and an eSTPK domain.
3. Identification of substrates for DR2518 kinase and the effect of protein phosphorylation on the known functions of PprA, a representative substrate.

CHAPTER 2

MATERIALS

AND

METHODS

2.1 Materials

2.1.1 General chemicals

Dehydrated components of culture media (Bacto-tryptone and Bacto-yeast extract) and agar-agar (Bacto-agar) powder were from Difco Laboratories, USA. Fine chemicals were from Sigma Chemical Company, USA or GIBCO-BRL, USA or USB-Amersham, UK, or E.Merck, Germany or Roche Biochemicals GmbH, Germany or Pharmacia LKB, Sweden or S.R.L. Ltd., India or Bangalore Genei (P) Ltd., India. Inorganic salts, urea, organic solvents and acids of Excel-R or Anal-R quality were from Qualigens (Glaxo) India Ltd. or E.Merck India Ltd. Molecular Biology Grade phenol was purchased from SRL, India. Diethyl pyrocarbonate (DEPC), SYBR Green 2X master mix, 2X PCR ready mix, sodiumdodecylsulphate (SDS), 2-mercaptoethanol (2-ME), sodium sarcosinate, phenylmethanesulphonylfluoride (PMSF), agarose, hydrogen peroxide, NADPH, triton X-100, ethidium bromide, Murashige and Skoog medium, ampicillin, Tri reagent RT and protenase K were purchased from Sigma Chemical Company, USA.

2.1.2 Enzymes and other molecular biology reagents

T4 Polynucleotide Kinase, T4 DNA ligase, restriction endonucleases, Exonuclease III, Alkaline Phosphatase, cDNAase I (from bovine pancreas), RNAase (from bovine pancreas), DNA polymerase (Klenow fragment) and random hexamer primers were from New England Biolabs, USA or Bangalore Genei (P) Ltd., India or Roche Biochemicals GmbH, Germany. Lysozyme (from chicken egg-white) was from Sigma Chemical Company, USA. *Taq* DNA Polymerase was obtained from Bangalore Genie Pvt Ltd, Bangalore. *Pwo* DNA polymerase and deoxynucleotidetriphosphates (dNTPs) were from Roche Biochemical Germany. DNA molecular weight standards were from Bangalore Genei (P) Ltd., India. PCR extraction kit, Gel

extraction kit and RNA extraction kit were from QIAGEN, Germany. All the primers used in this study were synthesized through Metabion, Germany.

2.1.3 Radionucleotides and photographic materials

[^{32}P] γ-ATP and [^{32}P] H_3PO_4 were from Board of Radiation and Isotope Technology (BRIT), India. X-ray films of medical type, polyester based, double-sided were from kodak films, colorado, USA.

2.1.4 Antibiotic stock solutions

Antibiotic	**Stock Solution (mg/ml)**	**Working Concentration (μg/ml)**
Ampicillin	100 in sterile DW	(100) a
Chloramphenicol	34 in Ethanol	(3-5) b
Kanamycin	25 in sterile DW	(5-30) a,c

Notes :

a - 100 μg/ml used for high copy vectors such as pET and pBluescript.

b - used for maintenance of plasmid in DEIRA cells and for growing knockout strains of DEIRA

c - for maintaining plasmid in DEIRA and its knockout derivatives

2.1.5 List of bacterial strains used in this study

Bacterial strains	Genotype	Source
Deinococcus radiodurans R1	Wild type	Schafer *et al* .,2000
D. radiodurans Y1	*pqqE* disruption mutant of *D. radiodurans* R1 Kan^R	This study
D. radiodurans Y2	*dr2518* knockout mutant of *D. radiodurans* R1 Kan^R	This study
D. radiodurans Y3	*dr1769* knockout mutant of *D. radiodurans* R1 Kan^R	This study
D. radiodurans Y4	*drC0015* knockout mutant of *D. radiodurans* R1 Kan^R	This study
D. radiodurans Y5	*dr0503* knockout mutant of *D. radiodurans* R1 Kan^R	This study
D. radiodurans Y6	*dr0766* knockout mutant of *D. radiodurans* R1 Kan^R	This study
D. radiodurans Y1RAD	pRADpqqE in *D. radiodurans* Y1, Kan^R , Cm^R	This study
D. radiodurans Y2RAD	pRAD2518 in *D. radiodurans* Y2, Kan^R , Cat^R	This study
D. radiodurans Y2P1	pRADK42A in *D. radiodurans* Y2, Kan^R , Cat^R	This study
D. radiodurans Y2P2	pRADS162A in *D. radiodurans* Y2, Kan^R , Cat^R	This study
D. radiodurans Y2P3	pRADT169A in *D. radiodurans* Y2, Kan^R , Cat^R	This study
D. radiodurans Y2P4	pRADS171A in *D. radiodurans* Y2, Kan^R , Cat^R	This study
E. coli DH5alpha	*F- / endA1 hsdR17 glnV44 thi-1 recA1 gyrA relA* Δ *(lacIZYA-argF) U169 deoR (Φ80dlac*Δ *(lacZ)M15)*	New England Biolabs
E. coli BL21	*F- ompT gal [dcm] [lon] hsdS_B DE3::T7RNA polymerase gene*	New England Biolabs

2.2 Methods for microbiological studies

2.2.1 Maintenance of the bacterial stocks

Bacterial stocks were maintained at RT and under frozen conditions. Room temperature stocks were made on soft agar LB medium containing 0.2 % glycerol. Stabs were prepared by piercing with needle wire containing bacterial cells on semisolid sterile LB agar medium and preserved at RT. Alternatively, bacterial culture stocks were preserved at freezing temperature in presence of cryoprotectants. The logarithmically growing culture was spun and resuspended in minimal medium and /or LB medium to cells density of 10^9 cells per ml. The cell suspension was mixed with 20% glycerol in polypropylene screw capped tubes and stored in freezing temperature tolerant boxes, at –70^{o}C. The stocks were numbered and entered in the bacterial culture book with full details.

2.2.2 Growth and CFU measurement of the bacterial strains

Different bacterial strains used in this study are summarized in Table 2.1.5 section. Bacterial culture was taken from long-term storage stocks directly on LB / TGY agar plates and or plates containing appropriate antibiotics if required. The plates were incubated at appropriate temperature overnight. Single isolated colonies were inoculated in broth with or without antibiotics and allowed to grow at 37^{o}C for *E. coli* and 30^{o}C for *Deinococcus radiodurans* at shaking speed of 150 rpm overnight. The overnight grown liquid culture was diluted to 1:100 in fresh broth with selection pressure if required, in the flasks having 10 times the air space to the volume of the medium. The culture was allowed to grow with shaking at 200 rpm at appropriate temperature. The aliquots were drawn at different time intervals and optical density was measured at 600 nm. In parallel an appropriate dilutions were plated on agar plates and the

numbers of colonies were counted. The growth curve drawn under defined set of conditions was used in determining the different growth phases of the culture for different experiments. Total number of colonies appeared on agar plates after different treatments of *Deinococcus radiodurans* cells were taken for calculating the colony forming unit (CFU).

2.2.3 Radiation treatment of *Deinococcus*

Deinococcus radiodurans cells were grown to early stationary phase and mid log phase as required, in TGY medium. The cells were collected and washed with sterile normal saline. The cells were resuspended in half volume of fresh TGY broth/ or saline and irradiated at different doses 6.82kGy per hr (Gamma 5000, ^{60}CO, Board of Radiation and Isotopes Technology, DAE, India). The cells were diluted appropriately and plated on TGY agar plates. For UV treatment, the cells plated on agar plates were directly irradiated with different doses of UV radiation. Mitomycin C (20µg/ml) as described in (Khairnar *et al.,* 2008). The plates were incubated at 30^{o}C for at least 36h for recording total viable counts in response to specific treatments.

2.2.4 Bacterial transformation

Escherichia coli

In *Escherichia coli*, the competence of DNA uptake can be artificially induced by chilling of the culture in presence of the $CaCl_2$. Competent cells were prepared using the standard protocol. In brief, the overnight grown culture of desired *E. coli* strain was diluted to 100 fold in fresh LB medium. The culture was allowed to grow at 37^{o}C till OD_{600} reaches to 0.3 to 0.4 and thereafter the cells were chilled on ice for 10 min. The culture was transferred to pre-chilled SS34 tubes and centrifuged at 4000 rpm for 5 min at 4^{o}C. The pellet was gently suspended in 10ml of

100mM $CaCl_2$ and suspension was incubated on ice for 30 min. Thereafter it was centrifuged at 5000 rpm for 10 min and competent cells were gently suspended in 0.1 culture volume of 100mM $CaCl_2$. The competent cells were stored on ice for two to three hours before they were used for transformation. These competent cells can be stored on ice till 16 h and at –70^oC in 20 % glycerol for one month without much loss of competence. *(Note: an eye shape structure in the center of the competent cells pellet indicates the good quality of competents*). The 100µl of the competent cells were aliquoted in 1.5 ml pre-chilled tubes and to it approximately 100ng of the plasmid DNA was added and mixed by tapping. The mixture was incubated on ice for 30 to 45 min and heat shocked at 42^oC for 1 min followed by 2 min on ice incubation. The transformation mixture was diluted with 800µl of 1x LB broth. For the expression of antibiotic resistance genes, the mixture was incubated at 37^oC for 45-60 min. The transformation mixture was plated on LB agar plates containing appropriate antibiotics. The plates were incubated at 37^oC for overnight and transformants were scored and characterized.

Deinococcus radiodurans

D. radiodurans is naturally transformable in all phase of cells growth. However treatment of *D. radiodurans* cells with 40mM $CaCl_2$ increases transformation efficiency drastically. Log phase culture was treated with $CaCl_2$, final concentration 40mM and allowed to grow at 30^oC with vigorous shaking for 30 min. 1 ml $CaCl_2$ treated culture mixed with 100ng of DNA and allowed to sit on ice for 10 min. dilute culture 10 fold and allowed to grow overnight with vigorous shaking. Next day the transformation mixture was plated on TGY agar plates containing appropriate antibiotics. The plates were incubated at 30^oC for 48-72 hr and transformants were scored and characterized.

2.3 Methods for molecular studies

2.3.1 Isolation of plasmid DNA (mini prep)

Plasmid DNA was prepared using alkaline lysis method as described in Sambrook *et al.*, 2001). In brief, the bacterial cells were grown overnight in 1.5 ml 2x LB broth with appropriate antibiotics. The cells were spun at 10,000 rpm for 20 sec and washed with TBS. The pellet was suspended thoroughly in 100 µl ice chilled GTE and 200 µl of freshly prepared alkaline SDS (0.2N NaOH, 1% SDS) was added. The mixture was incubated at RT for 5 min and 150µl pre-chilled potassium acetate (5M) was added and mixed thoroughly and quickly. The mixture was incubated on ice for 5 min and then spun at 10,000 rpm for 10 min. The supernatant was extracted with equal volume of chloroform: isoamylalcohol (24:1) and 0.6 volume of isopropyl alcohol were added. The contents were mixed and incubated at RT for 20 min before plasmid DNA was collected by centrifugation, washed and air-dried.

2.3.2 Large scale preparation of plasmid DNA

Large scale preparation of plasmid DNA was carried out as described in Sambrook *et al*., 2001. In brief, cells harboring high copy plasmid DNA were grown overnight with vigorous aeration in 50 ml 2x LB broth medium. The low copy number plasmid harboring bacterial culture was grown overnight in presence of antibiotics and then diluted 1:100 in 500 ml LB with antibiotic and allow to grow till OD_{600} was 0.4. Appropriate antibiotic was added in the mid exponential phase culture and allowed to grow for 12-16h at 37^{o}C with vigorous shaking. Cells were harvested and washed with TBS. The plasmid DNA was prepared using alkaline lysis method. The cell pellet was completely suspended in 4 ml GTE buffer and to this 8 ml alkaline SDS solution was added. Contents were gently mixed and incubated at RT for five min. The

nucleoprotein-SDS complex was precipitated with 6 ml of pre-chilled 5M potassium acatate (pH 5.6) solution on ice for 5 min. The supernatant was collected by centrifugation at 12K for 10 min at 4^{o}C and RNaseA (50µg/ml) was added and extracted with equal volume of CHCl3: IAA (24:1). The upper layer was taken and DNA was precipitated with 0.6 volume of isopropanol at RT for 20 min. The DNA was collected by centrifugation at 12 K for 10 min and washed with 70% ethanol followed by absolute alcohol. The pellet was dried and DNA was dissolved in sterile water. The plasmid DNA concentration and purity were determined by measuring the OD_{260} and OD_{280}, spectrophotometrically. The purity of DNA preparation was assured by finding the ratio of A_{260}/A_{280}. (A_{260} of 1.0 corresponds to 50µg/ml DNA, A_{260}/A_{280} ratio indicates the purity of the DNA preparation, the ratio more than 1.65 showed the DNA free from proteins).

2.3.3 Protocol for plasmid isolation from *Deinococcus*

The overnight grown culture was spun, washed with 70% ethanol and resuspend in 300 microlitre GTE containing 10mg/ml lysozyme. The mixture was incubated for 30 min at RT/37^{o}C. The 5mg/ml proteinase K was added and suspension was incubated at 37^{o}C for 10 min, 50^{o}C for 30 min, ice for 5 min and at 100^{o}C for 1 min. Lysis was achieved by addition of 600 microlitre of solution II (1% SDS and 0.2N NaOH). Chromosomal DNA and proteins were precipitated with ice chilled 450 microlitre of solution III on ice for 5 min. Mixture was centrifuged at 12K spin for 15 min and the supernatant was extracted twice with equal volume of phenol:chloroform: IAA (25:24:1), once with chloroform:IAA (24:1) and spun at 12k for 15 min. The upper phase was precipitated with 2 volume of ice chilled ethanol followed by washing with 70% ethanol and then 100 % ethanol. Dry the pellet and dissolve in 30µl sterile water. An aliquot of 3 µl was anlysed on agarose gel.

2.3.4 Genomic DNA isolation

Escherichia coli

Bacterial chromosomal DNA was isolated by using protocol as described earlier (Clark *et al.*, 1981). In brief, the cell pellet was washed with TBS and preserved at –70^{o}C if not processed immediately. The pellet was thawed at 4^{o}C and homogenized in 1/10th volume of 25 % sucrose and lysozyme (200 µg/ ml) was added and mixed properly. The mixture was incubated for 10 min on ice and 10mM EDTA (pH 8.0) was added and incubated for 5 min on ice. The cells were lysed with 0.5% SDS and mixed gently to get clear lysate. The RNase A (50µg/ml) was added and incubated at RT for 20 min. The lysate was extracted with equal volume of phenol (pH 8.0) till white precipitate stops coming. The aqueous phase was extracted once with phenol: $CHCl_3$: IAA (25:24:1) and once with CHCl3: IAA. The aqueous phase was mixed with 0.3M NaCl and 2.5 volume of chilled ethanol was added from the side of the tube to layer on the top of the DNA solution. The high molecular weight DNA was recovered by spooling on sterilized glass rod. The spooled DNA was washed with 70% ethanol and air-dried. The total DNA was recovered by centrifugation and washed with 70 % ethanol. The DNA pellet was dissolved in sterile 10 mM Tris-HCl, pH 8.0.

Deinococcus radiodurans

The 100 ml culture was overnight grown at 150 rpm at 30^{o}C. The cells were collected, washed with 70% ethanol and resuspended in 1ml TE Buffer; add 2mg/ml lysozyme (stock 10mg/ml) and incubated at 37^{o}C for 30 min. The 2mg/ml proteinase K and 2% SDS was added and incubated at 50^{o}C for 3 hours. Supernatant was extracted with equal volumes of phenol-chloroform, incubated on ice for 10 min. Mixture was centrifuges at 10K for 10 min. The

aqueous layer was extracted with equal volumes of 24:1 mixture of chloroform-isoamyl alcohol. The supernatant was mixed with $1/10^{th}$ volume of 3M sodium acetate pH 4.5 & 2 volumes of ethanol and incubated at $-20^{o}C$ for ½ hr. DNA was collected at 15K for 15 min and dissolved in sterile water. DNA concentration was ascertained with OD_{260} and on agarose gel.

2.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the qualitative and quantitative analysis of DNA. The agarose was dissolved in required buffer by heating in microwave oven and the solution was cooled to around $50^{o}C$ before ethidium bromide (0.5µg/ml) was added and poured. It was allowed to solidify at RT for 30 min. The gel along with the platform was taken and set in the running tank. The buffer was added to submerge the gel. The samples mixed with gel loading dye solution were loaded and electrophoresed. After the electrophoresis has completed, the DNA bands were visualized on UV transilluminator (UVP Model 3UVTM Transilluminator) at different wavelengths or with hand UV lamp (Model UVGL-58) depending on the necessity of the experiment. The gel was photographed under short wavelength UV on gel documentation system (SYNGENE GeneGenius).

2.3.6 PCR amplification

2.3.6.1 Primer design and synthesis

Primers were designed manually using the corresponding nucleotide sequences from *E. coli* and *Deinococcus radiodurans* genomes database. The sequence of the DNA to be amplified was taken and restriction enzyme analysis was carried out using Bioedit software. The unique restriction sites and restriction endonucleases were noted and suitable restriction endonuclease

sites were incorporated into the forward and reverse primers sequence. Normally forward primers were the sequences as that of the target sequence and reverse primer sequences were the complementary sequence of the target sequences. The palindrome sequences specific to the non-cutting restriction enzyme of this DNA fragment, were adjusted to the maximum sharing with the 5' end of the gene specific sequence. Further some more sequences that are required by the corresponding restriction endonucleases to bind on the restriction site were added at the upstream to the 5'end of both the primers. The composition of the primers was adjusted to have at least 50 % G+C content (wherever possible). In any case the minimum G+C content in the primer was not less than 40 %. The approximate Tm values were determined as Tm= 2x (A+T) +4 x (G+C). Care was taken to avoid repetitive sequences at the 3' end which might results in primer dimer formation and 3' base was necessarily kept A or T while C or G was preferred as penultimate base. The complete primer sequence was subjected for its match on the other sites of the chromosome. The mismatch was adjusted in such a manner that 6-9 nucleotide of the 3' end should not have perfect match on any other site on the chromosome. The primers were commercially synthesized and purified to best purity by manufacturers.

2.3.6.2 PCR reactions

Polymerase chain reaction is a very sensitive method for the amplification of a DNA fragment of a few kilobases from giant size genomes of single cell. Therefore, traces of contamination of impurity could give false positive results. Therefore special precautions are necessary while preparing the components for the PCR reactions. PCR amplification was mostly carried out in 50µl volume. A typical PCR reaction contains template (50ng), primers 400nM, dNTPs 200µM, Taq DNA polymerase buffer 1X, 1.5 mM $MgCl_2$ and Taq DNA polymerase 2.5U.

Taq DNA polymerase is relatively an error prone enzyme and catalyses DNA polymerisation with an average error of one nucleotide per 1000 bases, which increases if conditions are not optimal. It also leaves 'A' overhang at the 3' end during cyclic polymerization which sometimes becomes the technical problem for blunt end cloning and additional steps of end flushing is required. So *Pwo* DNA polymerase, which has high fidelity and devoid of 'A' overhang adding activity, was, used in several of the PCR reactions especially for PCR product used in sequencing and in blunt end cloning.

2.3.6.3 PCR product purification

PCR products were separated on molecular biology grade high melting point (HMP) agarose. After electrophoresis is complete, the DNA was visualized with handset UV lamp in long wavelength and DNA band was cut with sterile blade. DNA extraction from HMP agarose gel pieces was carried out either by commercially available gel extraction kits using manufacturer's protocols or by freezing – thawing method. The frozen gel pieces were spun at 12K for 10min and supernatant was transferred in a separate 1.5 ml tubes. The DNA was extracted with equal volume of phenol: CHCl3: IAA (25:24:1) till white precipitate stops coming and finally precipitated with salt and ethanol. Gel extraction kits were also used from QIAGEN, Roche Biochemicals, Sigma Chemicals Company and the respective kits protocols were used in this study.

2.3.7 DNA manipulation

2.3.7.1 Restriction digestion

The protocols followed for restriction digestion with a particular enzyme were largely as described by manufacturers. The restriction digestion of DNA was carried out in presence of the

specific buffers and minimum amount of enzymes required for the digestion of that DNA preparation. However, the complete digestion of the DNA was ensured by using 5-10 U enzyme for 1 μg of DNA. STAR activity of certain restriction enzymes has been kept in mind during restriction digestion of the DNA. The ratio of enzyme to DNA and glycerol concentration was kept less than 10%. Care was also taken specifically to avoid STAR activity. The double digestion of DNA with two different restriction endonucleases requiring same buffer was carried out separately, using one enzyme, for one hour and then the second enzyme was added and incubated for a further period of 3-5 h. An aliquot of each digest was taken and analysed on agarose gel along with double digestion mixture to make sure that both the enzymes have activity under selected reaction conditions. Restriction digestion has been mostly carried out in 10-30μl for chromosomal DNA and for vector preparation for cloning work; the reaction was carried out in 50-100μl. After every digestion reaction, the samples were analysed on agarose gel to make sure of enzyme activity.

2.3.7.2 Different vectors used in this study

Diferrent plasmids used in this study are summarized in following table.

List of plasmids used in this study

Sr No.	Name of the plasmid	Characteristics	Source
1	pBluescript SK+	Amplicilin, 2.930kb, *colE1*	Stratagene
2	pET28a+	Kanamycin, 5.320kb, colE1	Novagene
3	pRADgro	The *groESL* promoter cloned in pRAD1 , 6.885kb	Misra *et al*., 2006
4	pNOKOUT	900bp nptII in pBluescript SK+, 3.9kb	Khainrar *et al*., 2008
5	pET2518	*BamH1-EcoR1* fragment of *dr2518* CDS cloned	This study

		in pET28a+	
6	pNokpqqE	500bp upstream and downstream of *pqqE* gene in pNOKOUT, 6.0kb	This study
7	pNOK2518	1kb upstream and downstream to *dr2518* CDS in pNokOUT s, 6.0kb	This study
8	pNOK1769	1kb upstream and downstream to *dr1769* CDS in pNokOUT s, 6.0kb	This study
9	pNOK0503	1kb upstream and downstream to *dr0503* CDS in pNokOUT s, 6.0kb	This study
10	pNOK0766	1kb upstream and downstream to *dr0766* CDS in pNokOUT s, 6.0kb	This study
11	pNOKC0015	1kb upstream and downstream to *drC0015* CDS in pNokOUT s, 6.0kb	This study
12	pPQCAT	NptII cassette was replaced with *cat* cassette in plasmid pNokpqqE	This study
13	pET2518K42A	*BamH1-EcoR1* fragment of *dr2518K42A* cloned in pET28a+	This study
14	pET2518S162A	*BamH1-EcoR1* fragment of *dr2518S162*cloned in pET28a+	This study
15	pET2518T169A	*BamH1-EcoR1* fragment of *dr2518T169A* cloned in pET28a+	This study
16	pET2518S171A	*BamH1-EcoR1* fragment of *dr2518S171A* cloned in pET28a+	This study
17	pGroK42	*Apa1-Xba1* fragment of *dr2518K42A* cloned in pRADgro	This study
18	pGroS162	*Apa1-Xba1* fragment of *dr2518S162A* cloned in pRADgro	This study
19	pGroT169	*Apa1-Xba1* fragment of *dr2518T169A* cloned in pRADgro	This study
20	pGroS171	*Apa1-Xba1* fragment of *dr2518S171A* cloned in pRADgro	This study
21	p11559	Spectinomycin, 11kb	Lecointe *et al.*, 2004
22	p11 wt.pprA	NdeI-XhoI fragment of *pprA* gene cloned in p11559	This study
23	p11pprA T72A	NdeI-XhoI fragment of *pprA* gene cloned in p11559	This study

2.3.7.3 Ligation

The random collision of different molecules determines the efficiency of ligation. So to increase the rate of reaction the higher molar ratio of vector to insert was taken for ligation. For sticky end ligation, normally the insert to vector molar ratios were set to 2:1 to 3:1. The amount of the insert is fixed to around 100ng and then vector amount was adjusted to a particular ratio, according to the size of the vector. For the blunt end ligation reaction, the amount of the insert was increased to 500ng and then amount of vector was adjusted to meet the molar ratio of inserts to vector of 3:1 or 4:1. The required amount of the vector and inserts were mixed in one tube and precipitated with salt and ethanol. The precipitated DNA pellet was washed twice with 70% ethanol and once with 100 % ethanol. The air-dried DNA pellet was dissolved in minimum volume of sterile water. The mixture was heated at 55^{o}C for 5 min and chilled quickly on ice. The ligation conditions for sticky end ligation and blunt end ligation are different. The blunt end ligation is carried out at 20^{o}C for overnight in presence of low concentration of ATP and 2 % PEG6000. On contrary, cohesive ends ligation requires 1mM ATP at 16^{o}C for overnight. However, excess ATP inhibits ligation reaction. A typical ligation reaction mixture consist of 100ng insert and vector to give 3:1 molar ratio, 1x T4 DNA ligase buffer (Bangalore Genei (P) Ltd.), 1 Weiss unit T4 DNA ligase in 10 µl reaction and incubated at 16^{o}C for overnight.

2.3.8 Pulsed field gel electrophoresis (PFGE)

2.3.8.1 PFGE agarose gel preparation and electrophoresis

1.6 gm low EEO agarose was melted in 200 ml of 0.5X sterile TBE and solution was cooled to nearly 40^{o}C and poured in gel casting assembly set up as per the instruction given in

manufacturer's protocols. Sample plugs were carefully loaded in agarose wells and sealed with a layer of agarose on top of the wells. The gel was placed in tank containing 3 litres of 0.5X TBE in E/W orientation and electrophoresis was started as per the following programs.

Phase	N/S pulse (Seconds)	E/W pulse (Seconds)	Durans (hr)
1 of 6	05	05	4.00
2 of 6	10	10	3.30
3 of 6	30	30	3.30
4 of 6	50	50	4.00
5 of 6	90	90	5.00
6 of 6	120	120	4.00

2.4 Methods used in proteins biochemistry

2.4.1 Inducible expression of genes in *E. coli*

E. coli strain BL21 DE(3) pLys*S* harboring pET2518/or its point mutants were grown overnight in the presence of kanamycin (25μg/ml). The cells were diluted in fresh LB medium containing antiboitics and allowed to grow for 3-4 h to get density OD600 0.3 and then IPTG (0.5mM) was added and growth was continued for a further period of 5h at 20^{o}C. A small aliquot (200μl) was drawn for checking for the inducible synthesis of respective protein and remaining cells were harvested, washed and preserved at –70^{o}C for downstream processing. For protein analysis the cells were collected and suspended in 50μl TE buffer. To it 50μl of 2x Laemmellie SDS gel buffer was added and mixture was heated at 95^{o}C for 5min and spun at 10k for 10min. The supernatant was loaded on SDS-PAGE and electrophoresis was conducted.

2.4.2 SDS-PAGE analysis of proteins

Polyacrylamide gel electrophoresis is being used as a powerful tool to characterize unknown proteins from biological systems, on the basis of their mobility under electric field. In PAGE

these proteins migrate according to mass / charge ratio and structural topology of that species. However, if charge is unified then these proteins will migrate on the basis of their masses and that would help in determining their molecular weights. Sodium dodecyl sulfate (SDS) is an ionic detergent which denatures proteins by "wrapping around '' the polypeptide backbone. SDS binds fairly specifically in a mass ratio of 1.4:1 and confers a negative charge to the polypeptide. Individual polypeptide chains form a complex with negatively charged molecules of sodium dodecyl sulphate and therefore migrate as a negatively charged SDS-protein complex through the pores of polyacrylamide gel. In denaturing SDS-PAGE therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

2.4.3 Preparation of SDS-PAGE gel

SDS-PAGE gel was prepared in two different ways. (i) Ordinary SDS-PAGE having the single concentration of the gel and (ii) gradient SDS-PAGE from 7- 20 % acrylamide concentrations. The ordinary gel with uniform 10 % Acrylamide concentration was made as below.

Composition of 10 % resolving polyacrylamide gel

Acrylamide (30%)	6.7ml
1.5M Tris-HCl, pH-8.8	5.6 ml
10 %SDS	200 µl
10 % ammonium persulfate	200 µl
TEMED	15 µl
Water	5.3 ml
Total volume	20 ml

Composition of 5% stacking gel

Acrylamide (30%)	0.83 ml
1.0M Tris-HCl, pH-6.8	0.63 ml
10 %SDS	50 µl
10 % ammonium persulfate	50 µl
TEMED	10 µl
Water	3.4 ml
Total volume	5 ml

For making gradient PAGE gel, the components for 7 % and 14 % solution were mixed separately as per following compositions.

	7%	**14%**
Distilled water	9.5 ml	---
Sucrose	---	3.4 gm
Acrylamide (30%)	4.7 ml	11 ml
Tris-HCl, pH 8.8 (1.5M)	5.6 ml	5.4 ml
SDS (10%)	0.2 ml	0.2 ml
Ammonium per sulfate (10%)	0.080 ml	0.060 ml
TEMED (100%)	0.010 ml	0.010 ml
Total volume	20 ml	20 ml

2.4.4 Procedure of gel pouring

Glass plates were cleaned with liquid soap, rinsed with water and wiped with 70% ethanol. The plate sandwich made with 1.5 mm spacers and assembled with gel casting stand. Plates were tightened with clamps and make sure that the screws are properly fitted. Gaps were sealed with agar and SDS-PAGE mixture for separating gel was poured. It was over layered with water-saturated n-butanol. For making gradient gel, both the solutions were poured in separate chambers of the gradient maker and connecting valve was open. The dense solution was filled in chamber connected to glass plates and was stirred on magnetic stirrer. The outlet of the first chamber containing dense solution was opened and outlet tube was fitted to glass plate sandwich. The pouring was continued till all the content of both the chambers were finished. Following to that a thin layer of water-saturated butanol was layered on top of the gel. The gel was allowed to polymerise for 30 min. (The polymerizing time should be extended if required as partially polymerized gel does not give well separation and wells does not form correctly). After 20-30 min when gel is polymerised, the butanol was removed and washed with water. Stacking mixture was poured and comb was fitted in the gel. Wells were cleaned with water 3-4 times and then

samples were loaded. The samples were prepared by heating equal volume of sample and 2X SDS gel loading dye at 95^{o}C. The denatured samples were spun at 12 k for 10 min and clear supernatant was loaded in the gel. The electrophoresis tank was filled with electrophoresis buffer (10% SDS-Tris-Glycine, pH 8.8) and gel was run for 1 h at 100 volts and then at 200 volts for 3 h or until tracking dye reaches to the ends. Gel assembly was dismantled; gel was taken out in plastic container and stained with different dye depending upon requirement.

2.4.5 Coomassie Brilliant Blue staining

Coomassie blue dye binds with the basic amino acids of the polypeptide. This is an irreversible method for protein staining. The common procedure used for staining gel by this method is as below. In brief, the gel was fixed for 10 min in destain solution I (DSI) (50% methanol + 12.5 % Glacial acetic acid). DS I was discarded and Coomassie strain solution was added. Staining was continued for 10 min or till bromophenol blue dye changes color to yellow. The stain was poured back for repeated use and gel was submerged in destain solution II (DSII) and changed couple of times until the background become colorless.

2.4.6 Silver staining methods of protein gel

Silver staining is a highly sensitive method for the staining of the proteins and nucleic acids. The staining relies of the affinity of the silver ions for nucleophilic and aromatic groups. Silver ions are reduced to metallic silver, which under alkaline condition gives brilliant brown precipitate. This procedure can be used for staining of biomolecules in PAGE gels and agarose gels. This staining method is extremely sensitive and a protein band of 1ng can be detected. For staining, wash the gel twice for 30 min each in 10% acetic acid, 50% methanol and 0.05% HCHO solution

or once over night with gentle shaking. Replace the solution with the 50 % ethanol and wash it twice for 20 min each. After discarding of ethanol, the gel was rinsed with de-ionised water for 10 sec. Then add (0.2 gm/L) sodium thiosulphate solution and keep it on shaker for exactly 1 min. Further rinse the gel with deionised water for three times for 20 sec each. The gel was incubated with silver nitrate (2gms/L) and 0.04 % HCHO for 45 min in dark. Further, rinse the gel with deionised water three times for 5sec.-10sec each under dark conditions to wash the extra stain from the gel. Add developer solution (10 mg/L) sodium thio-sulphate, (0.25 ml/L) 30% HCHO in (60gms/L) of $NaCO_3$] to the gel and keep it on shaker until the spots develops. Further add [Tris (50 gms / L in acetic acid (25 ml / L)] for 5 min. The gel is stored in water followed by in DS-I.

2.4.7 Preparation of cell free extract of *E. coli*

The cell pellet was thawed on ice and 1 gm pellet was suspended in 2 ml lysis buffer A (20mM Tris-HCl, pH 7.5; 0.1mM EDTA and 1mM DTT). Lysozyme was added to a final concentration 200µg/ml and incubated on ice for 20 min. To it, Triton X-100 was added to a final concentration of 1% and sonicated at 4^oC and 50% duty cycle, for 3 min with the 1 min intermittent cooling on ice. Suspension was diluted to two folds and centrifuged at 12k (SS34, Sorvall) for 15 min. The clear supernatant was used for salt fractionation using ammonium sulfate.

2.4.8 Preparation of cell free extract of *D. radiodurans*

Cell pellet was resuspended in 2 ml of suspension buffer (20mM Tris-Cl pH-7.6, 50mM NaCl, 1mM EDTA, 0.5mM DTT, and 0.5mM PMSF). To this lysozyme was added to a final

concentration of 2 mg/ml and incubated at 37^{o}C for 30min. The cell lysate was subjected to freeze-thaw cycles in liquid nitrogen. After this, cells were sonicated using 30 sec. pulse cycles with intermittent cooling for 1 min each, at 40% duty cycle for total time of 10 min. Cell lysate was centrifuged at 12,000g for 20 min. and clear cell free extract was used as soluble protein fraction.

2.4.9 Metal affinity purification

Immobilized Metal ion affinity Chromatography exploits molecules affinity for cheated metal ions. The hexa-histidine tag present in recombinant protein forms complexes with transition metal ions such as Cu_2+, Zn_2+ and Fe_3+. Chelating sepharose fast flow with a suitable immobilized metal ion e.g.; Ni_2+ selectively retained the recombinant protein with exposed histidine. Exposed Cysteine and Tryptophan residues may also be involved in the binding to immobilized metal ion but their contribution to the binding is much lower than the contribution of exposed histidine residues. The strength of binding is affected by buffer pH and the metal ion selected. The most important factor affecting proteins binding is the pH; at more alkaline pH binding will most likely be via deprotonated amino groups. High concentration of salt should be present to quench any ion exchange effect; usually a concentration of 0.5 to 1 M NaCl is sufficient. For purification of protein under native condition the proteins were extracted by different methods, such as sonication of cells pretreated with non-ionic detergent, by using cell lytic express (Sigma Chemicals Co). The clear supernatant was loaded onto the columns packed, charged with $NiCl_2$, and washed, equilibrated with buffer in which the proteins were suspended. Proteins were loaded on the column at flow rate of 1 ml/ min. Columns were washed with

NINTA wash buffer for native proteins and eluted as described in NINTA agarose native protein purification protocols.

2.4.10 Protein estimation

Using different calorimetric and spectrophotometric methods, the presence of proteins in a particular solution was checked, both qualitatively and quantitatively. Absorbance maxima of proteins at 280 nm could be exploited for detecting the presence of proteins in eluted fractions. Other calorimetric method used for the estimation of proteins was dye- binding method (Bradfrod, 1976) using Bradford dye (BioRad) kit protocols. In brief, 200µl of the 5x dye solution was mixed with 800µl of total solution containing proteins and buffer. The content was mixed by vortexing and left at RT for 15 min before color development was monitored spectrophotometrically at 595nm. The OD_{595} was compared with a calibrated curve that was made using standard concentration of BSA. When the concentration of the protein in samples is beyond the linearity range (10µg) of the Bradford dye method, the protein was estimated using Lowry methods (Lowry *et al*., 1951). In brief, 100 µl of the sample (sample + buffer) was taken and 1.0 ml of Lowry stock reagent was added in each tube. Mixed the content and incubated at RT for 30 min. 100µl of Folin's reagent was added to each tube and incubated for 30 min at RT. The absorbance was read at 595 nm.

2.4.11 *In-vitro* protein kinase activity assays

Detection of autophosphorylation of *dr2518* kinase and K42A, S162A, T169A and S171A mutant derivatives, can be checked by either immunodetection using phospho-Ser/Thr epitope specifc antibody, or by autoradiography using [32p]-γ-ATP. Purified proteins were incubated

with cold ATP for immunodetection or with [32p]-γ-ATP for autoradiography, in a reaction mixture containing 10mM Tris-HCl, pH, 7.6, 20mM KCl, 0.5mM DTT, 2% Glycerol, 1.5mM $MgCl_2$ and 0.5mM ATP/ 50μci [^{32}P]-γ-ATP. Samples were heated with equal volume of 2 X Laemmli sample buffer at 95^{o}C for 5 min and separated on SDS-PAGE. The protein was transferred on PVDF (Millipore) membrane for immunoblotting with antibodies against phospho ser / thr epitops (Cell Signaling Technology, USA). For autoradiography, the gel was stained with coomassie brilliant blue and destained to ascertain equal amount of proteins loading in each lane, dried and exposed for autoradiogram.

CHAPTER 3

RESULTS

Chapter 3.1

Studies on the role(s) of pyrroloquinoline-quinone (PQQ) and five PQQ binding motifs containing proteins in radiation resistance of *D. radiodurans*

Pyrroloquinoline-quinone (4,5-dihydro-4,5-dioxo-1H-pyrrolo [2,3-5,6] quinoline-2, 7, 9 tricarboxylic acid: PQQ) is an important redox-active cofactor used by a number of bacterial dehydrogenases involved in mineral phosphate solubilization (MPS) phenotype in bacteria (Goldstein, 1994). PQQ has been reported to act as an antioxidant *in vitro* (Misra *et al.,* 2004.), having roles in oxidative stress in both mammals (He *et al.,* 2003) and bacterial system (Khairnar *et al.,* 2003) *in vivo,* and as a member of B group vitamins (Kasahara and Kato, 2003). He and colleagues (2003) have shown that the antioxidant nature of PQQ is concentration dependent. Higher concentrations of PQQ induce oxidative stress on mitochondrial activity in rats, which lead to both apoptotic and necrotic cell death. Further studies have shown that the necrotic cell death could be selectively inhibited in presence of antioxidants while apoptotic cell death continues by a still unknown mechanism (Shankar *et al.,* 2010). *D. radiodurans* genome contains *pqqE* gene, which encodes a functional PQQ synthase enzyme in transgenic *Escherichia coli* and these cells showed higher resistance to the photodynamic effect of rose bengal and improved resistance to UVC and γ radiation as compared to untransformed *E. coli* (Khairnar *et al.,* 2003). It has been reported that PQQ works as an inducer of a periplasmic membrane protein kinase (YfgL) in *E. coli* and subsequently the role of YfgL was demonstrated in radiation resistance and recombination repair of UV induced DNA damage. Therefore, the possible role of PQQ as an inducer for proteins kinases (YfgL) involved in distinctly different metabolic and physiological processes has been suggested (Khairnar *et al.,* 2007). *pqqE* gene, which encode PQQ synthase an enzyme, involved in cyclization of PQQ structural rings from precursor glutamate and tyrosine amino acids. Since *D. radiodurans* is MPS (mineral phosphate solublization) negative, therefore the significance of PQQ presence without mineral phosphate solublization function in *D. radiodurans*, would be worth investigating. In this chapter *in vivo* synthesis of PQQ in *D.*

radiodurans, its involvement in γ radiation resistance and DSB repair and the possible molecular mechanism underlying its function in *Deinococcus* have been demonstrated.

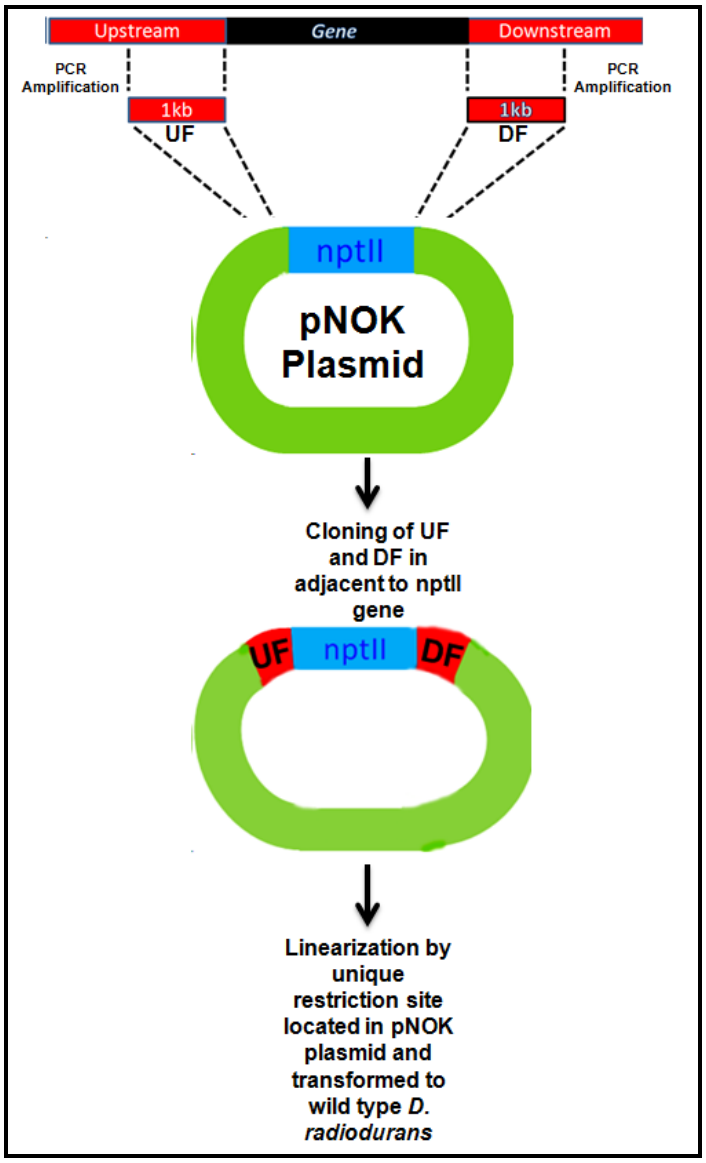
3.1.1 Methods

3.1.1.1 Generation of *pqqE* disruption mutant and deletion mutant's derivative of five PQQ motif containing proteins of *D. radiodurans*

The 1kb upstream fragment which contains 500bp of 5' region of *pqqE* coding sequences along with 500bp upstream to this ORF, was PCR amplified using forward primer (5' CTAGGGCCCCAGTGGGAGTACCTC 3') and reverse primer (5' GGAATTCCTACTGT TAGACTGTTG3'). PCR product was cloned at *ApaI* and *EcoRI* sites in pNOKOUT (Khairnar et al 2007) to yield pNok*pqqE*1. The 1455bp downstream fragment which contains 500bp of 3' half of *pqqE* coding sequences along with 955bp downstream to this, was PCR amplified using forward (5'CGGGATCCATGTCCAAATTTAAGCATC3') and reverse (5' CTCTAGACTGCGACTGGGAATGAAG 3') primers and cloned at *BamHI* and *XbaI* site in pNok*pqqE*1 to yield pNok*pqqE*. Recombinant plasmid pNok*pqqE* was linearized with *ScaI*, gel purified and transferred into *D. radiodurans*. The transformants were grown for several generations in TGY supplemented with Kan (10µg/ml) to obtain homozygous *pqqE* disruption mutant. Homozygosity was ascertained by scoring the amplification of 2.2kb PCR product, which includes both *pqqE* (1.1kb) and *nptII* cassette (1.1kb) using *pqqE* coding sequence specific forward primer (5'ATGGTGGCATTTCTCCGTGGC 3') and reverse primer (5'TCATGCGTGACTTACCAATGGA 3'). Clones showing the complete absence of normal

pqqE gene (1.1kb) were considered as homozygous *pqqE* disruption mutants and were used for further studies.

The plasmid constructs for generation of deletion mutants of *dr2518*, *dr1769*, *dr0766*, *dr0503* and, *drc0015*, were made in pNOKOUT as depicted below. In brief, the 1kb upstream and downstream to the coding sequences of each ORF, were PCR amplified using sequence specific primers (Table A) and cloned respectively at upstream and downstream to *nptII* cassette in pNOKOUT to yield pNOK2518, pNOK1769, pNOK0766, pNOK0503 and pNOK0015 plasmids (Fig. 3.1.8). These plasmids were linearised and used for generation of *dr2518*, *dr1769*, *dr0766*, *dr0503* and *drC0015* deletions, respectively. The transformants harboring respective plasmids were grown several generations in presence of Kanamycin (10µg/ml) to obtain homozygous deletion mutant. Complete replacement of wild type copy of these ORFs with *nptII* gene was ascertained by PCR amplification using gene specific internal primers (Table A). Clones showing complete absence of respective genes were designated as Δ*dr2518*, Δ*dr1769*, Δ*dr0766*, Δ*dr0503* and Δ*drc0015* mutants. For generating *pqqE* disruption mutation in Δ*dr2518* background, the *nptII* cassette flanking upstream and downstream sequences of *pqqE* including the part of neighboring genes in pNOKpqqE (Rajpurohit *et al.*, 2008), was replaced with chloramphenicol acetyl transferase (*cat*) cassette to yield pPQCAT. The linearised pPQCAT was transformed into Δ*dr2518* mutant and homozygous replacement of wild type *pqqE* with disrupted *pqqE:cat* was confirmed by PCR amplification. The resultant strain was designated as double mutant (Δ*dr2518 pqqE:cat).*



Schematic representation of generation of gene-knockout strategy

3.1.1.2 Construction of PQQ synthase expression plasmid

Genomic DNA of *D. radiodurans* R1 was prepared as published previously (Battista *et al.* 2001). The 1128bp DNA fragment was PCR amplified from the genomic DNA using *pqqE* gene specific primers (Forward- 5'CCGGGGCCCATGGTGGCATTTCTCCGTGGC3' and reverse 5'GCTCTAGATCATGCGTGACTTACCAATGGA3'). The identity and correctness of *pqqE*

gene, was ascertained by restriction analysis and partial nucleotide sequencing. PCR product was ligated at *ApaI* and *XbaI* sites in pRADgro (Misra *et al.,* 2006) to yield pGro*pqqE*. Recombinant plasmid was transformed into *D. radiodurans* as described earlier (Meima *et al.,* 2001) and chloramphenicol resistant clones were isolated on TGY agar plates containing chloramphenicol (5µg/ml). Plasmid DNA was prepared from these clones and the presence of insert in these plasmid samples was confirmed by restriction analysis.

3.1.1.3 Detection of PQQ in *D. radiodurans*

PQQ was extracted from stationary phase cells of *D. radiodurans* using a modified protocol published earlier (Suzuki *et al.,* 1990). In brief, the cells were sonicated and cell free extract was digested with 50% acetonitrile at 65^{o}C for 2h. The mixture was centrifuged at 15k for 10 min; the clear supernatant was collected and dried by concentrator under vacuum. Residues were dissolved in 50% n-butanol at 1mg/ml and PQQ was extracted at 50^{o}C overnight. The clear supernatant was dried under vacuum and dissolved in 100% methanol (HPLC grade). HPLC analysis was carried out using "C-18 µ bondapack" column and eluted with mobile phase comprises of 45% methanol in HPLC grade water at the flow rate 1ml / min. Each fraction was scanned through UV detector set at 289nm. The identity of the PQQ was ascertained by comparing the retention time of a peak with that of the standard sample and its possible absence in *pqqE* mutant cells.

3.1.1.4 The effect of DNA damaging agents on cell survival

Deinococcus cells were treated with different doses of UV and γ radiations as described earlier (Khairnar *et al.,* 2008). In brief, the mutant and wild type *D. radiodurans* and different mutant

cells were grown in TGY till late log phase at 32^{o}C. These cells were suspended in sterile phosphate buffered saline (PBS) and exposed to different doses of γ radiation at dose rate 7.2kGy per hr (Gamma 500, ^{60}CO, Board of Radiation and Isotopes Technology, DAE, India). Appropriate dilutions were plated on TGY agar plates and incubated at 32^{o}C. For UV effects, the cells were prepared as described above and different dilutions were plated. Cells were exposed to different doses of UV radiation at 254 nm (FUV) and incubated at 32^{o}C. MMC treatment was given as described in (Harris *et al.,* 2004). In brief, the late log phase cells were treated with MMC (20µg/ml) and aliquots were drawn at regular interval. The appropriate dilutions were plated on TGY agar plate supplemented with kanamycin (8µg/ml) when required, and the plates were incubated at 32^{o}C for colony formation. Hydrogen peroxide treatment was given as described earlier (Misra *et al.,* 2006). In brief, the late log phase cells were treated with different concentration of hydrogen peroxide for 30 min with vigorous aeration. Cells were diluted with phosphate buffered saline and different dilutions were plated on TGY agar plate. The colony forming units were recorded after 48h of incubation at 32^{o}C. For quantifying desiccation resistance of *D. radiodurans* Cells an exponential-phase culture was collected by centrifugation, washed in 4 volumes of 10 mM MgSO4, and resuspended in an equal volume of 10 mM MgSO4. A 100-ml aliquot of this suspension was spotted on a sterile glass coverslip, placed inside a sterile petri dish, and dried at 25^{0}C in a desiccator over anhydrous $CaSO_4$ containing a visual indicator. The desiccators were sealed, and the dried cultures were stored undisturbed at 25^{0}C for 2 weeks. Relative humidity within the desiccators was measured as less than 5% with a membrane hygrometer. Samples were revived by washing the cells free of the plate in 1 ml of 10 mM $MgSO_4$ and plating on TGY agar. Plates were placed in a 30^{0}C incubator and scored for survivors 5 days later.

3.1.1.5 Protein phosphorylation and DNA strand breaks repair studies

In vivo phosphorylation of proteins was studied in the cells grown in presence of 1mCi/ml [^{32}P]-orthophosphoric acid for overnight as described earlier (Mann *et al.,* 1991). Labeled cells were collected and lysed in 1X lamellae buffer at 95^{o}C for 10 min. The incorporation of [^{32}P] was monitored by TCA precipitation as described earlier (Misra *et al.,* 1998). SDS-PAGE analysis was carried out with equal amount of proteins from both the cells. Signals were detected by autoradiography. For DNA strand break repair studies, the cells were irradiated with 6kGy γ radiation (7.2kGy/h) at different time intervals during post irradiation recovery (PIR). Cell lysis and restriction digestion were carried out in-gel. The DNA fragments were separated by pulsed field gel electrophoresis using the modified protocol of an earlier method (Lecointe *et al.*, 2004). In brief, the cells were washed with 70% ethanol in PBS (pH 7.5) for 5 min. Agarose plugs containing these cells were incubated with lysis buffer I (2 mg/ml lysozyme in 5mM EDTA, pH 8.0) for 2 h at 37^{o}C, followed by overnight incubation at 55^{o}C in lysis buffer II (0.5 M EDTA, pH 8.0, 1% sodium sarcosine and 2mg/ml proteinase K). Plugs were washed four times with TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0) and lastly with restriction enzyme buffer for one hour each at 55^{o}C. For restriction digestion, the plugs were transferred to fresh tube having enzyme buffer and 100 units of *XbaI* and incubated for overnight at 37^{o}C. DNA fragments were analysed on 0.8% low EEO agarose (Bangalore Genei, India) for 25h.

3.1.2 Results

3.1.2.1 Disruption of *pqqE* makes deinococcal cells devoid of pyrroloquinoline-quinone

The genomic copy of *pqqE* (DRC0034) was disrupted with *nptII* cassette and used for generating *pqqE* disruption mutant of *D. radiodurans*, as mentioned in methods. Homozygosity of clones was confirmed by PCR amplification with gene specific primers (Fig 3.1.1). PCR product from wild type *D. radiodurans* genome, showed the amplification of single 1.1kb PCR product which is the predicted size of *pqqE* gene (Fig. 3.1.1, Lane 3). The PCR product size from genome of selected two clones was approx. 2.1 kb in size which was equal to the size of *pqqE gene* (1.1 kb) and *nptII* cassette (1.08 kb) (Fig. 3.1.1, Lane 1 and 2). PCR based screening of clones confirms the insertion of *nptII* marker gene in *pqqE* gene. Earlier studies showed that biosynthesis of pyrroloquinoline quinone (PQQ) in *Klebsiella pneumoniae* require the expression of six genes (*pqqA−F*). One of these genes (*pqqE*) encodes a 43 kDa protein PQQ synthase (PqqE) that catalyze the cyclization of PQQ ring structure from precursor peptide chain containing glutamate and tyrosine amino acids in conserved motif Glu-X-X-Tyr (Veletrop *et al.*, 1995). The requirement of PQQ synthase protein for synthesis of *in vivo* PQQ in *D. radiodurans* was confirmed by HPLC analysis. PQQ was extracted from cell free extract of both wild type and *pqqE* mutant's stationary phase cells and its presence was detected by HPLC. Commercially supplied PQQ from sigma give single peak at 3.27 min retention time (Fig. 3.1.2 A). The HPLC elution profile of wild type and *pqqE* mutant cells showed the similar pattern of eluted species except peak with retention time 3.29 min, which was absent in *pqqE* mutant. The 3.29 min peak present in wild type showed close matches with that of standard PQQ (3.27min) under similar chromatographic conditions (Fig. 3.1.2 A and B). The absence of similar retention time peak in extract of *pqqE* mutant cells strongly indicated that only wild type cells make extractable PQQ.

The appearance of new peak of 3.94 min in mutant compare to wild type (3.84 min), possibly suggests the accumulation of precursors of PQQ biosynthetic pathway. Since in *E. coli* lacking *pqqE* of *K. pneumoniae*, biosynthetic intermediates could not detected, therefore, it might also be possible that appearance of entirely new species in *pqqE* mutant, could be the result of activation of side chain metabolic pathways. Nonetheless, these results ascertain the synthesis of PQQ in *D. radiodurans*.

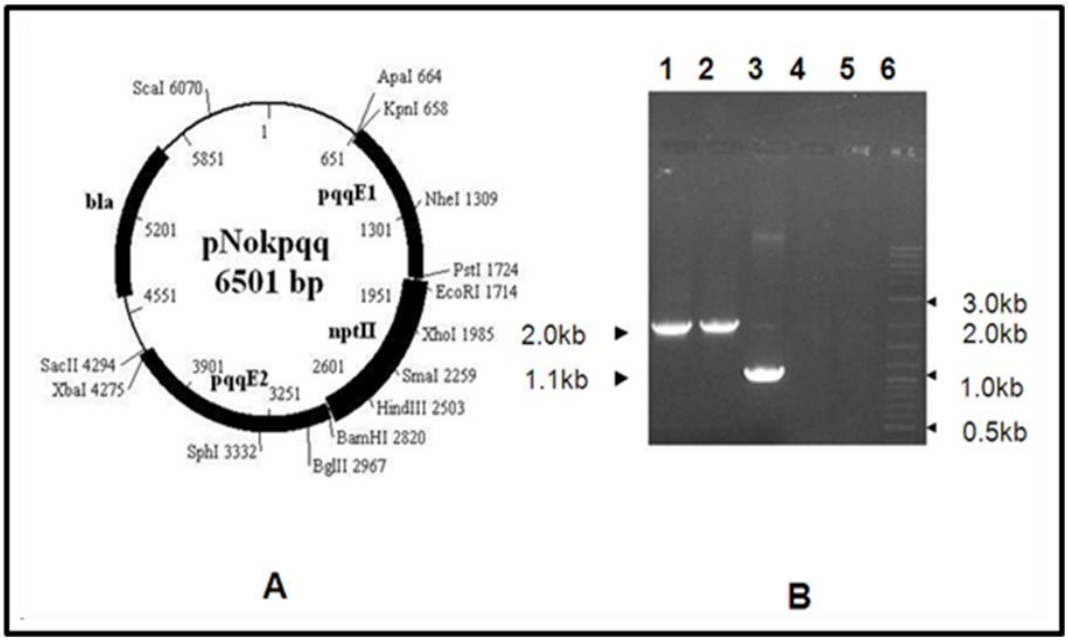


Fig 3.1.1 Construction of recombinant suicidal plasmid and generation of *pqqE* disruption mutant derivative of *D. radiodurans* R1 The *pqqE* gene sequence was divided from the middle of the gene, in two equal halves. Both the fragments were PCR amplified along with upstream (*pqqE*1) and downstream (*pqqE*2) sequences and cloned in pNOKOUT to yield pNOK*pqqE* **(A)**. Recombinant plasmid was linearised with *ScaI* and transformed into DEIRA. Recombinant clones were sub-cultured several generations for the complete replacement of normal copies with disrupted copy on the genome. Genomic DNA from two homozygous *pqqE* mutant clone-1 (1) and clone-2 (2) and wild type (3) was PCR amplified using *pqqE* coding sequences flaking primers **(B)**. Clone-1 was subsequently taken for further studies.

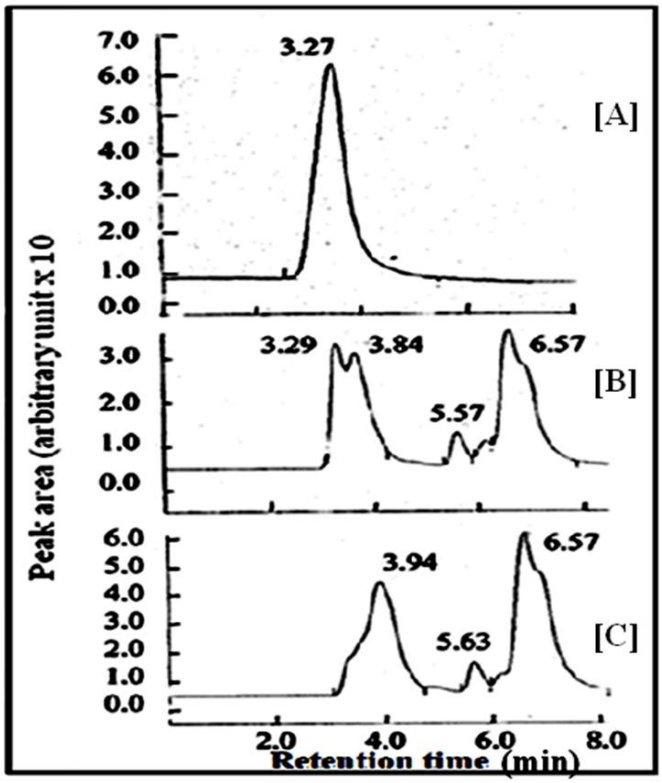


Fig 3.1.2 Detection of pyrroloquinoline-quinone in *D. radiodurans R1* PQQ was extracted from cell free extract of both wild type and *pqqE* mutant's stationary phase cells and its presence was detected by HPLC. The elution profile of commercially available pyrrolloquinoline-quinone (**A**) and PQQ extracted from cell free extract of wild type (**B**) and *pqqE* disruption mutant (**C**) cells.

3.1.2.2 The *pqqE* mutant showed sensitivity to DNA damaging agents

The involvement of PQQ in oxidative stress produced by photodynamic effect of rose bengal (Khairnar *et al.*, 2003) and in homologous recombination has been demonstrated in *E.coli* (Khairnar *et al.*, 2007). The importance of PQQ in *D. radiodurans* cell free extract was therefore, checked for its possible involvement in extreme resistance of *D. radiodurans* against DNA damaging agents. The *pqqE* disruption mutant cells were checked for their response to gamma and UVC radiations, MMC and hydrogen peroxide effects. Mutant cells showed 3-log cycle loss as compared to wild type levels of γ resistance (Fig. 3.1.3 A) and a 2-log cycle decreased MMC

tolerance (Fig. 3.1.3 B). The UV resistance of *pqqE* mutant cells was similar to that of wild type (Fig. 3.1.3 C). Both γ radiation and MMC treatment produces high density of double strand breaks on genome (Keller *et al.,* 2001) while UVC produces maximum single strand breaks and less than 1% double strand breaks on genome (Cadet *et al.,* 2005). The unique effect of PQQ in response to γ radiation and MMC clearly indicated the possible role of PQQ in the regulation of DNA double strand break repair. PQQ also has known for its antioxidant properties *in vitro* and *in vivo* (Misra *et al.,* 2004; Khairnar *et al.,* 2003). Therefore we tested the contribution of PQQ oxidative stress tolerance of *D. radiodurans.* Mutant cells showed nearly fivefold decrease in hydrogen peroxide tolerance as compared to wild type cells (Fig. 3.1.3 D) suggesting that PQQ also has a role in oxidative stress tolerance of *D. radiodurans*. However, the contributions of PQQ to oxidative stress tolerance in *D. radiodurans* were not as pronounced as its effect on DNA double strand break repair. Higher sensitivity of PQQ deficient *D. radiodurans* cells to γ radiation and MMC, clearly suggest the contribution of PQQ in DNA double strand break repair in bacteria apart from its antioxidant properties. Thus the functions of PQQ in both oxidative stress tolerance and DNA double strand break repair could be strongly suggested. Although, the molecular mechanism of PQQ action in DNA double strand break repair is not clear, its role as a cofactor for periplasmic protein kinase, which is involved in DNA strand break repair and homologous recombination, has been suggested in *E.coli* (Khairnar *et al.,* 2007).

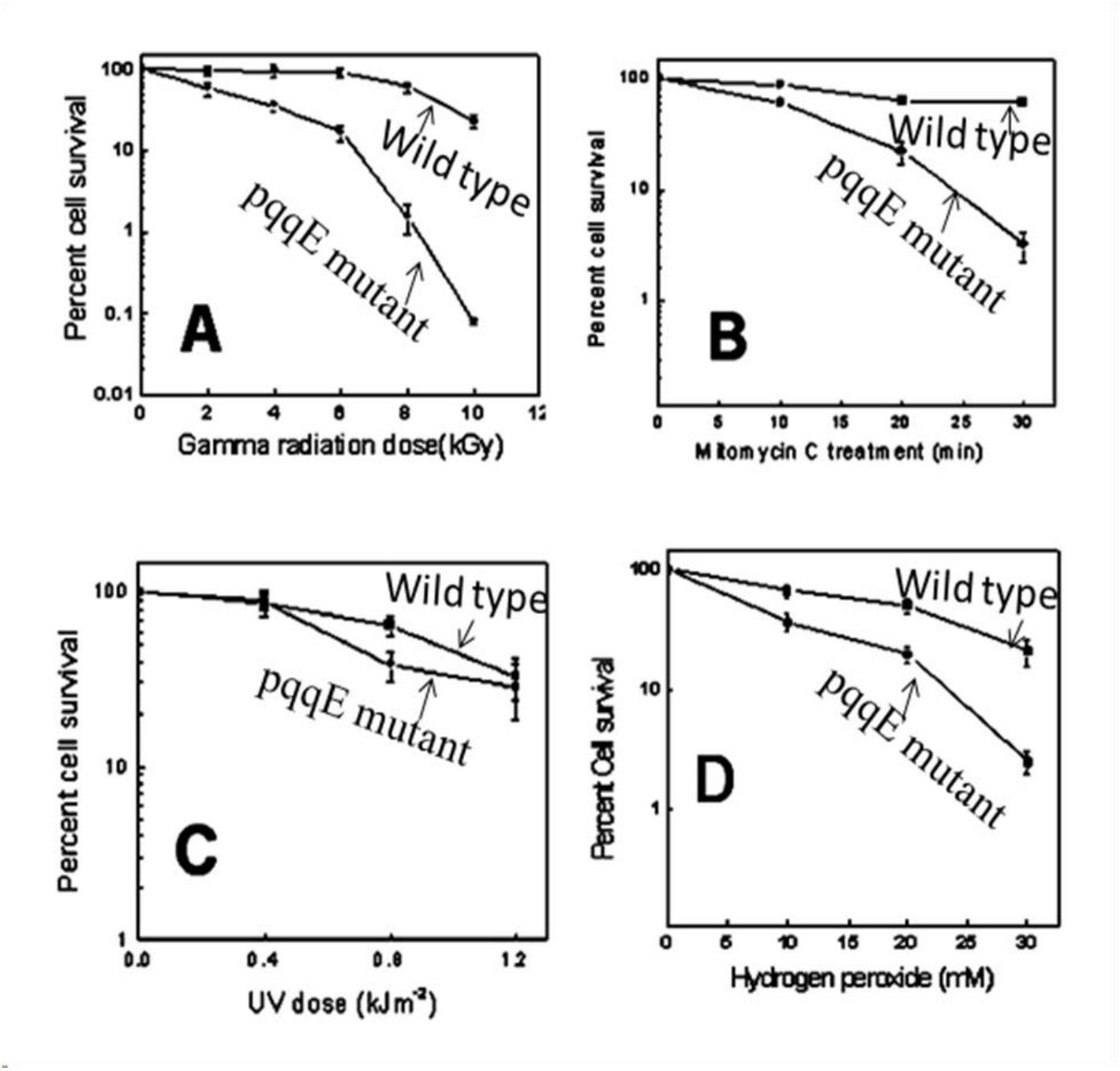


Fig 3.1.3 Cell survival response of *pqqE* disruption mutant to DNA damaging agents
D. radiodurans cells harboring wild type (wt) and disrupted copy (mutant) of *pqqE* gene, were treated with different doses of γ radiation (A), UVC radiation (B) different exposure time for MMC (20µg/ml) (C) and different concentration of hydrogen peroxide (D). Cell survival was monitored. Initial cell density of cultures used in all experiments was approximately 10^7cells/ ml.

3.1.2.3 PQQ synthase (*pqqE*) expressing on low copy plasmid complements the *pqqE* mutant phenotype

To ascertain that *pqqE* mutant phenotypes are due to lack of functional PQQ synthase *per se*, the enzyme was expressed *in trans,* by expressing the *pqqE* gene under constitutive promoter, P_{groESL} and responses of such cells to DNA double strand breaks producing agents were monitored. The *pqqE* mutant cells harboring pGro*pqqE* (Fig. 3.1.4 A,B) showed the recovery of lost γ radiation and MMC resistance phenotypes nearly to wild type levels (Fig. 3.1.4 C,D). The level of complementation was nearly 90 % of wild type level. This suggested that *pqqE* mutant phenotypes were not due to absence of any proteins downstream to *pqqE,* but absence of PQQ synthase *per se*. The expression of transgene under P_{groESL} promoter in pRadgro has been shown in earlier studies (Kota and Misra 2006 and Khairnar *et al.,* 2008). Mutant cells showing the functional complementation of lost phenotypes also confirmed the expression of *pqqE* gene on pGro*pqqE* plasmid. These results strongly suggest the role of PQQ in DNA double strand break repair and radiation resistance of *D. radiodurans.*

3.1.2.4 Sensitivity to γ radiation correlates with defects in DSB repair and lesser incorporation of [^{32}P] in *pqqE* mutant cells

Wild type and *pqqE* mutant cells of *D. radiodurans* exposed to 6kGy γ radiation were allowed to recover under normal growth conditions. Aliquots were drawn at different time interval and kinetics of DNA double strand break repair was monitored on pulsed field gel electrophoresis. Results showed a strong impairment of DSB repair mechanism in *pqqE* mutant cells while wild type cells showed normal pattern of DSB repair (Fig. 3.1.5).

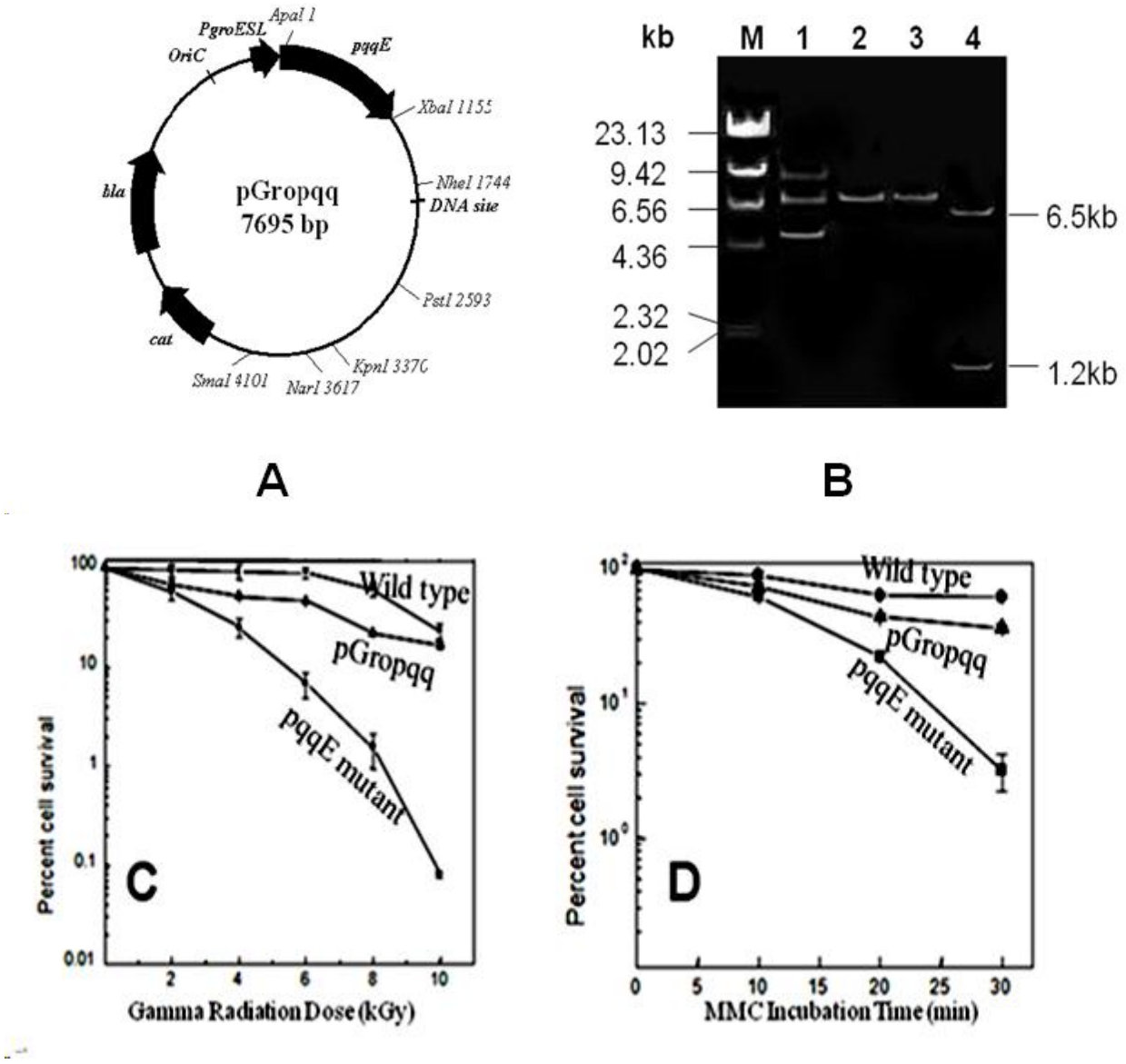


Fig 3.1.4 Functional complementation of *pqqE* mutant phenotypes with wild type PQQ synthase Wild type (wt) and *pqqE* mutant (mutant) cells were transformed with pGro and pGropqq respectively (A, B) and recombinant derivatives were scored on TGY agar plates in presence of chloramphenicol (3µg/ml). These cells were treated with different doses of γ radiation (C) and MMC (D) and cells survival was monitored. Initial cell density of cultures used in this experiment was approximately $2x10^8$cells/ ml.

Wild type cells showed recovery of normal size genome in 4h of post irradiation time. The mutant cells showed no sign of recovery up to 6h of post irradiation time. This result indicated in *pqqE* mutant, ability for assembly of shattered genomic DNA in to intact genome, follow slower kinetics of double strand break repair as compared to wild type cells. The repair of shattered genome caused by gamma radiation require ESDSA and homologous recombination repair process in this bacteria therefore explanation of this result cannot be explained based on antioxidant properties of PQQ. This result clearly indicates the novel role of PQQ in DSB repair in *D. radiodurans*.

Recently PQQ has been shown to involve in activity stimulation of membrane protein kinase YfgL *in vitro* and contribution of YfgL in homologous recombination suggested (Khairnar *et al.*, 2007). The possibility of PQQ work as an inducer for *D. radiodurans* protein kinases activity was tested by checking the total phosphoproteins of wild type and *pqqE* mutant cells growing in normal growth condition. Total phosphoproteins profiles of wild type and *pqqE* mutant cells of *D. radiodurans* were analysed *in vivo*. Results showed nearly 10 fold less incorporation of [^{32}P] in mutant cells as compared to wild type (Table 3.1.1). The average Cpm for *pqqE* mutant was 15049 Cpm/50µg proteins, where as wild type cells having 158898 Cpm/50µg proteins. The low levels of protein phosphorylation observed in *pqqE* mutant could be due to either PQQ is absolutely required for regulation of certain protein kinases activity and / or PQQ stimulates the residual activity of these protein kinases in the cells. However, together the low level of [^{32}P] incorporation and sluggish DNA double strand break repair in *pqqE* mutant cells compare to wild type, indicating the possible involvement of protein kinases in DSB repair and gamma radiation resistance of *D. radiodurans*.

Table 3.1.1 Quantitation of incorporation of [^{32}P] in *D. radiodurans* and *pqqE* mutant

S.No.	*D. radiodurans* R1 (Cpm/50µg of total Proteins)	*pqqE* mutant (Cpm/50µg of total Proteins)
1	175258	12937
2	144313	17422
3	157124	14789
Average Cpm	158898	15049

3.1.2.5 PQQ binding protein (s) mutants showed differential response to DNA damage

The mechanism of PQQ action in DSB repair was most intriguing observation of this study. Since PQQ does diverse role in different systems, as an antioxidant (Khairnar *et al.,* 2003), essential nutrient (He *et al.,* 2003), as an inducer of apoptotic and necrotic cell death in tumor cell lines (Shankar *et al.,* 2010), and as a member of B-group vitamins (Kasahara and Kato, 2003). These diverse roles suggest that PQQ might act as a signaling molecule and PQQ acting as a cofactor for regulatory protein kinases possibly important for DNA repair could be hypothesized. Since, PQQ is known as a redox cofactor for bacterial dehydrogenases (Matsushita *et al.,* 2002) and interact with these enzymes through the conserved β-propeller amino acid motif distributed in the primary structure of dehydrogenases. Database search of these motifs containing proteins showed that this motif is present in proteins kinases across the organisms (http://www.sanger.ac.uk and http://smart.embl-heidelberg.de).

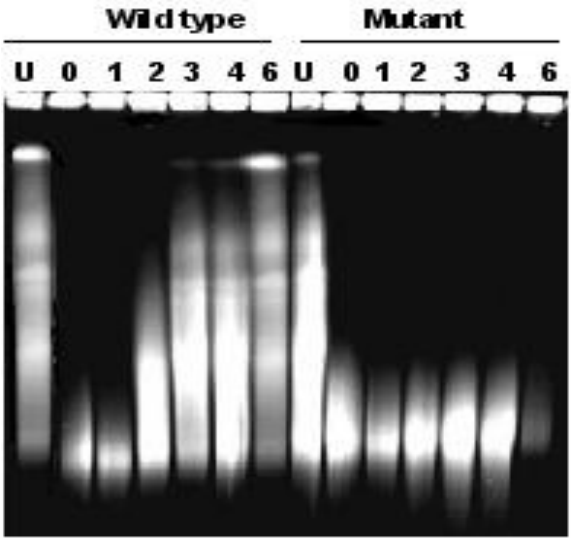


Fig. 3.1.5 DNA double strand breaks repair kinetics of wild type and *pqqE* disruption mutant derivatives of *D. radiodurans* during post irradiation recovery. Logarithmically growing *Deinococcus* cells (U) were irradiated with 6kGy γ radiation at dose rate (7.2kGy/h) and allowed for post irradiation recovery. Aliquots were drawn at different time (0, 1, 2, 3, 4, and 8 h) intervals and genomic DNA was digested with *XbaI* in agarose plugs. The extent of DNA strands break and its repair kinetics were monitored on pulsed field gel electrophoresis.

Using this information, one such protein, YfgL, has been characterized for its interaction with PQQ (Kharinar *et al.*, 2007) and it has been shown that PQQ interaction stimulates the autophosphorylation activity of this protein *in solution*. *D. radiodurans* genome encodes five ORFs for putative Ser/ Thr kinases, having PQQ binding β-propeller amino acid motif in their primary structure. The possibility of these enzymes requiring PQQ for their activity stimulation and in radiation stress tolerance of *D. radiodurans* were further investigated.

Domain search analysis (http://smart.embl-heidelberg.de/) showed that there are five genes named as *dr0503*, *dr0766*, *dr1769*, *dr2518* and *drc0015* encoding uncharacterized proteins having multiple though different numbers of PQQ interacting motifs (Fig. 3.1.6). Among these

five proteins, DR2518 also contains a well-characterized eukaryotic type Ser/Thr protein kinase (eSTPK) domain at its N-terminal while DR1769 contains a little less defined signature of STK domain. To ascertain the involvement of these genes in radioresistance of this bacterium, they were individually deleted from *D. radiodurans* R1 genomes using recombinant pNOK variant plasmids of all these five genes as discussed in methods (Fig. 3.1.7), and the homozygous replacement of wild type alleles with *nptII* antibiotic marker was confirmed by PCR amplification (Fig. 3.1.8). The individual mutant of all these genes showed the complete absence of respective genes, as internal gene specific primer for all these genes gave amplification of approx size of 500bp PCR product from wild type genome but same was absent in respective mutant. Further amplification of 1.1 kb PCR product with *nptII* cassette specific primers from these mutants but not from wild type genome confirms the homozygous replacement of these genes with *nptII* marker in respective mutant. The deletion mutants of *dr0503, dr0766, dr1769, dr2518* and *drc0015* genes were designated as Δ*dr0503*, Δ*dr0766*, Δ*dr1769*, Δ*dr2518* and Δ*drc0015*. These mutants were checked for γ radiation resistance. The cell survival of Δ*dr2518* mutant decreased nearly 3-log cycle at 8kGy γ radiation and at 1200Jm^{-2} of UVC radiation. ~2.5 log cycle loss of viability upon 14 days desiccation at 5% humidity (Fig. 3.1.9 A, B, C). The Δ*dr2518* mutant survival decreased by nearly 1.5-log cycles in presence of 20µg /ml mitomycin C treated for 30 min and by nearly 7 fold at 30mM hydrogen peroxide treatment (Fig.3.1.10 D, E), as compared to wild type. The levels of γ radiation tolerance in Δ*dr1769* mutant decreased to less than 1.0 log cycle at 10kGy as compared to wild type (Fig. 3.1.9 A). Other mutants showed no effect of DNA damage and their gamma radiation survival continued to be similar to wild type (Fig. 3.1.9 A). These results suggested the important role of DR2518 protein in extraordinary tolerance to DNA damages in *D. radiodurans*. Since DR2518 also contains

signature motif for PQQ interaction, therefore the possibility of PQQ effect in gamma radiation survival possibly through *dr2518* gene product was speculated and tested. The *pqqE* (*drC0034),* encoding PQQ synthase, was deleted from Δ*dr2518* mutant genome by homologous replacement of *pqqE (drC0034)* by chloramphenicol resistance cassette to yield *Δdr2518pqqE:cat* double mutant. The Δ*dr2518pqqE:cat* double mutant was isolated and checked for its gamma radiation cell survival and found to have γ-radiation sensitivity similar to Δ*dr2518* single mutant indicating that both PQQ and DR2518 function through common pathway(s) inside the cell for conferring the γ radiation resistance in *D. radiodurans* (Fig. 3.1.11 B). These results collectively ascertain the role of PQQ and quinoproteins (DR2518 and DR1769) in gamma radiation resistance of *D. radiodurans*. To ascertain that the loss of γ radiation resistance in Δ*dr2518* mutant was not due to the polar effect of this deletion but due to the loss of DR2518 enzyme *per se*, the wild type allele of *dr2518* was expressed into Δ*dr2518* mutant under $P_{gro\text{ESL}}$ promoter in pRADgro (Misra *et al.*, 2006). For that *dr2518* gene was PCR amplified and cloned in pRADgro plasmid at *ApaI* and *XbaI* rescriction sites (Fig. 3.1.10). After confirming the correctness of construct, construct was transformed to Δ*dr2518* mutant cells and gamma radiation survival was checked as described in methods. The γ radiation resistance of transgenic Δ*dr2518* mutant cells expressing DR2518 *in trans* was recovered nearly full to wild type levels (Fig. 3.1.11 A), indicating that the γ radiation sensitivity of Δ*dr2518* mutant was due to absence of this protein *per se*. These results suggested that amongst the five putative PQQ binding proteins of *D. radiodurans*, the DR2518 contributes maximum to the γ radiation resistance in this bacterium. Further, the DR2518 seems to be the most preferred protein through which PQQ possibly functions in radiation resistance and DSB repair of *D. radiodurans*.

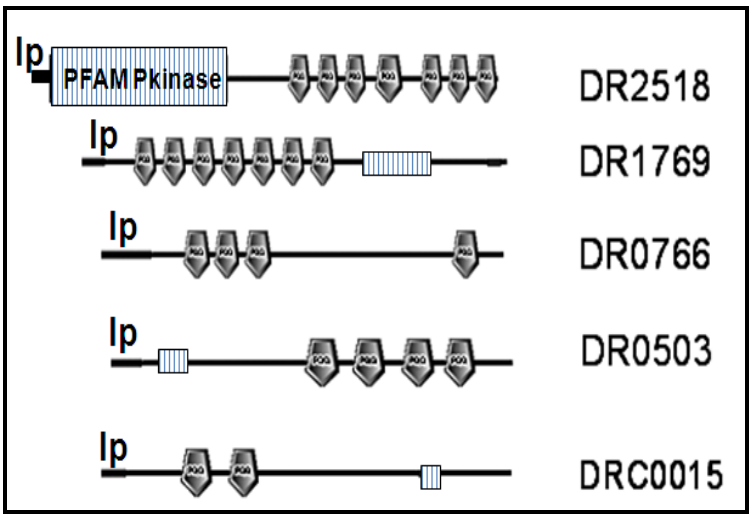


Fig. 3.1.6 Diagrammatic representation of individual open reading frames encoding putative quinoproteins in *D. radiodurans* R1. The DR0503, DR0766, DR1769, DR2518 and DRC0015 ORFs encoding uncharacterized proteins with multiple PQQ binding motifs and N-terminal leader peptides (**lp**) differ in protein kinase domains. DR2518 showed a well defined STKPs type protein kinase domain (PFAM:Pkinase) at N-terminal and PQQ binding motifs at C terminal while DR1769 contains relatively less defined kinase domain at C-terminal and PQQ binding domains at N-terminal.

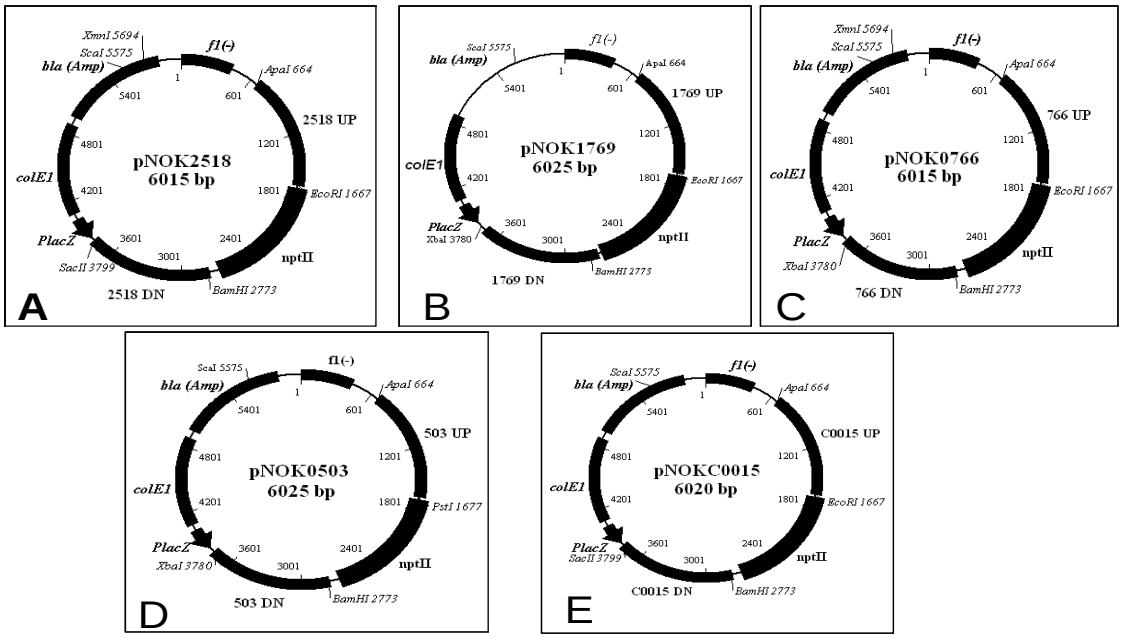


Fig. 3.1.7 Partial restriction map of constructs used for the generation of *dr2518* (**A**), *dr1769* (**B**), *dr0766* (**C**), *dr0503* (**D**) and *drc0015* (**E**) mutants of *D. radiodurans*.

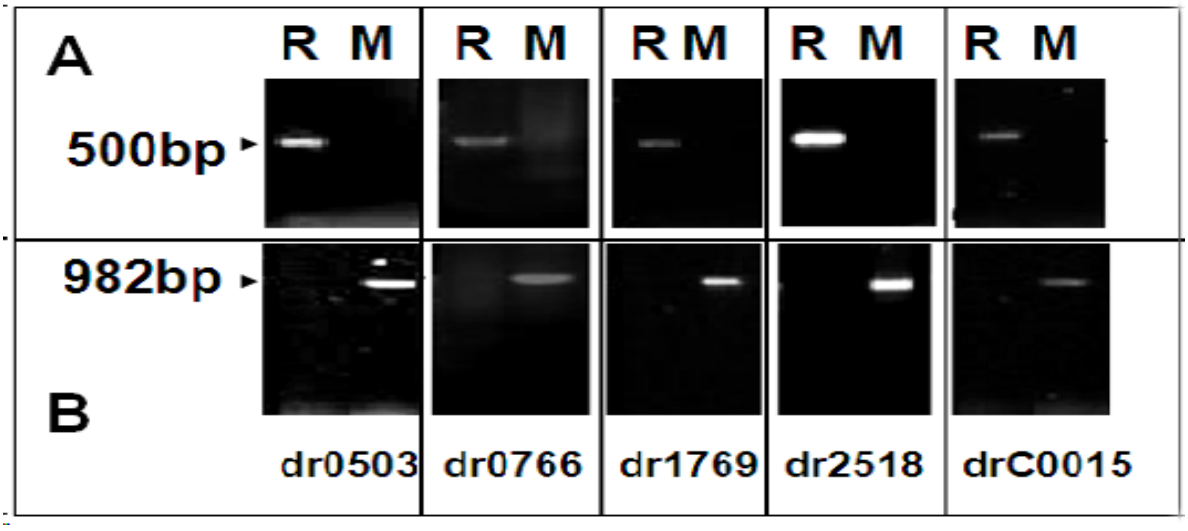


Fig. 3.1.8 Confirmation of gene replacement with *nptII* marker in respective deletion mutants of *D. radiodurans*. Genomic DNA from wild type (**R**) and putative deletion mutants of *dr0503*, *dr0766*, *dr1769*, *dr2518* and *drc0015* genes (**M**) was used for PCR amplification using gene specific (**A**) and *nptII* gene (**B**) primers and sizes of products were estimated with molecular size marker (**L**) on 1% agarose gel.

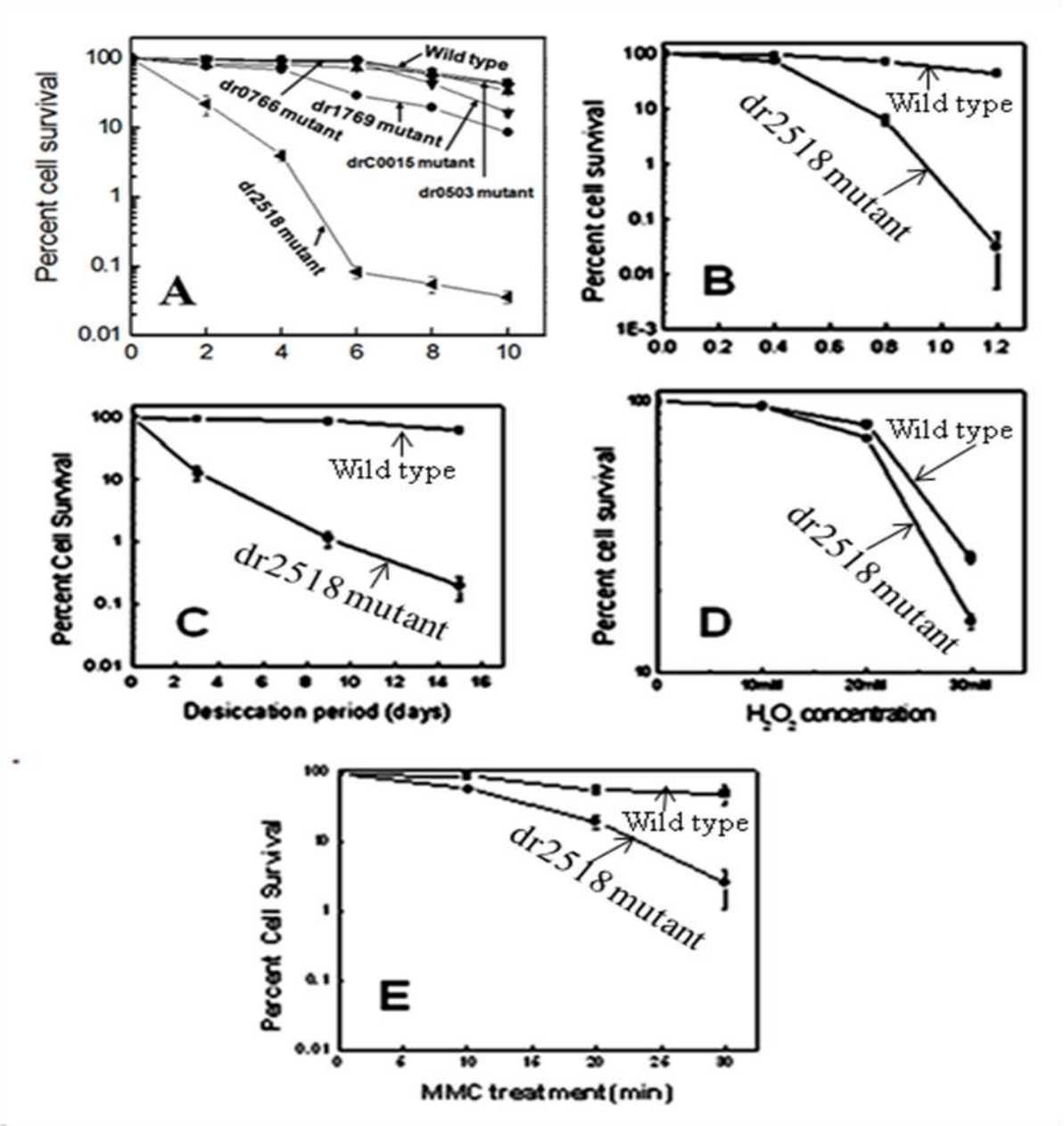


Fig. 3.1.9 DNA damage response of different mutants of *D. radiodurans* R1. Wild type (-■-) and Δ*dr0503* (-►-), Δ*dr0766* (-▲-), Δ*dr1769* (-●-), Δ*dr2518* (-◄-) and Δ*drc0015* (-▼-) mutants were exposed to different doses of γ radiation **(A)**. Similarly, the Δ*dr2518* deletion mutant (-●-) and wild type cells (-■-) were treated with different doses of UVC (B), for different period of desiccation at 5% humidity (C) and different concentration of H_2O_2 and the effects of these mutations on cell survival were measured.

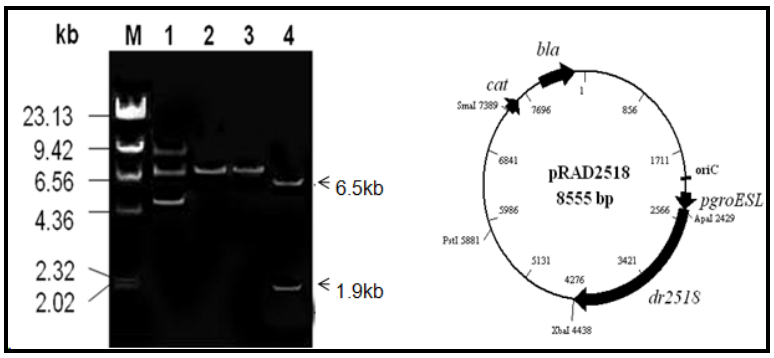


Fig. 3.1.10 Cloning of *dr2518* in *pRADgro* plasmid. The *dr2518* gene was PCR amplified using gene specific primers and cloned in *pRADgro* plasmid at *ApaI* and *XbaI* rescriction sites to yield pRAD2518 plasmid (8.55kb). (A) M-Marker, Lane 1- *pRAD2518* uncut plasmid, Lane 2- *pRAD2518* cut with *ApaI* restriction enzyme, Lane 3- *pRAD2518* cut with *XbaI* restriction enzyme and Lane 4- *pRAD2518* cut with *ApaI* and *XbaI* restriction enzyme. (B) Partial restriction map of *pRAD2518* plasmid.

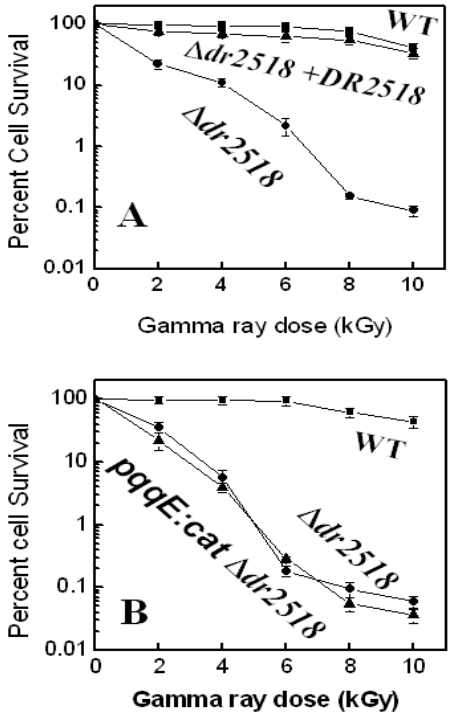


Fig. 3.1.11 Effect of *pqqE* disruption and *in trans* expression of wild type DR2518 on Δ*dr2518* mutant response to γ radiation. Wild type (-■-), Δ*dr2518* mutant (-●-) and Δ*dr2518* mutant expressing DR2518 on plasmid (-▲-) were treated with different doses of γ radiation (**A**). Similarly, the Δ*dr2518* (-●-) and Δ*dr2518 pqqE:cat* double mutant (-▼-) cells were treated with different doses of γ radiation **(B)** and cell survival was measured.

3.1.2.6 The Δ*dr2518* mutant showed altered phosphoprotein profile and impaired DSB repair

The sensitivity of *dr2518* mutant against gamma radiation prompted us to find out the cause of sensitivity. Apart from other form of DNA damage, DNA strand breaks is one of the major type of DNA damage caused by gamma radiation, therefore the effect of *dr2518* deletion on the kinetics of double strand break (DSB) repair was monitored during post irradiation recovery of mutant and wild type. The Δ*dr2518* mutant showed a major defect in DSB repair. No significant DSB repair was observed till 24 hr of PIR (post irradiation recovey) growth in *dr2518* mutant as genome size did not recover to its unirradiated *NotI* digested genome size even up to 24h PIR growth (Fig. 3.1.12 A). On the other hand the wild type cells represents normal pattern of DSB repair and its unirradiated *NotI* digested genome size was recovered in as early as 4h PIR. This indicated deletion of *dr2518* causes either impaired / or slow DSB repair in mutant.

The molecular genetics studies of *pqqE,* Δ*dr2518* and Δ*dr2518pqqE:cat* double mutant clearly indicate that PQQ mediated gamma radiation sensitivity of *D. radiodurans* was possibly due to inefficient functioning of *dr2518 in vivo*. In addition to this, quinoprotein DR2518 primary sequence contains a well-characterized kinase domain at its N-terminal region. The protein phosphorylation is reversible posttranslational modification and *in vivo* phospho-status of any given protein was controlled by antagonistic action of protein kinases and protein phosphatases. These observations encouraged us to check the total phosphoproteins profile of wild type as well as *dr2518* mutant during PIR. The γ irradiated *dr2518* mutant cells showed significant differences in both levels of total phosphoproteins and their profiles when compared with wild type control. The phosphoprotein profiles of both mutant and wild type cells were similar in unirradiated samples. After γ irradiation, the levels of protein phosphorylation decreased

significantly in 1h PIR in both wild type and mutant cells (Fig. 3.1.12 B, lane 1). Subsequently, wild type cells showed faster recovery of phosphoproteins as seen in 3 and 4h PIR, which was much slower in mutant cells and similar profile was not observed in these cells till 24h PIR (Compare lane 3 and 5 in Fig. 3.3.12 B). The molecular weights of these phosphoproteins were in the range of 29-50 kDa and above 97kDa, which were different from estimated size of DR2518 protein (~72kDa). It has been clear from this results that some of the phosphorpoteins sized between 29 to 50 kDa on ordinary SDS-PAGE, recovered very fast to their unirradiated levels in first 3-4 h PIR in wild type. These phosproteins however, having almost similar level of phosphorylation in unirradiated sample of *dr2518* mutant cells, could not be able to achieve their original level of phosphorylation in *dr2518* mutant till 24 h PIR, this indicated that the process of recovering the normal phosphorylation levels of phosphoproteins in mutant cells was delayed by more than 24 h PIR time. The slow recovery of phosphorylation of phosphoproteins in *dr2518* mutant might argue to the possibility of some of these proteins are phosphorylated by direct or indirect effect of kinase function of DR2518 in wild type, which were eventually absent in cells missing *dr2518* gene. This further suggested the possible connection of protein phosphorylation / dephosphorylation in radiation resistance and DSB repair of this bacterium.

In brief, Finding of this chapter showed that *D. radiodurans* R1 genome encodes PQQ synthease enzyme involved in *in vivo* PQQ synthesis in this bacterium. *D. radiodurans* cells devoid of PQQ exhibit sensitivity to ionizing radiation possibly due to inefficient functioning of putative protein kinase DR2518. In recent past PQQ role has been demonstrated in oxidative stress tolerance in bacteria and animal systems (Khairnar *et al.,* 2003; He *et al.,* 2003) and has been reported to act as an antioxidant *in vitro* (Misra *et al.,* 2004). It has been showed that PQQ also work as an inducer for protein kinase functions in bacterial and eukaryotic system and PQQ

stimulated kinase function required for oxidative stress tolerance mechanism in these organisms (Khairnar *et al.,* 2007; Rucker *et al.,* 2009) however, role of PQQ in DNA double strand break repair has not been studied in detail. The results of this chapter clearly suggest that PQQ and PQQ interacting putative protein kinase has important role in double strand break repair and possibly involving protein phosphorylation. Further study of possible mechanistic basis DR2518 function in gamma radiation resistance would be interesting and discussed in chapter 3.2 and 3.3.

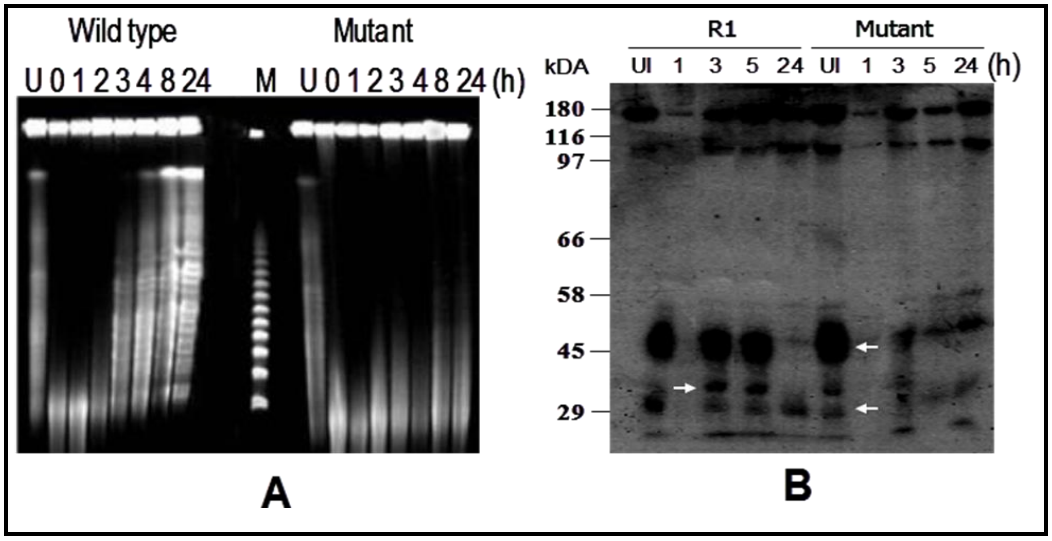


Fig. 3.1.12 Kinetics of DSB repair and phosphoproteins profiles change during post irradiation recovery of *D. radiodurans* R1. A. Wild type (wild type) and Δ*dr2518* mutant (mutant) cells were irradiated with 6kGy γ radiation dose and aliquots were collected at different post irradiation recovery period (0, 1, 2, 3, 4, 8 & 24 h) and DSB repair pattern was compared with respective unirradiated (U) controls by monitoring the recovery of wild type *NotI* pattern of genomic DNA from mutant cells. **B.** Both wild type (wild type) and Δ*dr2518* mutant (mutant) cells pre-labeled with [^{32}P] were treated with γ radiation and allowed to grow in TGY medium supplemented with [^{32}P]- phosphoric acids. Aliquots were collected at 1, 3, 5 24 h of post irradiation with 6kGy γ radiation and unirradiated (U) controls. Total proteins were analysed on SDS-PAGE and autoradiogram was developed. The phosphoproteins, which are missing in mutant cells, are indicated with arrow.

Chapter 3.2

Functional characterization of DR2518 protein having PQQ binding motifs and an eSTPK domain

Cells exposed to different DNA damaging agents survive by adjusting both qualitative and quantitative changes in their proteome. To achieve this goal, organisms have developed the efficient systems, which coordinate the DNA damage response with genome function and post translation regulation of proteins turnover. In bacteria, the molecular mechanism of DNA damage response has been better studied in *Escherichia coli* cells exposed to DNA damaging agents including UV, and / or produced by the blockage of normal replication (Kowalczykowski *et al.*, 1994; Kuzminov, 1999). These cells respond to DNA damage by invoking a repair mechanism called SOS response (Radman, 1975; Sassanfar and Roberts, 1990; Walker, 1996; Shimoni *et al.*, 2009). Under normal conditions the LexA a transcriptional autorepressor, represses the transcription of several genes involved in DNA damage repair, keeping the transcription of these genes at a basal level in *E. coli* (Little *et al.*, 1980; Little, 1983). Upon DNA damage LexA inactivation leads to derepression of a large number of SOS repair proteins including RecA and UmuD/C (Cox, 2007). Transcriptome analysis of *E. coli* cells exposed to DNA damage shows the involvement of more than 1000 genes in DNA damage response of this bacterium (Khil *et al.*, 2002). However, except the activation of co-protease activity of single strand DNA bound RecA inactivates LexA and thus expression of SOS genes other DNA damage regulatory mechanisms are not described in bacteria as well known in eukaryotes. In eukaryotes, the sensing of the DNA damage, signal transduction for controlling the expression of genes and modulation of regulatory proteins activity required for both DNA repair and cell cycle regulation, are extensively discussed (Zhou and Elledge, 2000). The activation of such signaling pathways leads to DSB repair before recommencement of cell cycle progression, and/or to the death of cells primarily by apoptosis, ensuring the genomic stability of the organism (Sancar *et al.*, 2004). Bacterial two component signal transduction system is made up of sensor kinases and

their cognate response regulators and stress response signals are transmitted through the cascades of phosphorylation / dephosphorylation. In histidine sensor kinase, first the phosphorylation of histidine residue of the kinase and then the transfer of phosphate from histidine kinases to the aspartyl residue of respective response regulators triggers the process of reversible protein phosphorylation by which the environmental signals are transmitted to genetic levels and thus the control the gene expression occurs in both prokaryotes and eukaryotes (Magasanik, 1995; Schaller *et al.,* 2011). Although the bacterial genomes encode the eukaryotic-like serine/threonine protein kinases (STPKs) (Leonard *et al.,* 1998), their involvement in DNA damage response has not been spoken. Furthermore, the LexA controlled SOS response, a well studied DNA damage response mechanism in *E. coli*, does not seem to be universal in all bacteria (Friedman *et al.,* 2005) and LexA controlled induced expression of RecA has been completely ruled out in *Deinococcus radiodurans* (Narumi *et al.*, 2001; Bonacossa *et al.,* 2002; Sheng *et al.,* 2004). The contribution of PQQ and PQQ interacting motif containing protein DR2518 a eukaryotic type STPK, in DNA strand breaks repair of *D. radiodurans* was suggested in chapter 3.1. The DR2518 protein was cloned, overexpressed and purified. This protein was further characterized for its *in vivo* and *in vitro* functional properties and requirement of its function in gamma radiation resistance of *D. radiodurans*.

3.2.1 Methods

3.2.1.1 Construction of recombinant plasmids

Genomic DNA of *D. radiodurans* R1 was prepared as published previously (Battista *et al.,* 2001). The coding sequence of *dr2518* gene was PCR amplified from genomic DNA of *D. radiodurans* using gene specific primer 2518F and 2518R (Table. B). PCR product was ligated at

NdeI and *BamHI* to yield pET2518. pET2518 plasmid was confirmed by partial sequencing. pET2518 plasmid was transferred to *E. coli* BL21 (DE3) pLysS and transgenic cells were induced with IPTG for the synthesis of recombinant DR2518 protein.

3.2.1.2 Purification and characterization of DR2518 for its localization, PQQ binding and autokinase activity

E. coli BL21 (DE3) pLysS harboring pETS162, pETK42, pETT169 and pETS171 and pET2518 were induced with 500µM IPTG and expression of recombinant proteins was confirmed by SDS-PAGE analysis. Recombinant proteins containing hexahistidine tag at N-terminal was purified using nickel affinity chromatography as with modified protocol described earlier **(**Tao *et. al.,* 2010**)**. In brief, the cells were incubated in buffer containing 50mM Tris-HCl, pH8.0 and 300mM NaCl containing 0.5mg/ml lysozyme for 30 min on ice. The mixture was sonicated for 5 min on ice with 30sec pulses at 1 min interval. Pellet containing insoluble DR2518 was collected by centrifugation at 12000x g and the recombinant protein was extracted from pellet with buffer containing 50mM Tris-HCl pH 7.6, 300mM NaCl, 10mM β-ME and 0.5% Sarkosyl and incubated overnight at 4^0C. The supernatant containing soluble protein was collected by centrifugation at 12000 x g and treated further with 1% Triton-X-100 and 10mM CHAPS for 10 min on ice. The mixture containing DR2518 in soluble form was purified using nickel affinity chromatography as described earlier (Kota *et al.*, 2010).

For determination of the cellular location of DR2518, *D. radiodurans* cells were fractionated in different cellular component as reported earlier with some modifications (Thompson *et al.,* 1981). In brief, 1 ml of overnight grown cells were collected and cell pellet washed twice with TE buffer and resuspended in 200 µl of 10% NaCl solution for 5 min. cells recovered by

centrifugation at 12000g. The supernatant contains outermembrane proteins of *D. radiodurans*. Recovered cells pellet dissolved in 200 µl of buffer A (50mM Tris-Cl pH 8, 20% Sucrose and 1mg/ml lysozyme) and incubated on ice for 30 min and centrifuged. The supernatant contains largely periplasmic proteins, was saved on ice till further use. Recovered pellet was dissolved in 200 µl of buffer 50mM Tris-Cl pH 8 and sonicated for 1 min and cell pellet was collected by centrifugation at 40000g. The supernatant contains mainly cytoplasmic proteins stored on ice till next use. The recovered pellet which largely contains membrane protein, cell debris and inclusion bodies. Membrane proteins were solublized by resuspending the pellet in 200 µl of 50mM Tris-Cl pH 8 supplemented with 1% Triton-X-100, sonicated on ice and centrifuged. Supernatant contains membrane proteins. Proteins precipitated with 5 volume of acetone and dissolved in 1X SDS-dye and separated on SDS-PAGE. The protein was transferred on PVDF (Millipore) membrane for immunoblotting with antibodies against DR2518 (Custom synthesized from Genei, India).

For PQQ-DR2518 protein interaction study, PQQ and DR2518 or DR2518 and PQQ mixture purified through G-25 spin columns, were used for recording the Circular Dichroism (CD) spectra (JASCO, J815, Japan).

The autokinase activity of purified DR2518 was assayed in presence of both cold ATP and [^{32}P] γ-ATP *in solution* and protein phosphorylation was detected by immunoblotting with polyclonal phosphor-Thr epitope specific antibodies in case of cold ATP and by autoradiography in case of [^{32}P] γ-ATP. The alkaline phosphatase treated protein was incubated with or without PQQ (1 µM) at 37^{o}C for 1h in a reaction mixture containing Tris-Cl 70 mM pH 7, DTT 5 mM, 10 mM $MgCl_2$and 0.5mM ATP/ 50µCi [^{32}P] γ-ATP with 10mM sodium fluoride. Samples were heated with equal volume of 2X Laemmli sample buffer at 95^{o}C for 5 min and separated on SDS-

PAGE. The protein was transferred on PVDF (Millipore) membrane for immunoblotting with antibodies against polyclonal phosphor-Thr epitops (Cell Signaling Technology, USA). For autoradiography, the gel was stained with coomassie brilliant blue and destained to ascertain equal amount of proteins loading in each lane, dried and exposed for autoradiogram. For checking the effect of damaged DNA on autokinase activity, the protein was preincubated with required DNA before either ATP or [^{32}P] γ-ATP was added in reaction mixture. The reaction was incubated for 1h at 37^{o}C and levels of DR2518 phosphorylation was detected by autoradiography followed by densitometric scanning of phosphosignals using Gene Genius tools (Syngene, UK).

3.2.1.3 Generation of site directed mutants of DR2518 protein and in vitro prortein kinase assay studies

Site directed mutagenesis for generating K42A, S162A, T169A and S171A mutants of DR2518 kinase was carried out using pET2518 (Rajpurohit and Misra, 2010) as template and site specific mutagenic primers (Table B) using site directed mutagenesis kit (New England Biolab, USA) following kit manufacturers protocols. For cloning these derivatives in pRadgro a *Deinococcus* expression vector (Misra *et al.*, 2006), the coding sequences of all the mutated derivatives of DR2518 kinase were PCR amplified from respective plasmid constructs using gene specific primer 2518F and 2518R (Table B) and cloned at *ApaI* and *Xba*I sites in pRADgro. The resulting plasmids containing *dr2518* gene with S162A, K42A, T169A and S171A mutations were named as pGroS162, pGroK42, pGroT169 and pGroS171 respectively. These recombinant plasmids were transformed into Δ*dr2518* mutant as described in chapter 2. The recombinant clones were scored in presence of chloramphenicol (5μg/ml) and presence of plasmid was confirmed by restriction analysis. All recombinant pET plasmids were also transferred to *E. coli* BL21 (DE3)

pLysS and expression of recombinant proteins was confirmed by SDS-PAGE analysis and purified as described above. All the mutations generated by *in vitro* mutagenesis, were confirmed by sequencing. For the detection of autophosphorylation of DR2518 kinase and K42A, S162A, T169A and S171A mutant derivatives, the cell free extract of *E. coli* expressing DR2518 and its various derivatives were separated on SDS-PAGE, transferred to PVDF membrane and probed with polyclonal phospho-threonine antibodies (Cell Signaling Technology, USA). For autophosphorylation activity, the ~200ng of purified recombinant DR2518 kinase and its mutated derivatives, were incubated in 25 µL of kinase buffer (Tris-Cl 70 mM pH 7, DTT 5 mM, 10 mM $MgCl_2$, 100 µM ATP) supplemented with 10µCi of [γ-^{32}P]-ATP for 30 min at 37^{o}C. For trans-phosphorylation activity, the ~200ng purified recombinant proteins were incubated with either 100ng myelin-basic protein (MBP) or 100ng PprA, as described above. The reaction mixtures were separated on SDS-PAGE and protein phosphorylation was detected by autoradiography.

3.2.1.4 DNA protein interaction studies

For DNA binding activity assay, the different amount of DR2518 (100ng, 300ng, 600ng and 1000ng) was incubated with 200ng linear DNA and 500ng circular DNA in sample buffer (10mM Tris-HCl, pH 7.6, 10mM KCl, 2mM $MgCl_2$ and 2% glycerol) for 20 min at 37^{o}C. The products were analysed on 0.8% agarose gel.

3.2.1.5 Transcriptional expression studies

Total RNA was prepared as previously described (Chen *et al.*, 2007). First-strand cDNA synthesis was carried out in 20 µl of reactions containing 1 µg of DNase I-treated total RNA and

3 µg of random hexamers. Level of *dr2518* gene transcript was checked by PCR amplification of *dr2518* gene specific forward primers (5'-CGTTCACAGTCACGGCA-3') and reverse primer (5'-CAGTTCCTGCATGTCGGA-3') using C-DNA from different PIR samples as a template. DR1343, deinococcal-glyceraldehyde 3-phosphate dehydrogenase (GAP) was used as an internal reference control.

3.2.1.6 Determination of *in vivo* phosphorylation of DR2518

Immunoprecipitation of DR2518 protein was carried-out with [^{32}P] labeled protein from unirradiated and 2kGy γ irradiated cells collected at different time interval of post irradiation recovery, using Seize X protein-G Immunoprecipitation Kit (Pierce, Illinois). The cell free extract of *D. radiodurans* cells was prepared. In brief, cells were treated with lysozyme (10 mg/ml) for 1 h at 37°C, followed by 0.5% NP-40 in cell lysis buffer (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM PMSF, 1 mM DTT). Treated cells were disrupted by either by sonication on ice bath for 1 min or by repeated freezing thawing for radioactive cells as described above, and cleared supernatant was obtained by centrifuging at 12000g for 30 min. Approximately, 500µg total proteins in cell free extract and 500ng equivalent purified DR2518 were incubated with DR2518 antibodies raised in rabbit in binding buffer (140mM NaCl, 8 mM sodium phosphate, 2mM potassium phosphate and 10mM KCL, pH 7.4). Mixture was incubated overnight at 4^{o}C and to this the Protein G agarose beads were added. Content was passed through Econopack column (Biorad, USA) and washed thrice with binding buffer and eluted with 500mM NaCl in binding buffer. Both column bound and flow through proteins were precipitated with 2.5 volume of ice-chilled acetone and precipitate was dissolved in 2X Laemmli buffer for SDS-polyacrylamide gel electrophoresis. Proteins were separated and autoradiogram was developed.

3.2.2 Results

3.2.2.1 DR2518 is a putative eukaryotic type Ser/Thr protein kinase (eSTPK)

Multiple sequence alignment of primary amino acid sequence of DR2518 protein has showed that the N-terminal of DR2518 contains a Hank type kinase domain (Hanks *et al.,* 1988) normally present in eukaryotic type Ser/Thr protein kinases (eSTPK), while C-terminal is having a sensory PQQ interacting domain. The amino acid sequences of the N-terminal catalytic domain of DR2518 kinase and PknB of *Mycobacterium tuberculosis* was aligned using Clustal X program and alignment was mapped with the 3-D structure of PknB (PDB code:1 MRU) using ESPRIPT 2.2. (Fig.3.2.1). It showed 40% identity with PknB at amino acid levels and a high degree of conservation at secondary structure levels that classified DR2518 a member of eukaryotic type Ser/Thr kinase sub family. The DR2518 had all the conserved motifs like P-loop, Helix-C, DFG motif, and P+1 motif and activation loop (Fig.3.2.1) as known in other eSTPKs. The activation loop is involves in determining substrate specificity and is containing variable number of phosphoacceptor serine/threonine residues. The activation loop, which receives the phosphate during autokinase activity, has varying number of phosphate receiving residues in different members of this sub-family. Our analysis showed that both DR2518 and PknB having two serine and two threonine residues in activation loop there position is different in activation loop. Many kinases are activated by phosphorylation on at least one Ser/Thr (Tyr for tyrosine kinases) residue in the activation loop, by either autophosphorylation or transphosphorylation by another kinase. The bioinformatic analysis of C-terminal region of DR2518 protein by SMART software (http://smart.embl-heidelberg.de/) predicted the presence of seven tandem β propeller repeat motifs at C-terminal sensory domain. Similar types of β-Propeller repeat motifs have been found in β-subunit of G protein representing the classic WD40 class protein; in low-density

lipoprotein receptor belongs to YWTD class, and in the NHL domain of BraT protein (Edwards *et al.,* 2003). To find out the subclass of DR2518 sensory C-terminal domain, the multiple sequence alignments was carried out with conserved seed sequences of each class of β-propeller family protein using Clustal X program. The alignment results showed that C-terminal sensor domain of DR2518 had seven WD repeats of approximately 40 amino acids that start with glycine (G) and end with tryptophan (W) (Fig. 3.2.2) in each repeat. Although, the presence of Glycine-Histidine (GH) and Trypthophan-Glutamate (WD) dipeptides are the characteristics of WD repeats subfamily proteins, neither of these are truly conserved among different members of this family (Smith *et al.,* 1999). This analysis classify DR2518 protein as a serine/threonine type protein kinase containing PQQ interacting WD type β-propeller repeats of WD subfamily. The WD-repeat proteins are important class of proteins perform wide range of functions, which includes regulatory functions in signal transductions, RNA synthesis and processing, chromatin assembly, vesicular trafficking, cytoskeletal assembly, cell cycle control and apoptosis (Li and Roberts, 2001).

Topology analysis (http://www.ch.embnet.org/software/TMPRED_form.html) predicted that DR2518 has membrane organization similar to PknB. DR2518 seem to be an "N- in- C-Out", membrane protein having N-terminal catalytic kinase domain and C-terminal PQQ binding domain lying in the cytoplasm (Fig.3.2.3 B, C). Since PASTA domain in PknB is a signal-receiving domain, the similar function of PQQ binding domain in DR2518 might be speculated. Our *in silico* analysis showed that DR2518 a membrane protein. In order to confirm membrane localization of DR2518, the outermembrane, periplasmic, cytoplasmic and membrane protein fractions were separated by differential centrifugation as described in methods. The silver stained

SDS-PAGE gel showed different protein pattern for all different fractions, however there are few proteins were common to seen in both periplasmic and cytoplasmic fractions. In order to find out the cellular localization of DR2518 protein, these different protein fractions transferred to PVDF membrane and probed with anti-DR2518 polyclonal serum. Results showed that control purified DR2518 protein crossreacted with antibody and produce intense signal on blot. DR2518 presence was detected in membrane fraction only. This suggests that DR2518 might localize in membrane of *D. radiodurans* (Fig.3.2.4).

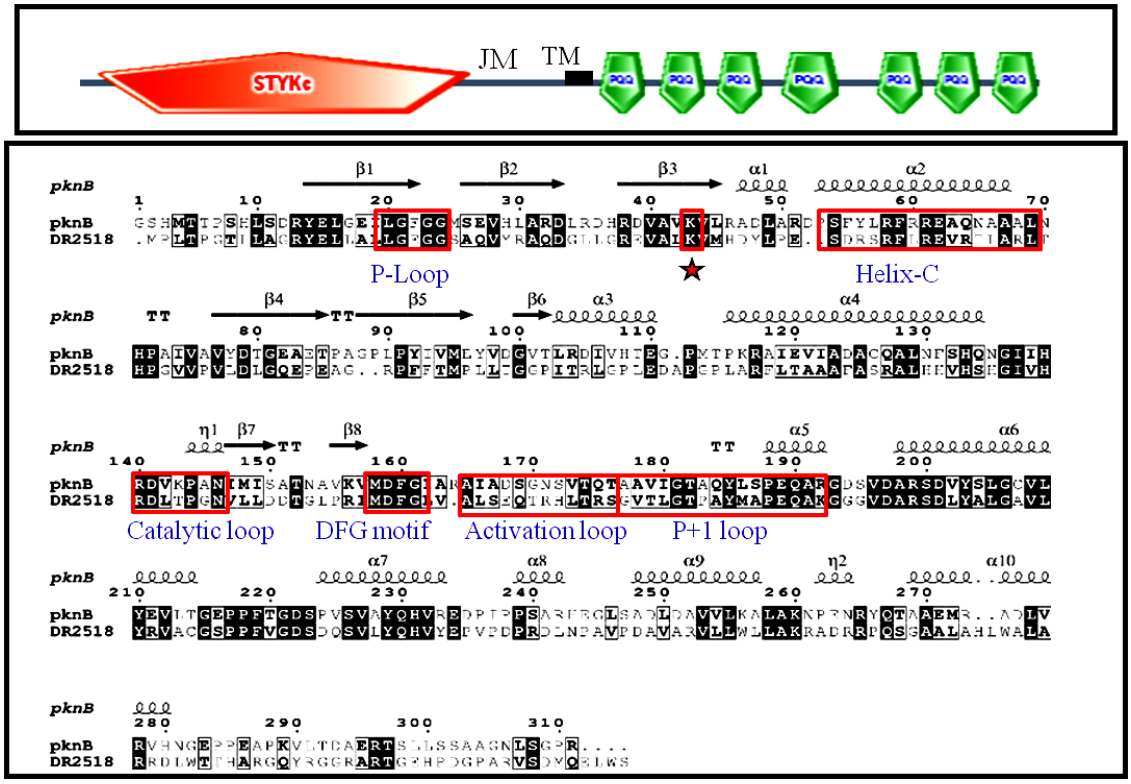


Fig. 3.2.1 Structural organization of DR2518 kinase

JM; juxta-membrane region, TM; trance membrane region, PQQ; PQQ binding motif. Primary sequence alignment of catalytic domain of DR2518 kinase and PknB of Mycobacterium. Conserved residues are boxed. Catalytically important Lysine is indicated with a star. Secondary structural elements are indicated above the sequence.

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PQQ_rep_1      -PLVWAGAAL QCDETGWLHALDA- RSGTP --------LWKVE--- 32
PQQ_rep_2      -PVISAGLVF LATEGGELLALDV- RNGEV --------RWTCR--- 32
PQQ_rep_3      --TVWGGRLL APSRDGHLHALSL- RTGEL --------AWAYR--- 31
PQQ_rep_4      PATLIGGHVL YGAWDGTLRRVEL- QSGSE --------VWRHQ--- 33
PQQ_rep_5      ASAAPGGVVV VAGWGGKVRGLRL- ADGED --------LWERT--- 33
PQQ_rep_6      -PLAASGTLY VAFMDGTLRAY----RNAHP --------EWRSEQEG 33
PQQ_rep_7      --PPGTATLV IATWEGEVHAIGLEVQNGRA ALAGEDAIRWTYD--- 41
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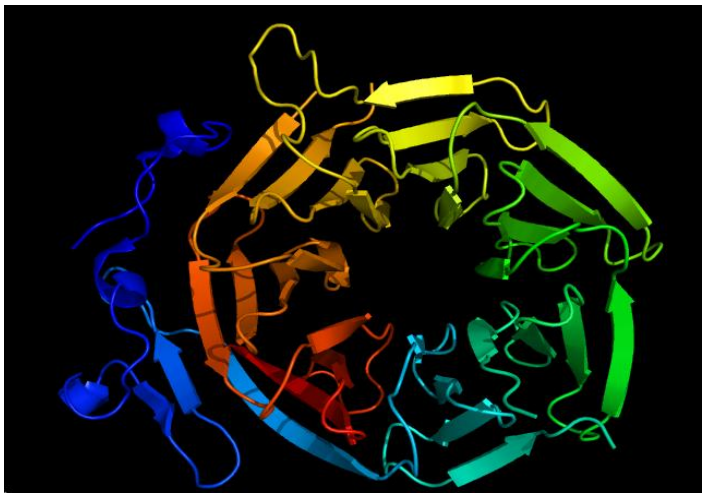
[A]



[B]

Fig. 3.2.2 Multiple sequence alignment of seven PQQ beta propeller repeats present at C-terminal of DR2518 kinase Conserved WD family residues are boxed [A] and the representative 3-D organization of beta propeller repeats (B) (Smith *et. al.,* 1999).

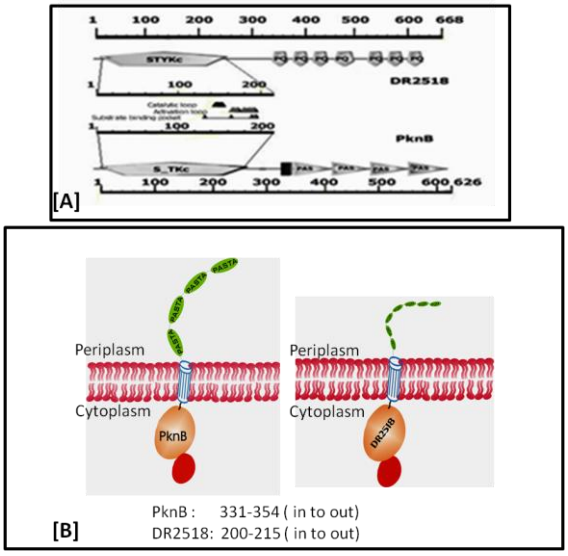


Fig. 3.2.3 Structural similarities of DR2518 with PknB sensor kinase DR2518 amino acids were compared with PknB for functional domains alignment (**A**). Topology search showed possible transmembrane domains distributed from amino acids 201-217 in DR2518 (**B**) distinctly dividing this protein into intracellular (STKc and seven PQQ binding motifs), transmembrane (201-215) and extracellular (217-668) containing seven PQQ binding motifs, which resemble closely to the corresponding regions of amino acids and ligand binding motifs of mycobacterial PknB sensor kinase.

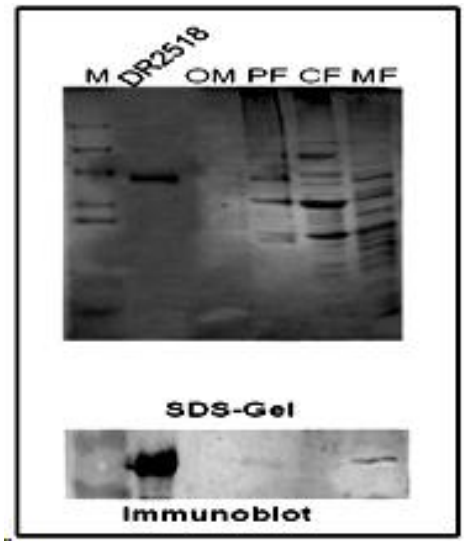


Fig. 3.2.4 Subcellular localization of DR2518 kinase detected by DR2518 speciifc polyclonal antibody. OM- Outer membrane fraction, PF- Periplasmic fraction, CF- Cytoplasmic fraction and MF- Membrane fraction.

3.2.2.2 *In vitro* functional characterization of DR2518 protein

3.2.2.2.1 Full length (DR2518) and its N-terminal kinase domain (DR2518KD) were cloned expressed and purified

In order to study the biochemical properties of DR2518 and the regulatory roles of C-terminal putative sensory domain, the full length DR2518 as well as its N-terminal kinase domain (DR2518KD) were cloned expressed in *E. coli* and recombinant proteins were purified by modified protocol for membrane protein purification using nickel affinity chromatography as described in methodology. The purity of eluted protein was checked on SDS-PAGE and found as a single homogeneous protein band of 72kDa on coomassie blue stained gel (Fig 3.2.5). Similarly DR2518KD was overexpressed and purified up to its homogeneity. The molecular weight of purified DR2518KD on SDS-PAGE was 33kDa (Fig 3.2.6). Both purified protein were stored in protein storage buffer (10mM Tris-Cl pH 7.6, 50mM KCl, 0.1mM EDTA, 1mM DTT and 50% glycerol) at -20^{0}C till further use.

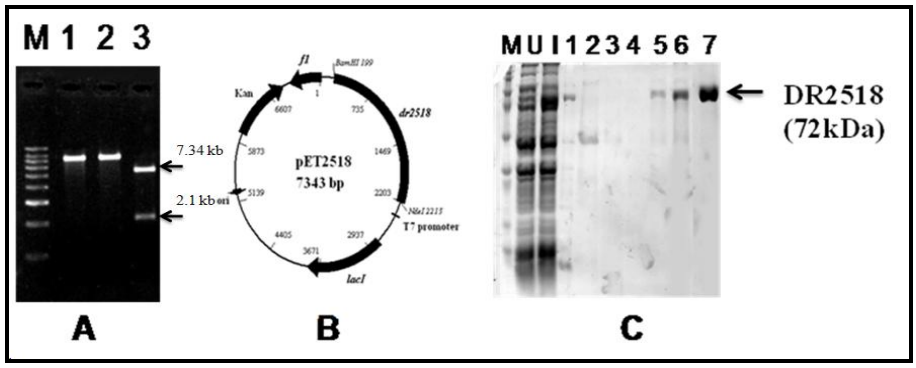


Fig. 3.2.5 Cloning and expression of *dr2518* in pET28a+ for the purification of recombinant protein from *E.coli*. PCR product was cloned in pET28a+ and recombinant plasmid was digested with *NdeI* (1), *BamHI* (2) and *NdeI-BamHI* (3) (**A)** and release of insert confirmed the cloning on *dr2518* gene in pET28a+ (**B**). Recombinant plasmid was transferred to BL21 *E.coli* and transformed cells (U) were induced with IPTG (I). Cell free extract was purified by metal affinity and fraction (1-7) were analysed on SDS-PAGE.

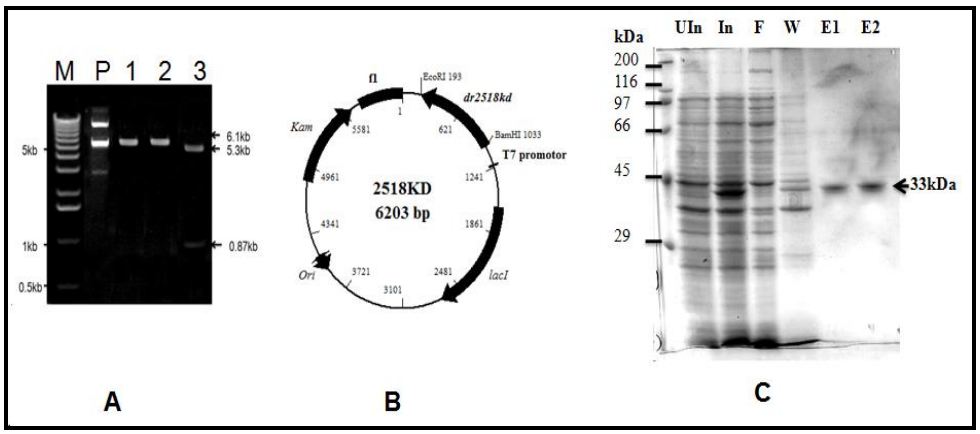


Fig. 3.2.6 Cloning and expression of *dr2518kd* in pET28a+ for the purification of recombinant protein from *E. coli*. PCR product was cloned in pET28a+ and recombinant plasmid (P) was digested with *EcoR1* (1), *BamHI* (2) and *EcoR1-BamHI* (3) (**A)** and release of 0.87 kb insert confirmed the cloning of *dr2518kd* (kinase domain) gene in pET28a+ (**B**). Recombinant plasmid was transferred to BL21 *E.coli* and transformed cells (UIn) were induced with IPTG (In). Cell free extract was purified by metal affinity and flow through (F), wash (W) and eluted (E1 and E2) were analysed on SDS-PAGE.

3.2.2.2.2 DR2518 is a functional kinase showing PQQ and DNA ends inducible autophosphorylation

The primary sequence of DR2518 protein contains signature for eukaryotic type serine/threonine protein kinases at its N-terminal and 7 β-propeller repeats at C-terminal for PQQ interaction. The phospho-nature of DR2518 polypeptide and the autokinase activity of this protein were ascertained using phospho-Ser/Thr epitops specific polyclonal antibodies and by *in vitro* phosphorylation using [^{32}P] γ-ATP. Purified recombinant DR2518 protein showed cross reactivity with phospho-Ser /Thr epitops antibodies (Fig. 3.3.7) suggesting this protein is a phosphoprotein. Since the DR2518 contains putative PQQ binding motifs and a well-defined eSTPK domain, the possibilities of this protein interact with PQQ and having autokinase activity was checked. The purified recombinant DR2518 treated with alkaline phosphatase (AP) did not

give any cross-reactivity with phsopho-Thr epitopes specific antibody (Fig. 3.2.7 A, lane 1), however DR2518 purified from *E.coli* give signal on immunoblot suggests that DR2518 gets phosphorylated during its synthesis. The alkaline phosphatase (AP) treated recombinant DR2518 was allowed for phosphorylation in presence of ATP with and without 1µM PQQ and it was found that the autophosphorylation of DR2518 increased 2.5 fold in presence of PQQ (compare lane 3 and 4, Fig. 3.2.7 A). Similarly, the AP treated protein when incubated with [^{32}P] γ-ATP, it showed phosphorylation, which was stimulated with PQQ by 2.679 ± 0.324 fold as detected by autoradiography and phosphosignals quantified by densitometric scanning using "Gene Genius tools" (Syngene, UK) (Fig.3.2.8 A). These results confirmed that DR2518 is a phosphoprotein having PQQ stimulated autophosphorylation activity, also suggested the functional interaction of PQQ with DR2518 *in vitro*. Functional interaction of DR2518 with PQQ, suggested that both these species may interact physically *in solutions*. Circular Dichroism (CD) spectroscopy was utilized to probe the change in secondary structure of DR2518 upon physical interaction with PQQ. The CD spectra of DR2518 (0.1µM) incubated with increasing concentration of PQQ were different from DR2518 protein alone (Fig. 3.2.7 B). Since PQQ alone did not show circular dichroism, the change in spectral characteristics as a function of PQQ concentration indicated the conformational change in protein upon PQQ interaction. Interestingly the change in CD spectra was not observed when the PQQ concentration was increased from 100nM to 1000nM, this may suggest that at 100nM of PQQ concentration all protein molecules gets saturated and did not respond on further increase of PQQ concentration. This stoichiometry suggested one protein molecule of DR2518 kinase requires one PQQ molecule for binding. Obtained CD spectra were in accordance with the typical characteristics of CD spectra generally obtained during ligand-protein interaction *in solution*. The structural similarities of DR2518 with eukaryotic type STPKs

and WD family proteins and stimulation of autophosphorylation activity of DR2518 kinase, together suggested that DR2518 protein could be a sensory quinoprotein kinase.

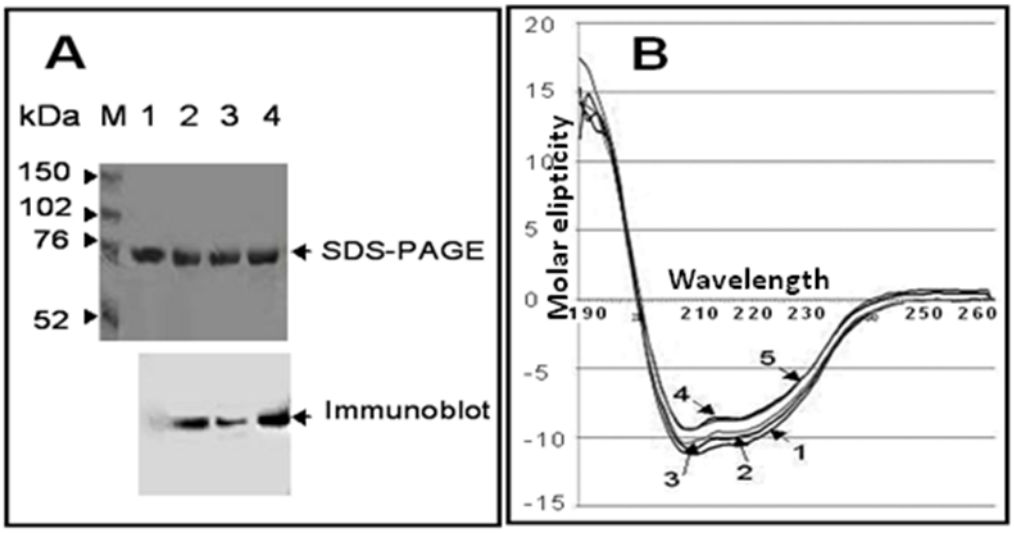


Fig. 3.2.7 PQQ interaction with DR2518 protein and its activity characterization *in solution* **A.** Purified recombinant protein was treated with alkaline phosphatase (1) and incubated with cold ATP in absence (3) and presence of (4) of PQQ. These samples were separated on SDS-PAGE along with untreated protein (2) and phosphosignals were detected with phospho-Ser/Thr antibodies (immunoblot). **B.** Circular Dichroism spectroscopy of purified protein incubated in absence (1) and in presence of an increasing concentration 1nM (2), 10nM (3), 100nm (4) and 1000nM (5), of PQQ was carried out. Data presented here is from a reproducible typical experiment.

Earlier findings have shown that PQQ reacts with reactive oxygen species and forms a non-reactive ROS adduct of PQQ (PQQ-ROS) *in solution* (Misra *et al.*, 2004). Here we observed that recombinant DR2518 is an autokinase and this activity is stimulated with PQQ *in solution* (Fig. 3.2.7A). Therefore, the possible role of PQQ as a sensor of oxidative stress forming PQQ-ROS, and the effect of this non-reactive species on kinase activity of DR2518 were evaluated. The autokinase activity was checked in presence of γ radiation exposed PQQ (PQQ-ROS) *in solution.* PQQ exposed with γ radiation did not improve in the autophosphorylation of DR2518 compare

to unirradiated PQQ (Fig. 3.2.8 A). This suggested that the PQQ stimulation of *in vitro* DR2518 activity was due to its interaction with this protein, as a cofactor irrespective of the history of PQQ exposure to γ radiation. Since the expression of DR2518 was induced with γ radiation that also produces high density of DNA strand breaks, the effect of unirradiated double-stranded DNA (dsDNA) and gamma radiation irradiated dsDNA on autokinase activity of DR2518 was examined by *in vitro* phosphorylation with [^{32}P] γ-ATP. The phosphosignal intensity was quantified by densitometric scanning using "Gene Genius Tools" (Syngene, UK). The autokinase activity of DR2518 increased by 2.134 ± 0.321 fold in presence of 1kb linear dsDNA fragment irrespective of γ radiation exposure and presence of PQQ (Fig. 3.2.8 A). Since γ radiation can cause different type of oxidative damage to DNA, considering this fact, gamma radiation irradiated and unirradiated 1kb dsDNA was also checked for its effect on autophosphorylation of DR2518. However, both type of 1kb dsDNA produces similar type of effect on stimulation of autophosphorylation of DR2518 kinase, strongly indicated the requirement of DNA ends for DR2518 activity stimulation. The absence of the cumulative effect of linear dsDNA, PQQ or γ irradiated PQQ and dsDNA when compared with linear dsDNA alone, on stimulation of autophosphorylation activity, argued in favor of a possible DNA ends mediated stimulation of DR2518 activity rather than oxidative damage of DNA. To check this possibility, the DNA ends mediated stimulatory effect of DR2518 kinase was evaluated in presence of *D. radiodurans* genomic DNA isolated from unirradiated and irradiated *D. radiodurans* cells. Results of this experiment showed the stimulatory effect on autokinase activity of DR2518 kinase by both type of DNA (Fig. 3.2.8 B). However it is apparent that in presence of genomic DNA isolated from irradiated cells could produce more stimulatory effect than DNA isolated from unirradited cells. The stimulation of DR2518 activity with total genomic DNA isolated from both unirradiated and

γ irradiated cells, might be due to open ends generated during preparation of genomic DNA even from unirradiated cells. Further to conclude weather the stimulatory effect of DNA on DR2518 kinase autophosphorylation was due to presence of DNA or DNA ends *per se*, more precise experiment was carried out where the autophosphorylation of DR2518 kinase was checked in presence of linear and covalently closed circular (CCC) plasmid DNA (pSK+). Nearly 2 fold increase in autophosphorylation with linear plasmid DNA but not with covalently closed circular (CCC) plasmid DNA (Fig. 3.2.8 C) strongly supported the conclusion that DR2518 activity was stimulated with DNA fragments having open ends. In order to understand the interaction of this protein with dsDNA, the DNA binding activity of purified protein was checked with circular form of plasmid DNA and linear dsDNA substrates. Both types of dsDNA substrates showed interaction with DR2518 *in vitro* (Fig. 3.2.9). Since this protein does not contain any known DNA binding domain, the mode of its interaction and stimulation of autophosphorylation of DR2518 by linear dsDNA are unclear. These results however, provided a strong evidence to suggest that PQQ and DNA ends could stimulate DR2518 autokinase activity *in vitro*.

Since PQQ can interact with DR2518 kinase and stimulate its autophosphorylation *in vitro* and our bioinformatic analysis reveals the PQQ interaction site in DR2518 kinase is located at C-terminal. To confirm this observation we made DR2518KD mutant of DR2518 protein which contains only N-terminal catalytic kinase domain amino acids (1-280) and lacking of C-terminal domain (Fig. 3.2.1, 3.2.6). The autophosphorylation of full length DR2518 kinase and DR2518KD was checked in presence of PQQ and dsDNA similarly as evaluated earlier, the stimulation of DR2518 activity by nearly 2 fold in presence of PQQ and dsDNA was consistent with earlier data. Interestingly, the autophosphorylation of DR2518KD when checked in

presence of PQQ and dsDNA fragment, it showed activity, which did not change significantly in presence of PQQ and dsDNA (Fig. 3.2.10). The Recombinant DR2518KD the protein missing C-terminal PQQ binding domain, did not showed the stimulation by PQQ indicating the regulatory role of PQQ and its interaction through C-terminal domain of this protein. Collectively these results indicated that DR2518 is a quino-phosphoprotein kinase exhibiting the PQQ and dsDNA ends stimulated autophosphorylation activity *in vitro*.

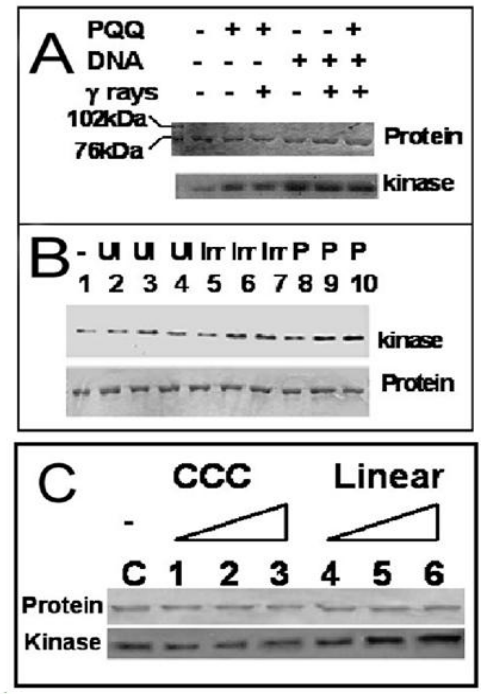


Fig. 3.2.8 ***In vitro*** **autophosphorylation of DR2518 kinase Recombinant** DR2518 was incubated with PQQ and PCR product in different combinations and the autokinase activity was detected by autoradiography **(A)**. Similarly, purified protein (1) was incubated with 10ng (2, 5, 8), 100ng (3, 6, 9) and 1000ng (4, 7, 10) of genomic DNA prepared from unirradiated (UI), 6kGy γ irradiated (Ir) cells and PCR amplified (P) DNA, and the autokinase activity was detected by autoradiography (**B**). DR2518 was also incubated with 100ng of each superhelical form (1, 2, 3) and linear form (4, 5, 6) of plasmid DNA and autokinase activity was detected by autoradiography (**C**).

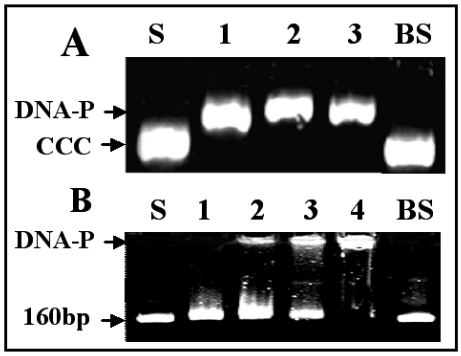


Fig. 3.2.9 DNA binding activity of recombinant DR2518 protein *in vitro*. The 500ng of superhelical form of plamid DNA (S) was incubated with 300ng (1), 600ng (2) and 1000ng (3) recombinant DR2518 for 20 min **(A**). Similarly the 160bp linear double stranded DNA was incubated with 100ng (1) 300ng (2), 600ng (3) and 1000ng (4) purified DR2518 for 20 min (**B**). Both types of DNA substrates were incubated with 500ng BSA (**BS**) in place of DR2518, as controls under identical conditions. Products were analysed on 0.8 % agarose. Data provided was from a reproducible representative experiment.

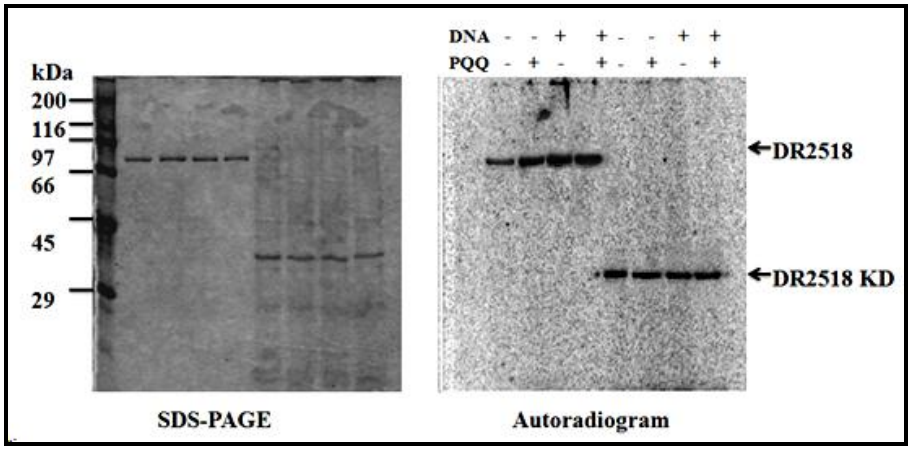


Fig. 3.2.10 *In vitro* autophosphorylation of DR2518 and DR2518KD proteins. The recombinant full length DR2518 and truncated DR2518KD proteins were incubated with PQQ and 1kb dsDNA in different combinations and the autokinase activity was detected by autoradiography.

3.2.2.2.3 Lysine 42 in catalytic cleft while Threonine 169 of activation loop is important for DR2518 kinase activity

Multiple sequence alignment with eukaryotic type kinases indicated the lysine 42 was located in N-lobe of kinase domain of DR2518 protein, was an important conserved catalytic residue similar to lysine 40 in PknB of *M. tuberculosis*. Also three phosphoacceptor residues; S162, T169 and S171 were predicted in the activation loop (Fig. 3.2.1). These amino acids were replaced with alanine by site directed mutagenesis and pETK42A, pETS162A, pETT169A and pETS171A mutants constructs were generated independently. These proteins were overexpressed in *E. coli* and endogenous phosphorylation of total proteins was checked using polyclonal antibodies against polyclonal phospho-threonine epitopes antibodies. Numerous phosphoproteins were seen in cells expressing wild type DR2518 and S162A mutant. IPTG induced cell free extract of K42A expressing cells did not detect serine / threonine phosphorylation while others (T169A and S171A) showed reduced phosphorylation with *E. coli* extract (Fig.3.2.11). Since all the proteins were expressed in *E. coli*, where no endogenous Ser/Thr kinase activity is reported except a periplasmic protein kinase (Khairnar *et al.*, 2007), the phosphorylation of *E. coli* proteins by wild type DR2518 might indicate that *E. coli* genome also encodes several proteins that could be phosphorylated by STPKs. The evolutionary functional significance of serine / threonine phosphorylation in *E. coli* proteins is not clear. The Involvement of lysine 42, serine 162, threonine 169 and serine 171 in protein kinase function of DR2518 was further studied with purified proteins by checking the autophosphorylation and the trans-phosphorylation of myelin basic protein (MBP) and a pleiotropic protein promoting DNA repair, PprA (*D. radiodurans* PprA was identified as a putative substrate for DR2518 kinase and will be discussed in chapter 3.3) (Narumi *et al.*, 2004). The wild type DR2518 showed kinase activity while mutated proteins

showed differential effect on its kinase function (Fig.3.2.12). Both wild type and S162A mutant showed nearly similar levels of autophosphorylation, and the transphosphorylation of MBP and PprA respectively (Fig.3.2.12 A, B). The K42A mutant did not show protein kinase activity and the levels of autophosphorylation and transphosphorylation of MBP and PprA by T169A and S171A mutants of DR2518 reduced by nearly 60% and 40% respectively, as compared to wild type (Fig.3.2.12 C). These results suggested that (i) the recombinant DR2518 kinase is functionally active in *E. coli* and could phosphorylate a number of *E. coli* proteins (Fig. 3.2.11), (ii) the lysine 42 was essential catalytic residue for its both autokinase and transkinase activities *in solution*, and (iii) the T169 and S171 were potentially important residues as putative phosphoacceptor sites in the activation loop of kinase domain, while S162 was redundant.

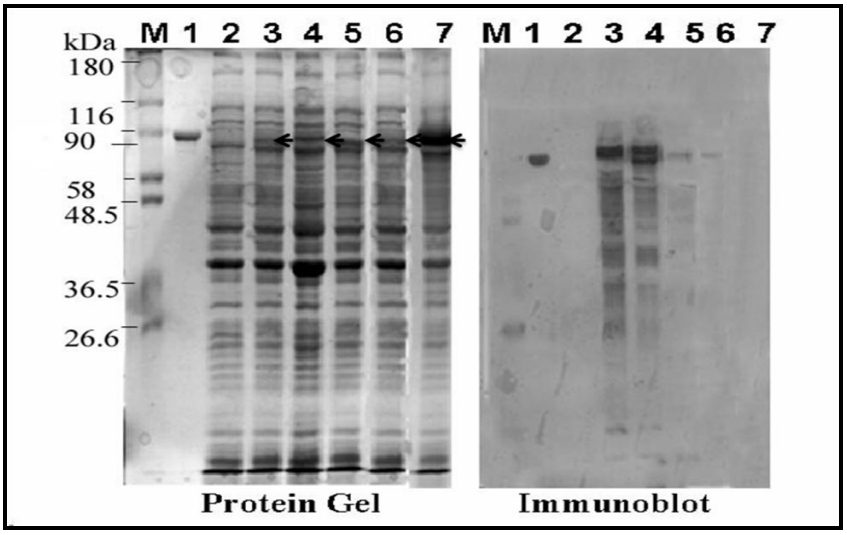


Fig.3.2.11 Immunodetection of autokinase activity of DR2518 kinase and its point mutants in induced cell free extract. M- Marker, purified DR2158 (1), Cell free extract of induced cells containing pET28a plasmid (2), Cell free extract of induced wild type DR2518 protein (3), Cell free extract of induced S162A mutant protein (4), Cell free extract of induced S171A mutant protein (5), Cell free extract of induced T169A mutant protein (6), Cell free extract of induced K42A mutant protein.

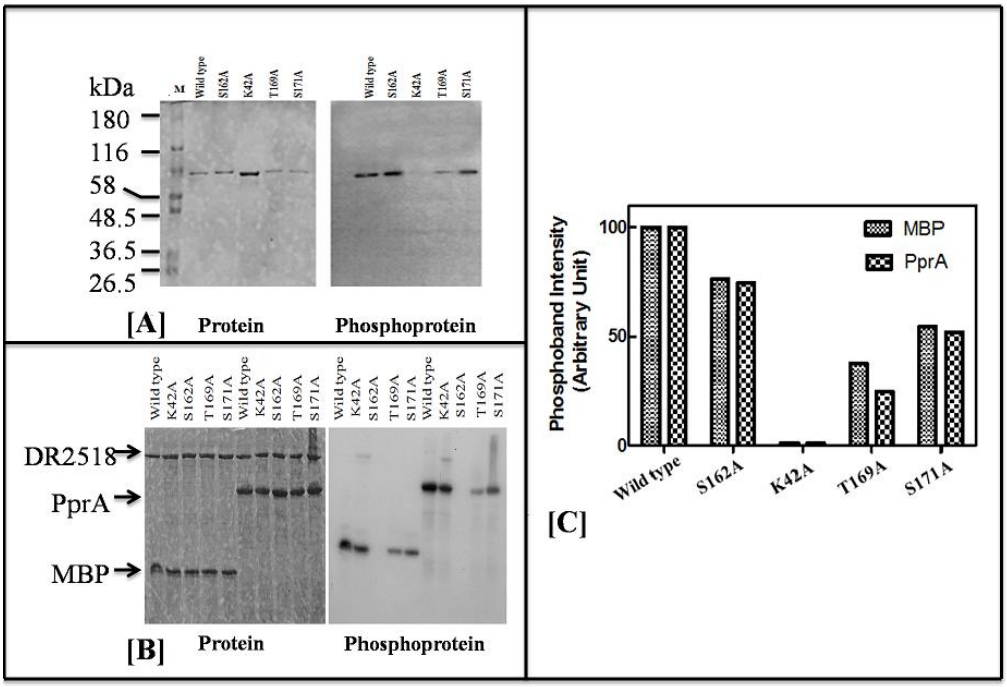


Fig. 3.2.12 Protein kinase activity characterization of different mutant derivatives of DR2518 *in vitro* Recombinant DR2518 and its mutants like K42A, S162A, T169A and S171A were purified to near homogeneity and autokinase activity and transkinase activity were checked by incubated with PprA (PprA) and myelin basic protein (MBP) in kinase buffer supplemented with [^{32}P] γ ATP. Products were analysed on SDS-PAGE, stained with coomassie blue (SDS-PAGE) and phosphorylation was detected by autoradiography (Autoradiogram).

3.2.2.3 *In vivo* functional characterization of DR2518 protein

3.2.2.3.1 Gamma radiation induces the transcription of *dr2518* gene

The effect of γ radiation on transcription of *dr2518* gene was studied by RT-PCR. For measuring the post irradiated expression kinetics of *dr2518* gene, the *dr2518* specific internal primers were used to amplify the PCR product by reverse transcription polymerase chain reaction(RT-PCR) using C-DNA from different PIR samples (UI, 0.5, 1, 2, 3, 5 and 24hr). The constitutively expressing glyceraldehydes 3- phosphate dehydrogenase (*gap*) gene amplified as an internal control (Fig. 3.2.13 A). after normalize the band intensity of *dr2518* gene in PIR samples with respective PIR intensity of GAP gene, it was evident that the transcript level of *dr2518* gene

increases nearly 15.355 ± 0.179 fold within 30 min PIR and maintained closely to this level till 3h PIR and then decreased to nearly unirradiated level in 24h PIR (Fig. 3.2.13 B). This indicated the stimulation of *dr2518* transcription in response to γ radiation is a significant increase in first 5hr of post irradiation time and suggests the possible important role of DR2518 in early phase of post irradiation recovery time.

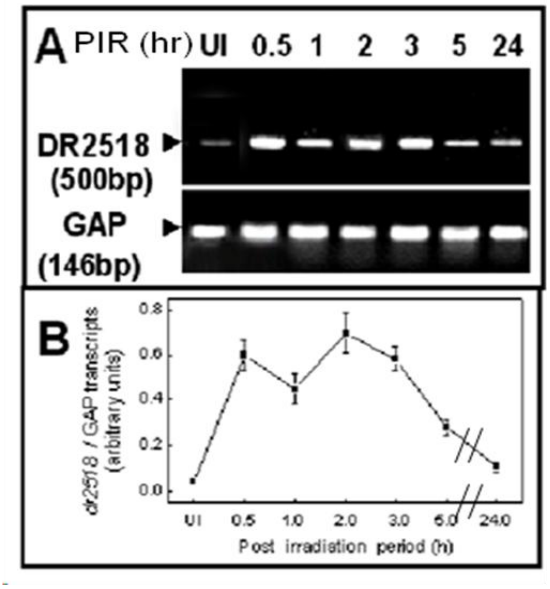


Fig. 3.2.13 Effect of DNA damage on *dr2518* gene expression and autokinase activity *in vitro*. The RT-PCR analysis of total RNA of wild type cells collected at different PIR (0.5, 1, 2, 3, 5 & 24h) period and unirradiated (UI) control (A) was carried out using internal primers of *dr2518* gene. The fold increase was calculated by densitometric scanning of DNA bands and normalized with a constitutively expressing gene (GAP) as internal reference (B) at every point independently.

3.2.2.3.2 Gamma radiation treatment induced autophosphorylation of DR2518 *in vivo*

Gamma radiation increases 15.355 ± 0.179 fold levels of *dr2518* transcript within 30 min PIR and that was maintained closely to this level till 3h PIR and then decreased to nearly unirradiated level in 24h (Fig.3.2.13). The phosphorylation status of DR2518 in *D. radiodurans* cells and the

effect of γ-radiation on its *in vivo* phosphorylation were also determined by coimmunoprecipitation with antibodies raised against purified recombinant DR2518. The exponentially growing [^{32}P] labeled cells were treated with 6.5kGy γ radiation and aliquots were drawn at different PIR. [^{32}P]-DR2518 protein was coimmunoprecipitated with DR2518 antibodies from the cell free extracts of labeled cells. The immunoprecipitates were purified through Protein-G affinity column chromatography and analyzed on SDS-PAGE along with the proteins that did not bind to column. Antibodies immunoprecipitated purified DR2518 (Fig 3.3.14 A, lane C). Unirrradiated sample showed very low intensity of phosphosignal precipitated with DR2518 antibodies while cell free extract of 1-3h PIR cells showed enhanced phosphorylation of DR2518 (Fig 3.3.14 A). Interestingly, the flow through fraction of PIR immunoprecipitates passed through Protein G Sepharose column, showed higher levels of phosphoproteins that did not bind to column, indicating the higher levels of protein phosphorylation in response to γ irradiation. This was further ascertained by immunoprecipitating the cell free extract of 1h PIR, its corresponding unirradiated control and purified [^{32}P]-DR2518 with antibodies. Phosphoproteins profiles of immunoprecipitate and unbound proteins as eluted in flow through were analyzed. Results showed immunoprecipitation with purified DR2518 (Fig 3.3.14 B, lane C) and higher levels of DR2518 phosphorylation upon γ irradiation (Fig 3.3.14 B, compare lane PU and PI). Interestingly, Protein-G flow through fraction from γ radiation treated 1h PIR sample showed an extensive phosphorylation of an additional protein of around 97kDa (Fig 3.3.14 B, lane SI). The corresponding signal was also seen though low levels, in unirradiated control (Fig 3.3.14 B, lane SU). These results confirmed *in vivo* phosphorylation of DR2518 and stimulation of this protein phosphorylation in response to γ irradiation. Abundance of higher size phosphoprotein band in flow through of Protein-G

affinity column chromatography from irradiated sample along with several minor signals, seems to be the putative substrates for this kinase getting phosphorylated in this bacterium in response to DNA damage. Further studies using *dr2518* deletion mutant would be useful in ascertaining the facts that these are true substrates for DR2518 kinase in this bacterium.

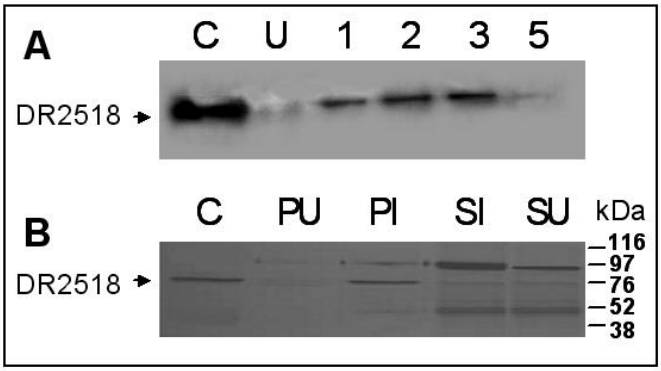


Fig. 3.2.14 Detection of *in vivo* phosphorylation of DR2518 in *Deinococcus radiodurans*. Polyclonal antibodies raised against purified recombinant DR2518 was incubated with purified [^{32}P] phosphorylated DR2518 (**C**) and with cell free extract of exponentially growing unirradiated cells (U) and 1, 2, 3 and 5h post irradiation recovery period **(A)** at 4^{o}C overnight**.** Similarly, the purified [^{32}P] phosphorylated DR2518 **(**C**),** and cell free extract of unirradiated (PU, SU) as well as 1h post irradiation recovery (PI, SI) cells were incubated with DR2518 antibodies at 4^{o}C overnight **(B).** Reaction mixtures were passed through Protein-G column. Both immunoprecipitates (C, U, 1, 2, 3, 5 as in A panel) and (C, PU, PI as in B panel) eluted from the columns and unbound proteins collected in flow through (SI, SU) were precipitated with acetone and separated on SDS-PAGE. Gel was dried and auroradiogram was developed. Data from a typical reproducible experiment was presented.

3.2.2.3.3 DR2518 kinase requires PQQ for γ radiation stimulation of its *in vivo* autophosphorylation

In chapter 3.1 we have shown that both *pqqE* gene and *dr2518* gene mutant of *D. radiodurans* were sensitive to gamma radiation and disruption of *pqqE* gene in Δ*dr2518* mutant did not

change the gamma radiation sensitivity of *pqqE:cat* Δ*dr2518* double mutant suggested both PQQ and DR2518 function through common pathway(s) in conferring the γ radiation resistance in *D. radiodurans*. We have also to show that C-terminal region of DR2518 kinase is PQQ interacting region and played important role in regulating PQQ mediated stimulation of autophosphorylation of DR2518 kinase *in solution*. The importance of PQQ in regulating the kinase function of DR2518 inside the cell was evaluated by coimmunoprecipitation of DR2518 protein using DR2518 specific polyclonal antibody from the wild type and *pqqE:nptII* mutant cells of *D. radiodurans* labeled with [^{32}P] and samples were collected at different PIR period.

The [^{32}P]-phosphoprotein signal was very low in immunoprecipitate of cell free extract from unirrradiated wild type cells, which was increased to maximum in 3h PIR and then reduces to unirradiated level in 5h PIR (Fig. 3.2.15 R1). On contrary to wild type pattern of *in vivo* phosphorylation pattern during PIR, the increase in phosphoprotein signal intensity in response to γ radiation was not noticed in *pqqE:nptII* mutant cells. Although DR2518 showed *in vivo* autophosphosrylation in *pqqE:nptII* mutant, these cells however, showed no change in phosphor signal in first 5h PIR, rather its reduced slowly during late PIR (Fig. 3.2.15 *pqqE:cat*). These findings suggested that the DR2518 undergoes phosphorylation *in vivo* and its both transcription and phosphorylation are induced by γ radiation in wild type. The PQQ plays a critical but yet uncharacterized regulatory role for the kinase activity stimulation in response to γ radiation *in vivo*.

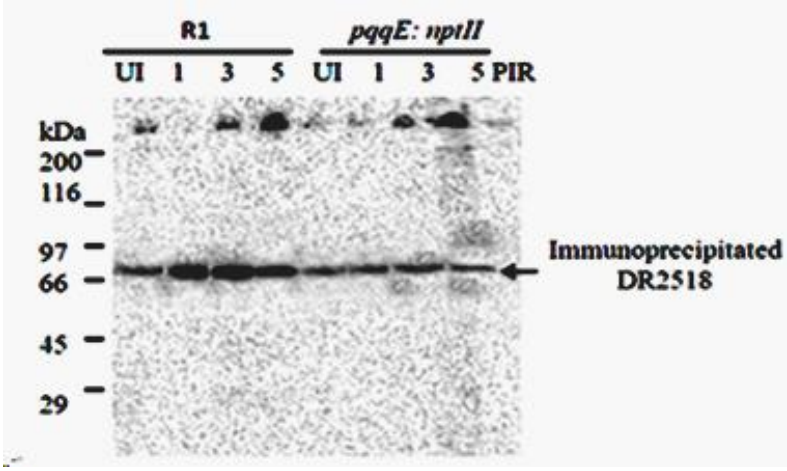


Fig. 3.2.15 Detection of *in vivo* phosphorylation of DR2518 in wild type and *pqqE:nptII* mutant of *Deinococcus radiodurans*. Polyclonal antibodies raised against purified recombinant DR2518 was incubated with [^{32}P] labeled Post irradiation recovery time (as indicated) cell free extract of wild type and *pqqE:nptII* mutant and immunoprecipitaed protein were separated on SDS-PAGE. Signal detected by autoradiography.

3.2.2.3.4 A protein kinase activity of DR2518 kinase is required for radiation resistance of *D. radiodurans*

DR2518 kinase has been characterized as a radiation inducible quinoprotein kinase contributing an important role in radiation resistance and reassembling of shattered genome during post irradiation recovery of *D. radiodurans* (Rajpurohit and Misra, 2010). However, the molecular mechanism underlying for its function in radiation resistance and DSB repair was not understood and has been further elucidated by mutational studies. Here we have obtained various point mutants of this protein having different levels of kinase activities. These derivatives of DR2518 kinase were expressed *in trans* in Δ*dr2518* mutant separately under the control of $P_{gro\text{ESL}}$ promoter. For that all these mutants derivative of *dr2518* were cloned in p*RADgro* plasmid and transformed to Δ*dr2518* mutant, and the γ radiation response of recombinant cells was monitored. The Δ*dr2518* cells expressing T169A and S171A proteins *in trans* improved γ

radiation tolerance of Δ*dr2518* cells by nearly 40-60% as compared to wild type kinase respectively (Fig.3.2.16). However, *in trans* expression of K42A an inactive kinase negative derivative of DR2518 kinase, did not show functional complementation in Δ*dr2518* mutant and these cells were as sensitive as Δ*dr2518* transformed with expression vector only. The T169A protein, which is almost 50% less active as compared to wild type kinase, supported nearly 60% to γ-radiation survival to Δ*dr2518* mutant. These results collectively suggest that the protein kinase activity of DR2518 kinase required for its role in radiation resistance and DSB repair in *D. radiodurans*.

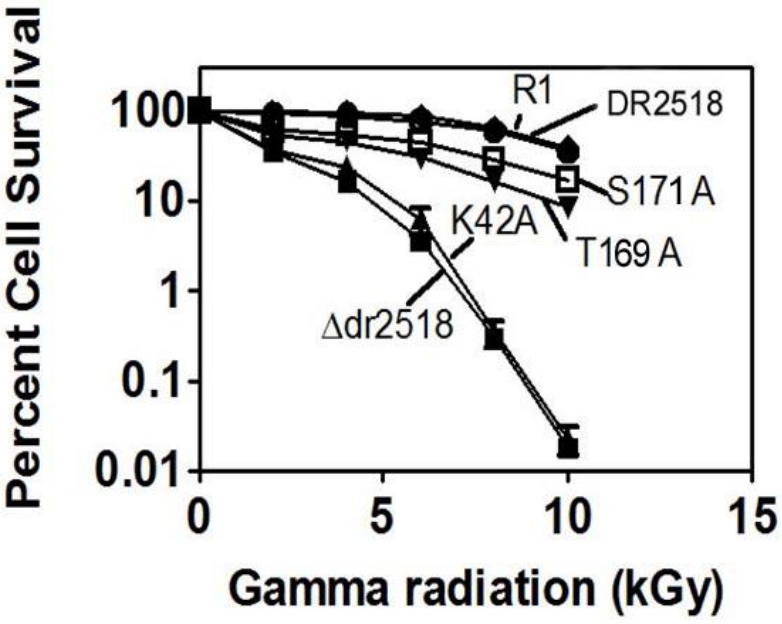


Fig. 3.2.16 Effect of kinase activity of DR2518 on functional complementation in Δ*dr2518* mutant. Wild type (-♦-) and the Δ*dr2518* mutant harboring pRADgro expression vector (-■-) and expressing wild type dr2518 (-●-), K42A (-▲-), T169A (-▼-) and S171A (-□-) mutant derivatives of DR2518, on pGroK42A, pGroT169A and pGroS171A plasmids respectively, were treated with different doses of γ-radiation and cell survival was measured. All the experiments were repeated three times and variation in the results are calculated as standard deviations (SD) and shown as error bars.

Chapter 3.3

Identification of DR2518 kinase substrates and effect of protein phosphorylation on the known functions of PprA, a representative substrate

Preceding chapters have described the involvement of PQQ, an antioxdant by nature and DR2518 a quinoprotein kinase roles in radiation resistance of *Deinococcus radiodurans.* Structure-function studies have indicated the requirement of the kinase functions of DR2518 per se for its role in radiation resistance. As protein kinases are known to regulate the phosphorylation of other proteins that leads to either activation or inactivation of target proteins. Therefore, the molecular basis of DR2518 with its interaction to PQQ that determines the crucial role of DR2518 kinase activity in radiation resistance and DSB repair was hypothesised through the modulation of DNA repair proteins in this bacterium. In this chapter, we identified a number of putative protein substrates and studied the effect of phosphorylation on various known functions of PprA (Pleiotropic protein promoting DNA repair) as a representative substrate of this enzyme.

3.3.1 Methods

3.3.1.1 Cloning and Mutagenesis

The site directed mutagenesis of *pprA* was carried out on plasmid pETpprA using site specific mutagenic primers (Table C). For generating T48A, T72A, S28A and S112A mutants of PprA was carried out using pETPprA (Swati and Misra, 2006) as template and site specific mutagenic primers (Table C) using site directed mutagenesis kit (New England Biolab, USA) following kit manufacturers protocols. All the mutations generated by *in vitro* mutagenesis, were confirmed by sequencing. For cloning of these derivatives in vector p11559, an IPTG inducible shuttle expression vector in *Deinococcus* (Lecointe *et al.,* 2004). Both wild type and mutant alleles were PCR amplified using PprAF and PprAR primers and cloned at *NdeI* and *XhoI* sites in p11559 (Lecointe *et al.,* 2004) and the recombinant plasmids were transformed into *pprA:cat* mutant.

Transformants were induced with IPTG and inducible expression of recombinant protein was confirmed by immunoblotting using PprA antibodies.

3.3.1.2 *In-vitro* protein kinase activity assays

For KESTREL (kinase substrate tracking and elucidation) proteomic approach (Cohen and Knebel, 2006), approx 500 µg heat inactivated cell free extract of *D. radiodurans* cells was incubated with 1µg DR2518 kinase in kinase buffer (Tris-Cl 70 mM pH 7, DTT 5 mM, 10 mM $MgCl_2$, 100 µM ATP) supplemented with 50µCi of [γ-^{32}P]-ATP for 30 min at 37^{o}C. In negative control, only cell free extract incubated with 50µCi of [γ-^{32}P]-ATP and for Positive control instead of DR2518 kinase, Protein kinase A was used.

3.3.1.3 Phosphorylation of PprA

Approximate 100 µg purified PprA protein was incubated with DR2518 protein kinase in 100:1 molar ratio in 300 µL of kinase buffer (Tris-Cl 70 mM pH 7, DTT 5 mM, 10 mM $MgCl_2$, 0.5mM ATP for 30 min at RT. Similar concentration of either PprA or DR2518 kinase in reaction buffer were kept as unphosphorylation PprA and DR2518 kinase control respectively. unphosphorylated PprA, phosphorylated PprA and DR2518 kinase control protein were concentrated in YM10 spin column and stored in protein storage buffer (Tris-Cl 10mM pH 7.6, KCL 50mM, EDTA 0.1mM, DTT 1mM and Glycerol 50%) till next use.

3.3.1.4 Electrophoretic mobility shift assay

Random sequence oligonucleotides (33 bases) (Table C) were annealed to make dsDNA of 33bp length, labeled with [^{32}P] by polynucleotide kinase, and purified by G-25 column. The 0.2 pmole

of labelled probe was incubated with increasing concentration (50nM to 3.2μM) of both phosphorylated and unphosphorylated PprA in 10μl of reaction mixture containing 10mM Tris-HCL, pH 7.5, 10mM $MgCl_2$, 50mM NaCl and 1mM DTT for 20 min at 37^0C. Products were analysed on a 12.5% native polyacrylamide gel, dried and radioactive signals were visualized by autogardiography. Intensity of DNA in free form as well as bound to protein was quantified by Image J software. The fraction of DNA bound to protein was plotted as a function of the protein concentration using Graphpad Prism 5. The Kd (dissociation constant) for curve fitting of individual plot was determined by the software working on the principle of least squares method applying the formula Y= Bmax *[X]/Kd+[X], where [X], is the protein concentration and Y is the bound fraction as described earlier (Das and Misra, 2011). For compairing the binding affinity of unphosphorylated and phosphorylated PprA for labeled DNA substarte, compitation assay was performed where binding of DNA with unphophorylated and phosphorylated PprA was challenged with molar excess of unlabelled DNA. The Log equilibrium dissociation constant of compititer unlabelled DNA, was calculated by curve fitting using nonlinear regression of competition binding equation of one site Fit Ki in Graphpad PRISM software.

3.3.1.5 DNA ligation assay:

The 400ng *Bam*HI digest of pBluescript SK^+ plasmid DNA was incubated with increasing concentration (0.2 to 3.2 μM) of both phosphorylated and non-phosphorylated PprA in 10mM Tris-HCL pH 7.5, 50mM NaCl, and 1mM DTT in 100μl reaction volume for 30 min at 37°C. To this, the 10μl of 10X T4DNA ligase buffer and 2.5 units of T4 DNA ligase were added and incubated for 30 min at 16^oC. Reactions was stopped by adding 80μL of a termination buffer containing 20 mM Tris-HCl (80% cation), 20 mM EDTA, and 0.5% SDS and deproteinized with

100 µg of predigested proteinase K. Products were extracted with a phenol: chloroform: isoamyl alcohol (25:24:1), precipitated with ethanol and analysed on 1.0 % agarose gels. DNA bands were visualized by ethidium bromide and quantified by densitometric scanning using Gene Genius tools (Syngene, UK).

3.3.1.6 Phosphorylation studies

For checking *in vivo* phosphorylation of PprA, the wild type, *Δdr2518* and *pprA: cat* cells were labelled with [^{32}P]-phosphoric acid under both γ irradiated at 6kGy dose and unirradiated conditions as described earlier (Rajpurohit and Misra, 2010). The irradiated cells were allowed to recover for in for 1.5h while unirradiated cells were kept one ice for 1.5h as control. The cells were collected and lysed by heating the cells in buffer containing 0.3% SDS, 1% β-mercaptoethanol and 50mM Tris-HCl (pH 8) at 95^{o}C for 20 minutes. The cell free extracts was obtained by centrifugation at 20000 x g and were treated with DNaseI (50µg/ml) and RNaseI (50µg/ml) for 1h at 37^{o}C. PprA protein was immunoprecipitated with PprA specific antibody; using Seize X protein-G Immunoprecipitation Kit (Pierce, Illinois) as described earlier (Rajpurohit and Misra, 2010). In brief, approximately 500µg proteins equivalent cell free extract was incubated with PprA antibodies (Narumi *et al.,* 2004) in binding buffer (140mM NaCl, 8 mM sodium phosphate, 2mM potassium phosphate and 10mM KCL, pH 7.4). Mixture was incubated overnight at 4^{o}C and to this the Protein G agarose beads were added. Content was washed thrice with binding buffer and eluted with 500mM NaCl in binding buffer. Eluted proteins were precipitated with 2.5 volume of ice-chilled acetone. Immunoprecipitate was dissolved in 2X Laemmle buffer and proteins were separated on 10 % SDS-PAGE and autoradiogram was developed.

3.3.3 Results

3.3.3.1 Target Substrate identification of DR2518 kinase kinase

2-D Gel electrophoresis based KESTREL (kinase substrate tracking and elucidation) proteomic approach was employed for identification of target substrate of DR2518 kinase (Cohen and Knebel, 2006). For that the heat inactivated cell free extract of 2hr PIR *D. radiodurans* cells was incubated with and without DR2518 kinase in kinase reaction buffer containing 50µCi [^{32}P] γ-ATP. Proteins were precipitated and analysed on 2-D gel electrophoresis as described in methods. Protein kinase A (PkA) instead of DR2518 kinase has been taken as a positive control. Proteins phosphorylated by either DR2518 kinase or Protein kinase A were detected by autoradiogram (Fig. 3.3.1). Results showed that the approximately 40 proteins were phosphorylated by DR2518 kinase. The numbers and autoradiogram pattern were different for both kinases (DR2518 and PkA) suggest the specificity of DR2518 kinase for its substrate selection. Further the identification and phosphopeptide mapping would be undertaken separately. Nonetheless the results of this experiment clearly demonstrated that *D. radiodurans* cell possessing several phosphoproteins and those could be phosphorylated by DR2518 protein kinase. Parallel to this, bioinformatic approach was also used for prediction of putative protein substrate for DR2518 kinase. As shown in chapter 3.2, DR2518 kinase is a functional eSTPK and represents the higher level of structural domains similarities with eukaryotic type STPKs. Extensive phosphopeptide based analysis for identification of phosphoacceptor motif of Mycobacterium Pkn kinases to which DR2518 shows considerable homology, have shown that XααααTX(X/V)$(P/R)I phosphomotif is a preferable motif for these STPKs, where T is phosphoacceptor site, α is an acidic residues $ a large hydrophobic residue and X any amino acid (Prisic *et al.*, 2010). It was also confirmed that large hydrophobic residues at +3 and +5 are

dominant components of a common phosphorylation motif for PknA, PknB, PknD, PknE, PknF, and PknH. Acidic residues from −2 to −5 has add up effect on phosphorylation by most of these kinases and the presence of glutamine, glutamate and aspartic acid amino acid at +1 position generally increases the substrate phosphorylation. Taking these facts for eSTPK substrate preference, we have designed motif "X-X-T-Q/D/E-X/V-$-X-$" as a probable preferred motiffor DR2518 kinase, and presence of this motif in deinococcal proteome was searched using www.scansite.mit.edu motif search tool. Analysis showed a large number of proteins having either exactly or slightly different structure of this phosphomotif (Table 3.3.1). Majority of the listed protein are either important DNA repair proteins or related to cell division. Among these proteins, some of them that are important for maintaining the genome integrity and DNA repair were either cloned, expressed and purified for this thesis work or obtained from other colleagues in the laboratory. The phosphorylation of these proteins was checked with purified DR2518 kinase *in vitro*. The most abundant phosphorylation was observed with three deinococal proteins RecA, PprA and ParB type proteins on chromosome 1 (C1B) (Fig. 3.3.2). DNA binding protein (DRA0282) and ParB type protein of megaplasmid (M1B) were relatively less phosphorylated. Two proteins DR2417, DrSsb from *D. radiodurans* were taken as homologous negative control and bovine serum albumin was taken as a heterologous negative control (Fig. 3.3.2). Results of this experiment confirmed that DR2518 having highly specific transphosphorylation activity and requires the presence of specific phosphomotif for its substrate phosphorylation. Among highly phosphorylated proteins (RecA, PprA and C1B proteins), the PprA (pleotropic protein promoting DNA repair) was studied further for evaluation of the effect of protein phosphorylation on PprA activities. PprA protein is a important novel DNA repair protein which is present exclusively in deinococcaceae family and characterized for DNA ends binding, protection of DNA from

exonucleases cleavage and assisting DNA ligases for DNA ends joining *in vitro* and also it has been shown to important for extreme radiation resistance and DSB repair *D. radiodurans* (Narumi *et al.,* 2004). The transexpression of PprA in *E.coli* increases the survival of *E.coli* cells against radiation and it induces the oxidative resistance of host cells by stimulating the catalase activity (Kota *et al.,* 2006). Here we revisit the important DNA metabolic activities of PprA with unphosphorylated and phosphorylated PprA *in vitro*. Also the phosphoacceptor site for DR2518 phosphorylation was identified and role *in vivo* PprA phosphorylation in gamma radiation resistance was evaluated.

3.3.3.2 Phosphorylation of PprA protein improved its DNA binding *in vitro*

In previous studies, the role of PprA has been shown in radiation resistance and DSB repair (Narumi *et al.,* 2004). Subsequently, it was demonstrated that PprA can preferentially binds to double-stranded DNA (dsDNA) ends and could stimulates the DNA end-joining activity of both ATP-dependent and NAD-dependent DNA ligases (Narumi *et al.,* 2004). Here, we confirmed that the wild type DR2518 kinase could phosphorylate PprA *in vitro* (Fig. 3.3.2). Therefore, the effect of PprA phosphorylation on their DNA binding activity was checked *in vitro*. The recombinant PprA protein was phosphorylated as described in methods. For checking the DNA binding activity of unphosphorylated and phosphorylated PprA, 33-mer dsDNA was labeled with [^{32}P] at 5' end and used for electrophoretic mobility shift with increasing concentration of both forms (phospho- and unphospho-) of PprA (0.05 to 3.2 µM). PprA phosphorylation stimulated the DNA binding activity of this protein as 0.8 µM of unphosphorylated PprA required for visual nucleo-protein complex, whereas only 0.2 µM concentration of phosphor PprA was sufficient for binding to DNA and showing visual nucleo-protein complex (Fig. 3.3.3A).

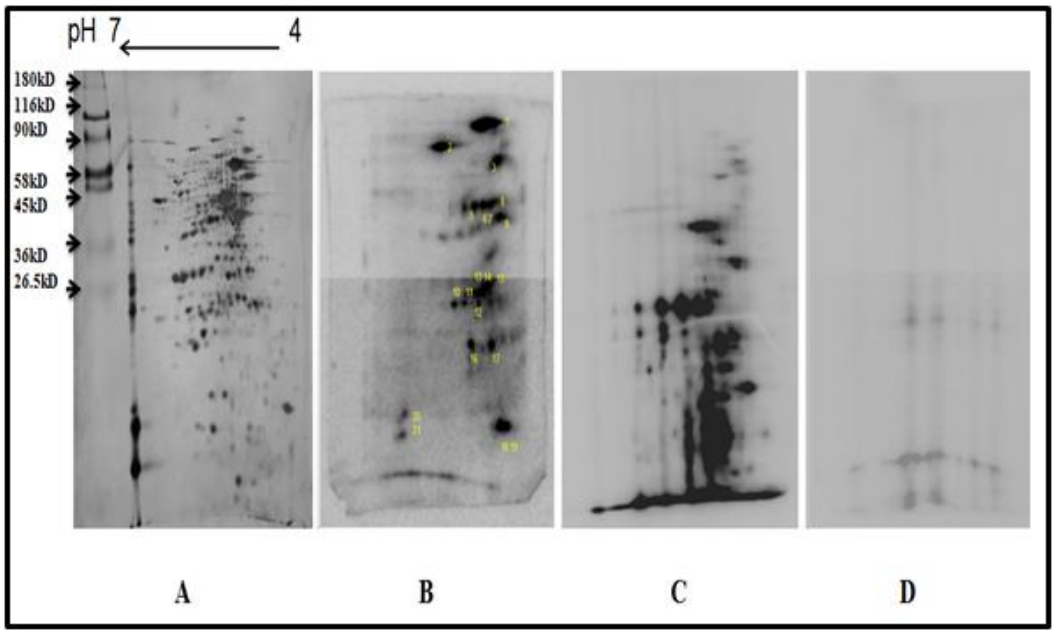


Fig. 3.3.1 KESTREL (kinase substrate tracking and elucidation) proteomic approach for identification of downstream substrate of DR2518 kinase. Heat inactivated 2 hr PIR cell free extract of *D. radiodurans* cells was incubated with recombinant DR2518 kinase , PkA (positive control) and without any exogeneous kinase (negative control) in kinase buffer containing 50µCi [^{32}P] γ-ATP. Reaction mixture was acetone precipitated and analyzed on 2D gel electrophoresis. (A) silver stained SDS-PAFE gel, (B) *D. radiodurans* Proteins phosphorylated by DR2518 kinase, (C) *D. radiodurans* Proteins phosphorylated by PkA kinase, and (D) Cell free extract without any exogenous kinase.

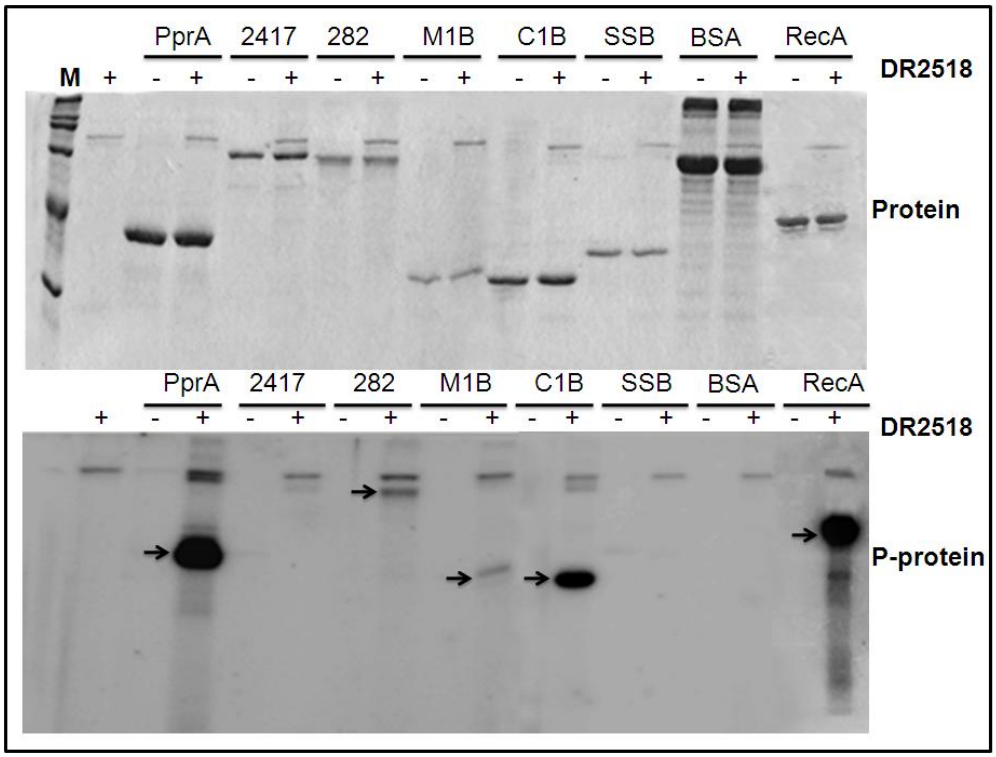


Fig. 3.3.2 Confirmation of predicted target protein substrates for DR2518 kinase. Purified recombinant proteins like PprA, ParB of chromosome 1 (C1B), ParB type protein of megaplasmid (M1B), DNA binding protein (DRA0282) and Recombinase A (Dr-RecA) of *D. radiodurans* having different phospho-motif structures were incubated in absence and presence of DR2518 kinase in kinase buffer containing [32P]-γ-ATP. Single stranded DNA binding protein (Dr-SSB), hypothetical protein (DR2417) and BSA were taken as a negative control as these proteins do not contain DR2518 specific phospho-motif. Products were separated on SDS-PAGE, stained with comassie blue (SDS-PAGE) and autoradiography was done for imaging protein phosphorylation (P-protein). Results shown are from a reproducible representative experiment that was repeated two times.

Table 3.3.1 Selected proteins of *D. radiodurans* showing putative phosphorylation motif "X-X-T-Q/D/E-X/V-$-X-$"), where T is phosphoacceptor site and α is an acidic residues, $ a large hydrophobic residue and X any amino acid (Prisic *et al.,* 2010).

Protein ID	Protein Name	Putative phosphomotif	Occurrence (No. of time)	Size kD
DR_A0065	HU protein	VAK**T**QLVEMV	1	12.289
DR_1424	DnaJ	VE**T**QQVCPTC	1	40.23
DR_2069	DNA ligase	LD**T**DDFTFTG AE**T**EAAPAES LV**T**QLLHEG	3	75.5
DR_0507	DNA polymerase III subunit alpha	LAM**T**DHGNM	1	149.2
DR_2263	DNA protection during starvation protein 1	DAR**T**QVADLV	1	23.03
DR_0493	Fpg (MutM)	RN**T**ERAHGRQ	1	30.8
DR_1984	Thymidine kinase	TR**T**QRLIGGQ	1	22.2
DR_A0344	LexA	QV**T**DRARAA	1	22.3
DR_A0346	PprA	AL**T**QSLQEA DQ**T**DGIYAA	2	32.2
DR_2340	Protein RecA	VN**T**DELLV	1	38.14
DR_1089	RecF	GE**T**EAYVRA LG**T**EIMLFRR	2	39.14
DR_0198	RecR	LEY**T**DEVTLG	1	23.7
DR_0939	Rex	LQ**T**QDLHLPE	1	25.09
DR_0912	RpoB	VVLQ**T**QDLHLPEA GDI**T**EVIPLP	2	128.7
R_0911	RpoC	KPK**T**QAVVAD RSL**T**DLLGGK	2	171.3
DR_0440	RuvC	LT**T**ESAWLMP	1	19.6
DR_2509	Hypothetical	RFT**T**QRARALGA	1	14.86
DR_1922	SbcC	DIE**T**QAAEAGR	1	100
DR_0689	UDG	EL**T**EDIPGFVA	1	27.7
DR_1771	UvrA	SEV**T**DRLLAG	1	112.1
DR_1354	UvrC	GDK**T**DLIEMAQ	1	68.9
DR_0012	ParB1	TG**T**QVQTL	1	31.8
DR_B0002	ParB type of Megaplasmid	GL**T**EVPVIV	1	32
DR_A0282	Hypothetical (Hu 80 type)	GE**T**QILSNLQG	1	54.8

The dissociation constant (Kd) was calculated by plotting the average values of percent bound fractions as a function of protein concentration in given lane using Graphpad Prism 5 software. The Kd values of phosphorylated and unphosphorylated PprAs were 0.348 ± 0.081 μM and 1.766 ± 0.70 μM, respectively. The Kd values signifies that protein phosphorylation of PprA increases the binding with [^{32}P] labelled 33-mer dsDNA probe by 5 fold, as compared to unphosphorylated PprA (Fig. 3.3.3B). These observations were further supported by competition binding assay of both unphosphorylated and phosphorylated PprA, where DNA binding of PprA protein with [^{32}P] labelled 33-mer dsDNA probe was competed by similar sequence unlabelled 33-mer dsDNA as described in methods. The log equilibrium dissociation constants (log Ki) of both forms of PprA with dsDNA, was calculated by curve fitting using nonlinear regression of competition binding equation of one site Fit Ki, in Graphpad PRISM software. The log Ki for phosphorylated PprA (63.82 ± 0.612) was nearly 4 fold higher than unphosphorylated PprA (16.3 ± 0.672) (Fig. 3.3.4). Which mean that phosphorylated PprA release the radiolabelled probe at approximately four fold higher concentration of unlabelled competitor dsDNA concentration compare to unphosphorylated PprA. These results suggested that the phosphorylation of PprA could enhance the affinity of this protein for dsDNA as compare to unphosphorylated PprA.

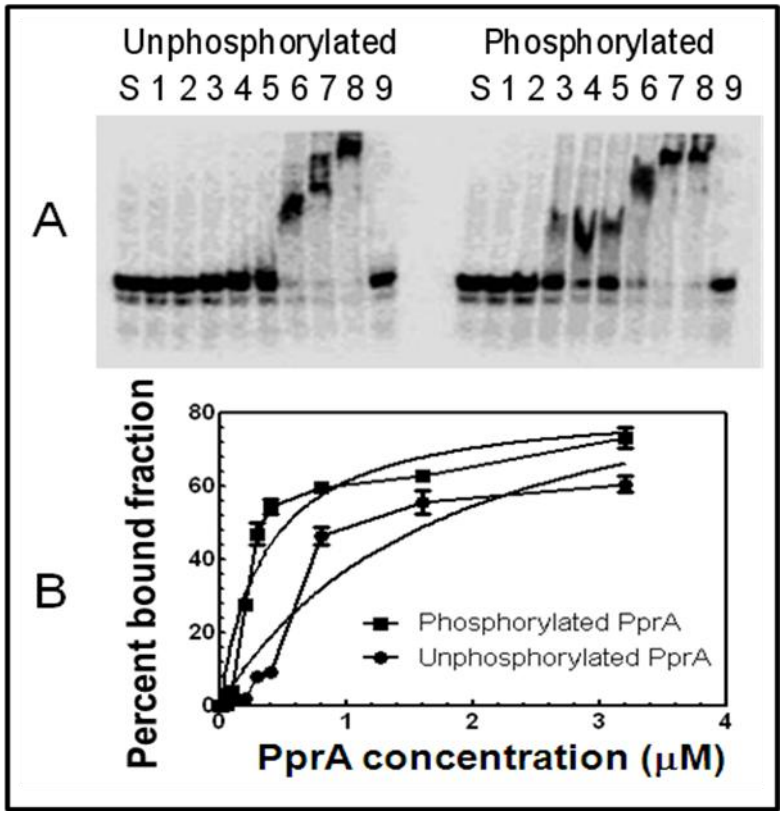


Fig. 3.3.3 Effect of phosphorylation on dsDNA binding affinity of PprA of *D. radiodurans*. The 0.2pmol of [^{32}P] labeled 33mer dsDNA was incubated with increasing concentration 0.05 (1), 0.1 (2), 0.2 (3), 0.4 (4), 0.8 (5), 1.6 (6) and 3.2 (7) µM of unphosphorylated and phosphorylated PprA, separately. The BSA in unphosphorylated (9) and DR2518 (9) in phsophorylated panels were negative and protein kinase controls, respectively. Products were analysed on native PAGE and autoradiogram was developed (A). Intensity of free DNA and DNA complexes with protein was determined by densitometric scanning. Percent fraction of DNA bound to proteins was calculated and plotted as a function of PprA concentration (B) using Graphpad Prism 5. The Kd values were determined for curve fitting of individual plot using nonlinear regression equation in Graphpad PRISM software, as described in methods. Experiments have been repeated three times and data shown here is from a representative experiment.

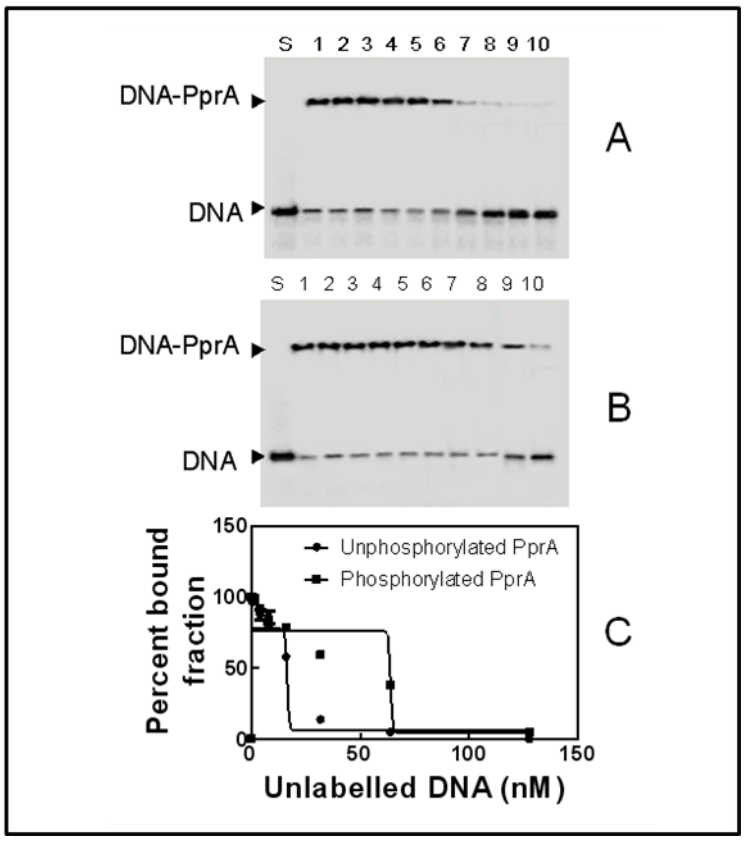


Fig. 3.3.4 Determination of log equilibrium dissociation constant (log Ki) of PprA with dsDNA. The 150nM unphosphorylated (**A**) and phosphorylated (**B**) PprAs were incubated with 0.2 pmoles of [^{32}P] labeled 33mer dsDNA in 10µl reaction mixture and chased with increasing amount like 0.25 (1), 0.5 (2), 1.0 (3), 2.0 (4), 4.0 (5), 8.0 (6), 16.0 (7), 32.0 (8), 64.0 (9) and 128 nM cold DNA and products were analysed on 12.5 % native PAGE. Autoradiograms were developed and the intensity of individual bands was determined by densitometric scanning. The percent bound fraction of DNA to protein was calculated and plotted as a function of protein concentration (C). The log Ki values were determined for curve fitting of individual plot using nonlinear regression of competition binding equation of one site Fit Ki in Graphpad PRISM software, as described in methods. Each experiment has been repeated at least two times and results were reproducible. Data shown is from a reproducible representative experiment.

3.3.3.3 PprA phosphorylation enhanced intermolecular ligation by T4 DNA ligase

Earlier it was shown that PprA could stimulate the DNA ligase activity of both ATP type and NAD type DNA ligases (Narumi *et al.*, 2004). The effect of phosphorylation on this role of PprA

was therefore, examined. Results showed that both phosphorylated and unphosphorylated PprA could improve the total DNA end joining activity of T4 DNA ligase *in vitro* (Fig. 3.3.5A). However, the amount of ligation products produced from intermolecular ligation (IMLP, intermolecular ligated products) by T4 DNA ligase was several folds higher with phospho-PprA than unphospho-PprA at 0.4μM concentration (Fig. 3.3.5B). This might suggest that the phosphorylation of PprA makes it favoring intermolecular end joining activity of DNA ligase over intramolecular ligation. Interestingly, it was observed that the levels of T4 DNA ligase activity stimulation by unphosphorylated PprA increased gradually with concentration and then become almost static, while this activity increased very rapidly at lower concentration of phosphorylated PprA and thereafter decreases as well, and the levels of ligase activity with 3.2 μM unphosphorylated PprA was more than similar amount of its phospho-form. Although, the reduction in ligase activity in presence of higher concentration of PprA has been reported earlier (Narumi *et al.*, 2004), the cause of this effect is yet unknown. Since, it is known that PprA forms multimer at higher concentration (Murakami *et al.*, 2006) *in solution*; this could possibly explain the inhibition of ligase activity at higher concentration of PprA by reducing the availability of free DNA ends for joining. Nonetheless these results suggested that phospho-PprA works more efficiently in supporting DNA end joining activity of T4 DNA ligase. These results suggested that PprA undergoes phosphorylation by STPKs at least by DR2518 kinase, of *D. radiodurans* and the phosphorylation of PprA could change its catalytic efficiency *in vitro*.

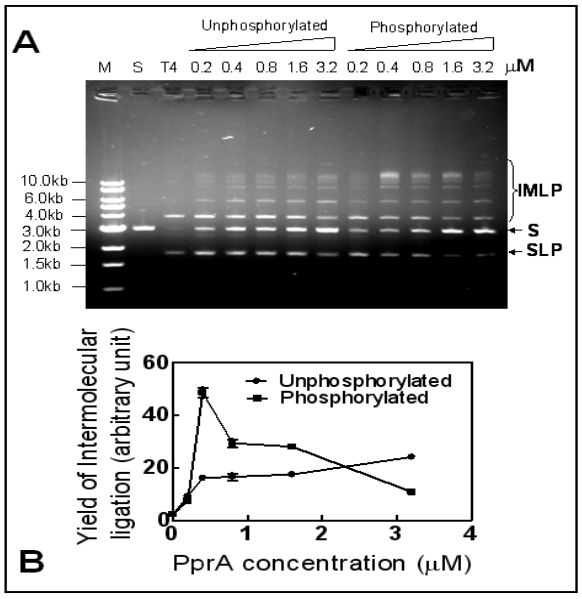


Fig. 3.3.5 Effect of PprA phosphorylation on T4DNA ligase activity stimulation. The 400ng *Bam*HI digested pBluescriptSK+ (**S**) was ligated with T4 DNA ligase (T4) in presence of increasing concentration (0.2μM to 3.2μM) of unphosphorylated and phosphorylated PprAs in ligation buffer at 16^{o}C. Products were analysed on agarose gel **(A)**. The intensity of both self ligated product (SLP) and intermolecular ligated products (IMLP) were determined by densitometric scanning and plotted as a function of protein concentration (**B**) using Graphpad PRISM 5. Data shown without statistical attributes are from a typical reproducible experiment, which were repeated three times.

3.3.3.4 Protein phosphorylation of PprA has negative effect on DNA ends protection

Protection of DNA from nucleolytic degradation is another distinguished role of PprA reported earlier (Narumi *et al.*, 2004). We checked the effect of PprA phosphorylation on *E. coli* Exonuclease III protection of linearized *pSK*+ plasmid as a function of time and concentration. ExoIII protection assay was carried out with increasing concentration of (0 to 2μM) PprA. There was no significant improvement in protection efficiency of phospho-PprA over unphospho-PprA (Fig.3.3.6). Subsequently we checked time dependent ExoIII protection for these two forms of

PprA by keeping PprA and ExoIII concentration constant and samples were collected at different time interval as depicted in Fig. 3.3.7. Surprisingly, we observed that unphosphorylated PprA could protect dsDNA from exonuclease III, better than phosphorylated PprA on longer incubation (Fig.3.3.7, compare panel A and B). This was an intriguing result because phosphorylated PprA has higher DNA binding affinity as compared to unphosphorylated PprA, therefore, better ExoIII protection by phosphorylated PprA was anticipated. The possible explanation could be that the phosphorylation of PprA might have changed the PprA DNA binding preference from DNA ends to wrapping around the DNA. This could lead to less ends protection as compared to unphosphorylated protein. In earlier studies, others have shown that PprA could bind DNA both at ends as well as internally with relatively reduced affinity (Murakami *et al.*, 2006). This argument may get further support from the results obtained on switching of T4 DNA ligase activity by phosphorylated PprA (Fig.3.3.5). As phosphorylated PprA may prefer internal DNA binding over DNA ends and stiffen the DNA backbone in such a way that it does not allow the DNA to bend, consequently that might favor more intermolecular end joining over intramolecular and less ExoIII protection of DNA ends however, this speculation requires independent investigations.

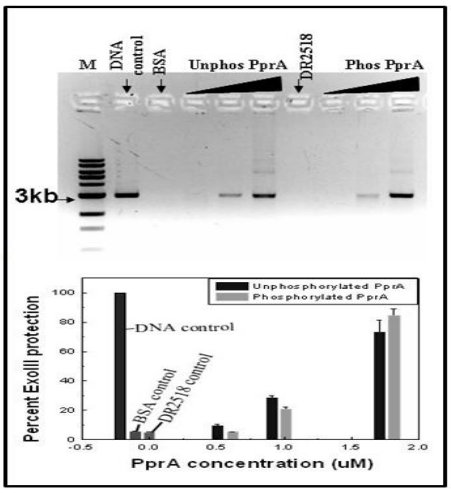


Fig. 3.3.6 Effect of PprA phosphorylation on inhibition of *E. coli* exonuclease III activity (Concentration dependent). 1.2 µM bp of 3.0kb *BamHI* linearized plasmid DNA (*pSK+*) was pre-incubated with increasing concentration (0.5, 1.0 and 1.8 µM) of unphosphorylated and phosphorylated PprA protein in buffer A at 37^{o}C for 30 min. *E. coli* exonuclease III (18 U) was added and the reaction was stopped after 30 min. incubation at 37^{o}C, by phenol-chloroform treatment and the samples were subjected to agarose gel electrophoresis.

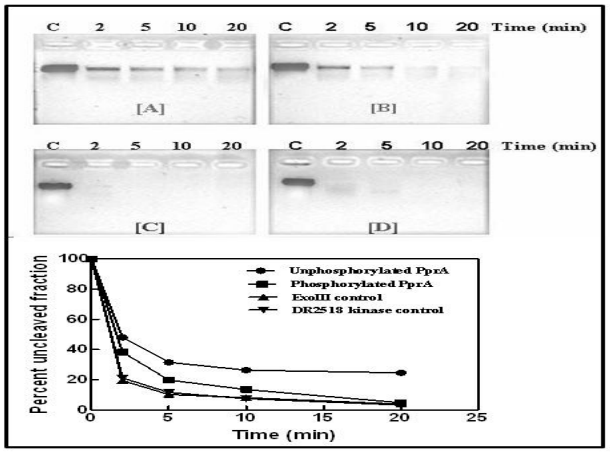


Fig. 3.3.7 Effect of PprA phosphorylation on inhibition of *E. coli* exonuclease III activity (Time dependent). 1.2 µM bp of 3.0kb *BamHI* linearized plasmid DNA (*pSK+*) was pre-incubated with fixed concentration (1.6µM) of unphosphorylated and phosphorylated PprA protein in buffer A at 370C for 30 min. *E. coli* exonuclease III (18 U) was added and the reaction was stopped at the indicated times by phenol-chloroform treatment and the samples were subjected to agarose gel electrophoresis. Unphosphorylated PprA(panel A) (-●-) protect dsDNA 30% more than phosphosrylated PprA (panel B) (-■-). DR2518 kinase (panel C) (-▼-) control did not protect linear dsDNA from *E. coli* exonuclease III at the indicated times.

3.3.3.5 PprA undergoes *in vivo* phosphorylation that is important for its contribution in radioresistance of *D. radiodurans*

Bioinformatic analysis using "X-X-T-Q/D/E-X/V-$-X-$" motif as described above, predicted Four putative phosphoacceptor sites such as Serine 28 (S28), Threonine 48 (T48), Threonine 72 (T72) and Serine 112 (S112), in the primary structure of PprA. These amino acids were individually replaced with alanine and pETS28A, pETT48A, pETT72A and pETS112A mutant constructs were generated by site directed mutagenesis. These constructs were transformed to BL21 *E.coli* strain and all mutant proteins were overexpressed with 0.5mM IPTG and purified by His-tag affinity chromatography separately (Fig. 3.3.8A). Purified wild type and mutated PprA proteins were checked for protein phosphorylation by recombinant DR2518 *in vitro*. The T72A mutation showed approx. 80% reducion in phosphorylation of PprA by DR2518 kinase (Fig. 3.3.8B). It was also found that S28A, T48A mutation did not affect phosphorylation while S112A mutation showed slightly reduced phosphorylation by DR2518 kinase as compared to wild type PprA. These results suggested that DR2518 kinase phosphorylate PprA at T72 *in vitro*.

In order to understand the possible role of PprA phosphorylation in radiation resistance of *D. radiodurans*, wild type, T72A mutant alleles (phosphoablative mutant) and T72D mutant alleles (phosphomimitic mutant) of *pprA* were cloned into IPTG inducible expression vector p11559 (Lecointe *et al.,* 2004) and transformed into *pprA:cat* disruption mutant (Narumi *et al.,* 2004). The *pprA:cat* distruption mutant grow slow in normal growth condition and showed highly sensitive phenotype to ionizing radiation. The IPTG inducible expression of recombinant PprA and wild type, T72A and T72D derivative was confirmed by immunoblotting with PprA specific polyclonal antibody. The IPTG inducible cell free extract from *pprA:cat* mutant containing p11559 vector only did not give crossreactivity. The IPTG induced cell free extract from

pprA:cat containing wild type, T72A and T72D mutant alleles, showed increased expression of respective PprA protein (Fig. 3.3.8 C and D). After confirming the IPTG inducible nature of cloned wild type T72A and T72D *pprA* allele in *pprA:cat* mutant background the γ radiation survival of these cells was monitored. The wild type PprA could help nearly complete recovery of γ radiation resistance of *pprA:cat* mutant (Fig. 3.3.8E) while phosphoablative T72A protein showed only partial recovery when compared with wild type and *pprA:cat* mutant. The T72A expressing cells showed ~40 fold less γ radiation resistance at 12kGy dose, as compared to *pprA:cat* cells expressing wild type allele, while nearly 1 log cycle improvement at 12kGy as compared to the *pprA:cat* cells harbouring expression vector as control. Furthermore the phosphomimitic T72D mutant showed sensitivity similar to *pprA:cat* mutant and did not support the function PprA. This suggested that PprA was phosphorylated by DR2518 kinase at T72 position has a significant role in γ radiation resistance of *D. radiodurans*.

The possibility of PprA undergoing *in vivo* phosphorylation was monitored in cell free extract of radiolabelled wild type, Δ*dr2518*, and *pprA:cat* mutant cells, by immunoprecipitation with antibodies against PprA. The PprA-immunoprecipitate picked up phospholabelled protein (s) from wild type and Δ *dr2518* mutant cell extracts but not from the cell free extract of *pprA:cat* mutant (Fig. 3.3.9), indicating the presence of phospho-PprA in both wild type and Δ*2518* mutant but not in *pprA* mutant. Quite surprisingly and reproducibly, it was observed that the levels of PprA phosphorylation as observed in immunoprecipitate, were phenomenally high in unirradiated Δ*dr2518* mutant when compared with wild type under both growth conditions, and Δ*dr2518* mutant under irradiated condition. Although the molecular basis of this result is not clear, it may be speculated that the PprA may be phosphorylated on other residues by some other kinases and the phosphorylation of T72 of PprA may be reducing its phosphorylation at other

sites. The extensive phosphorylation of PprA in absence of DR2518 kinase and its differential regulation upon γ irradiation are intriguing observations. Nonetheless, these results could suggest that (i) PprA undergoes phosphorylation *in vivo*, which can also be phosphorylated by other indigenous kinases, (ii) PprA phosphorylation at T72 residue seems to be important for radiation resistance.

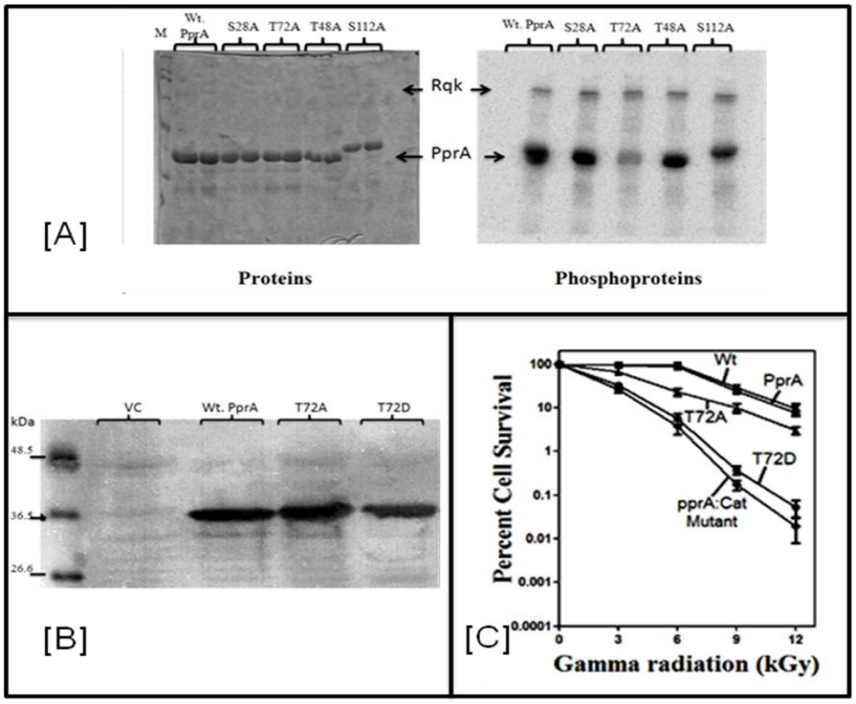


Fig 3.3.8 Mapping of phosphorylation site on PprA and determination of PprA phosphorylation role in radiation resistance of *D. radiodurans*. The threonine 48 (T48A), serine 28 (S28A), serine 112 (S112A) and threonine 72 (T72A) sites of PprA (WT) were replaced with alanine and recombinant proteins were purified and incubated with DR2518 kinase in presence of [^{32}P]- γ-ATP and phosphorylation was detected on autoradiogram (A). Wild type PprA as well as mutant alleles (phosphoablative T72A and phosphomimitic T72D) were cloned in *Deinococcus* expression vector and IPTG inducible expression was checked on western blot by PprA specific antibody; VC (*pprA:cat* (Mutant) containing p11559 vector), Wt.PprA, T72A and T72D represents the *pprA:cat* (Mutant) harbouring p11559 vector containing wild type PprA, T72A and T72D mutant PprA. *D. radiodurans* R1 (WT), *pprA:cat* (Mutant) and *pprA:cat*

cell expressing wild type allele of *pprA* (PprA), T72A and T72D mutant allele of *pprA* were exposed to different doses of gamma radiation and cell survival was monitored (C).

Fig 3.3.9 *In vivo* phosphorylation of PprA. The [^{32}P] labelled phosphoproteins of wild type (WT), *dr2518* deletion mutant (Δ*dr2518*) and PprA mutant (*pprA:cat*) cells grown under normal conditions (UN) and treated with 6kGy γ radiation (IR), were precipitated with antibodies against PprA. Immunoprecipitates were analysed on SDS-PAGE and the presence of [^{32}P] PprA was detected by autoradiography.

CHAPTER 4

DISCUSSION

All live organisms exposed to radiation (UV, γ & X-rays) emanating either from natural sources (cosmic radiation, solar radiation, external terrestrial sources, radiation in the human body and radon) or from artificial sources (industrial radiography, medial radiology and nuclear medicine etc.) causes DNA damage. The repair of damaged DNA is crucial for life and defects in DNA repair pathways can lead to genetic disease and cancer. Ionizing radiation exerts both oxidative stress and direct damage by direct deposition of energy in biomolecules in living organisms. Oxidative stress causes damage of proteins, lipids and nucleic acids. Direct effect of ionizing radiation on proteins and lipid are not understood, however nucleic acids are most susceptible bimolecule for direct deposition of energy and suffered both single strand DNA break and DNA double strand breaks (Ward, 1975; Kuzminov, 1999; John *et al.*, 2011). Conversely non-ionizing radiations like ultraviolet (UV) affect biomolecules in energy dependent manner. UVC (254nm) has been shown to damage DNA maximally by direct base modification, formation of bulky adducts, and minimally via oxidative stress. UVB and UVA do damage DNA by base modification but also act indirectly through oxidative stress. Base cross-linking due to UV radiation leads to both single strand breaks by way of unrepaired lesion while double strand breaks are produced due to replication arrest when replication machinery encounters base adducts formed on DNA (Cadet *et al.*, 2005). Therefore, both types of radiation produces all types of DNA damage but their density and spectrum of a specific type of DNA damage vary in non-ionizing and ionizing radiation.

Deinococcus radiodurans has extraordinary robustness against various DNA damaging agents including ionizing and nonionizing radiation (Minton, 1994; Battista, 2000). Surprisingly, this bacterium has extraordinary resistance to gamma radiation and UV radiation close to 254nm (UVC) but it is very sensitive to low energy UV components like UVB and UVA. Its

mextraordinary radioresistance has been primarily attributed to its efficient DNA strand break repair (Zahradka *et al.,* 2006; Blasius *et al.,* 2008) and a strong oxidative stress tolerance (Markeille *et al.,* 1999, Slade and Radman, 2011). DNA strand break repair in this bacterium showed biphasic repair (Daly *et al.,* 1994; Daly and Minton, 1996) which gets accomplished by two distinct mechanisms like extended synthesis dependent strand annealing (ESDSA) and a slow process of maturation by homologous recombination (Zahradka *et al.,* 2006, Blasius *et al.,* 2009). The stability and protection of shattered genome from nucleolytic degradation, recession of protected ends by deprotecting proteins and synthesis as well as assembling of various DNA repair proteins in macromolecular complexes are some of the essential pre-requisites for efficient DSB repair of this bacterium. Although, the molecular mechanisms supporting these pre-requisites are not known, the γ radiation changes the expression of a large number of genes (Liu *et al.,* 2003, Tanaka *et al.,* 2004), recycling to proteomes (Joshi *et al.,* 2004), and the involvement of two DNA protecting proteins such as PprA (Narumi *et al.,* 2004) and DdrA (Harris *et al.,* 2004) in radiation resistance, have been shown in this bacterium. Effect of DNA damage on expression of DNA repair and recombination genes is regulated by SOS response in most of the bacteria studied for this mechanism (Sutton *et al.,* 2000). Surprisingly, this bacterium does not express the classical SOS response mechanism (Bonacossa de Almeida *et al.,* 2002; Narumi *et al.,* 2001) known as only studied DNA damage response in many bacteria (Shimoni *et al.,* 2009; Walker, 1996). In spite this, in response to DNA damage *D. radiodurans* adjusts its transcriptome and proteome by regulating the differential gene expression (Liu *et al.,* 2003; Tanaka *et al.,* 2004) and DNA repair related and other proteins recycling and syntheisis (Joshi *et al.,* 2004 and 2011). It has been demonstrated that *D. radiodurans* exposed to γ radiation stops its growth presumably till DSB repair is accomplished. This indicated a strong possibility of an

uncharacterized DNA damage induced checkpoints regulating DNA repair and cell division in this bacterium (Cox and Battista, 2005).The genome of this bacterium however encodes a large number of putative transcription factors, response regulators and different types of protein kinases (White *et al.,* 1999; Makarova *et al.,* 2001) including histidine kinases where some of these belong to type IIA group stress response factors (Dong and Steven, 2001) and various eukaryotic type protein kinases (eSTPKs) (Makarova *et al.,* 2001). Therefore, the possibility of some alternate DNA damage response mechanism could be speculated and had been one of the aims of this study.

Historically pyrroloquinoline-quinone (PQQ) has been known as a redox cofactor for periplasmic as well as cytosolic dehydrogenases contributing in the mineral phosphate solubilization (MPS) phenotype in bacteria (Goldstein, 1994). In recent past PQQ role has been demonstrated in oxidative stress tolerance in bacteria and animal systems (Khairnar *et al.,* 2003; He *et al.,* 2003) and has been reported to act as an antioxidant *in vitro* (Misra *et al.,* 2004), also as a member of B group vitamins (Kasahara and Kato, 2003). *D. radiodurans* R1 genome encodes PQQ synthease and five ORFs encoding polypeptides with beta propeller signatures constituting PQQ binding pocket in 3-D structure of proteins. It has been showed that PQQ also work as an inducer for protein kinase functions in bacterial and eukaryotic system and PQQ stimulated kinase function required for oxidative stress tolerance mechanism in these organisms (Khairnar *et al.,* 2007; Rucker *et al.,* 2009) however, role of PQQ in DNA double strand break repair has not been studied in detail. *D. radiodurans* with its extraordinary capability to repair of hundreds of DNA double strand breaks and with very high oxidative stress resistance offer excellent system to study the PQQ and PQQ interacting putative protein kinases role in gamma

radiation resistance and DSB repair particularly in absence of classical bacterial DNA damage response SOS response mechanism.

Here we have shown that *D. radiodurans* cells make PQQ *in vivo* and the cells devoid of PQQ loose both oxidative stress tolerance and DNA double strand break repair potential. PQQ is known as an antioxidant and its contribution in γ radiation stress could have accounted to its antioxidant roles. However, the severe effect of *pqqE* deletion on the DSB assembly during PIR, and PQQ stimulation of *E. coli* resistance to UVC that contributes very little to oxidative stress (Khairnar *et al.,* 2007), together argued for a possibility of this compound functioning in DNA strand break repair perhaps through the modulation of DNA repair proteins functions. Interestingly, *D. radiodurans* cells grown in presence of [^{32}P] phosphoric acid showed nearly 10 fold less incorporation of [^{32}P] in *pqqE* mutant compare to wild type. Since the cellular phosphoproteins level is being regulated by protein kinases function, therefore the low levels of protein phosphorylation observed in *pqqE* mutant was hypothesized the possibility that either PQQ is absolutely required for certain protein kinases activity and / or PQQ stimulates the residual activity of these protein kinases in the cells. This suggested the important role of PQQ in radiation resistance and DNA strand break repair in *D. radiodurans*, possibly through protein (s) phosphorylation. In conjunction with transcriptional regulation, post translational modification (PTM) of proteins is important regulatory mechanism. Till the date, more than 200 different type of Post translational modification (PTM) have been identified of which only few of them are reversible and important for regulation of biological processes (Delom and Chevet, 2006). Protein phosphorylation is most vital type of PTM, and is ubiquitously recognized as an important regulatory and signaling mechanism which involved in rapid and reversible modification of physio-chemical properties of a protein, triggering a number of possible

consequences: change of enzyme activity, oligomerization state, interaction with other proteins, subcellular localization or half-life (Kobir *et al.,* 2011). The most common type of protein phosphorylation (namely on Serine, Threonine, Tyrosine, Histidine and Aspartate residues) have been identified and present ubiquitously in all three kingdoms of life. The involvement of signal transduction and protein phosphorylation in DNA strand break repair and post repair resumption of bacterial growth have not been demonstrated. Cox and Battista have brought the discussion on the linkage between the DNA damage checkpoint, DSB repair and cell division in *Deinococcus* (Cox and Battista, 2005). They have argued that *Deinococcus* cells would not divide until cells have repaired the DNA double strand breaks however, how cells sense the signal for the onset of cell division has not been commented. Here we could demonstrated that PQQ, an antioxidant showing crucial role in DNA double strand break repair in *D. radiodurans* possibly through activation of protein kinase activity. Therefore the identification of protein kinase(s) and the mechanism of protein phosphorylation contributing to DSB repair and radiation resistance in bacteria would be interesting to investigate. Recently the involvement of protein phosphorylation has gained significant importance in bacterial DNA metabolism for example in *D. radiodurans,* a novel response regulator DrRRA regulate the expression of *recA* and *pprA* expression (Wang *et al.,* 2008), a DNA damage response switch regulator protein PprI involved in regulation of phosphorylation of at least two proteins (DRA0283 and DR1343) (Lu *et al.,* 2009) and the co-existence of DNA repair proteins with protein kinases and phosphoproteins in a multiprotein DNA processing complex (Kota and Misra, 2008) were showed. In *E. coli*, the functional interaction of PQQ with a periplasmic protein kinase having a role in radiation tolerance (Khairnar *et al.,* 2007), and the role of two component system in expression of DNA recombination and repair genes under normal growth conditions (Oshima *et al.,* 2002; Zhou *et*

al., 2003), have also been reported. In *Bacillus subtilis*, the phosphorylation of single strand DNA binding protein (SSB) and the effect of phsophorylation on switching of substrate preference of SSB has been demonstrated (Mijakovic *et al.,* 2006). We argued that low levels of protein phosphorylation and impairment of DSB repair could be due to low protein kinase activity in *pqqE* mutant. Molecular mechanisms underlying PQQ regulating either protein phosphorylation, DSB repair and radiation resistance were further investigated. The *in silico* search for PQQ binding motif as defined in dehydrogenases, showed *D. radiodurans* genome possessing five ORFs (namely *dr2518, dr1769, drC0015, dr0503* and *dr0766*) , encoding polypeptides containing PQQ binding signatures in their primary protein sequence. The possibility of PQQ role in DSB repair and gamma radiation resistance of *D. radiodurans* might involve these polypeptide was speculated and tested by generating individual deletion mutants and these deletion mutants and Δ*dr2518 pqqE:cat* double mutant were evaluated for their gamma radiation survival. Results showed that DR2518 protein contribute maximally in gamma radiation resistance and suggesting the regulatory role of DR2518 in radiation resistance and DSB repair of *D. radiodurans*. Although both *pqqE* and *dr2518* mutants were sensitive to gamma radiation, it may be noted that *pqqE* and *dr2518* mutants showed differential response to UVC radiation and different levels of gamma radiation sensitivity. Unlike *pqqE* mutant, *dr2518* mutant was sensitive to UVC radiation. Since, there are five different proteins having PQQ binding signature motifs, therefore it might be possible that separate roles of PQQ beyond DR2518 function. DR2518 that showed a role in gamma radiation resistance in *D. radiodurans* was finally characterized as a eukaryotic type Serine/Threonine type protein kinase (eSTPK).

The primary amino acid sequence analysis of DR2518 protein has showed that the N-terminal of DR2518 contains a Hank type kinase domain (Hanks *et al.,* 1988) normally present in eukaryotic

type Ser/Thr protein kinases (eSTPK), while C-terminal is having a sensory PQQ interacting domain. The N-terminal of DR2518 had all the conserved motifs like P-loop, Helix-C, DFG motif, and catalytic loop as known in other eSTPKs and C-terminal contains seven tandem β-propeller repeat motifs. Similar types of β-Propeller repeat motifs have been found in β-subunit of G protein representing the classic WD40 class protein. The autophosphorylation of DR2518 kinase increases in presence of PQQ in solution suggested that PQQ work as a inducer of its protein kinase function *in solution*. It has been showed that transcript level of both *pqqE* and *dr2518* genes induced in response to γ radiation (Liu *et al.,* 2003); in addition to this γ radiation also produces several DNA breaks. Since we found that *dr2518* gene function required for γ radiation resistance thus the possible effect of DNA ends on stimulation of autophosphorylation of DR2518 kinase was evaluated and found that DNA ends also has positive stimulatory effect on kinase function of DR2518 *in vitro*. The DNA ends mediated stimulation of DR2518 autophosphorylation was most intriguing observation as primary sequence of DR2518 kinase does not contain any known DNA binding region. *In silico* fold prediction studies using FUGUE server package, also revealed that the 273-401 amino acids region located between STPK and PQQ binding domain could be a site for DNA interaction of DR2518, which matches with nucleotide interacting domain located between STK and PUG (a sensor domain) of a dual function (a transmembrane sensor protein kinase and ribonuclease) human-Ire1 (Lee *et al.,* 2008). Further although both PQQ and DNA ends has positive stimulatory effect on kinase function of full length DR2518, the same effect was absent on the autophosphorylation of only kinase domain (DR2518KD) *in solution* suggested the possible regulatory function of C-terminus PQQ interacting domain on kinases function of DR2518. Similar ligand based activation of PknB kinase has been demonstrated (Barthe *et al.,* 2010). Our *in vivo* observation

suggested that PQQ mediated stimulation of DR2518 kinase function is main contributor in stimulation of DR2518 kinase function as *in vivo* autophosphorylation of DR2518 during PIR time did not increase in *pqqE:nptII* mutant compare to wild type cells. Interestingly, it was observed that levels of both *pqqE* and *dr2518* transcript increase in response to γ radiation was much higher than the γ radiation stimulated *in vivo* phosphorylation of DR2518. Differences in the increase of *dr2518* transcription and *in vivo* DR2518 autophosphorylation activity by γ radiation are intriguing. It could account either to the activation of this gene expression by other components of γ radiation effect or due to completely different mechanisms involved in regulation of these two processes *per se*. However similar levels of DR2518 phosphorylation stimulation by DNA ends *in vitro* and by γ radiation *in vivo* might further indicate the possibility of a threshold in the levels of autophosphorylation of this protein, which may be regulated by requirement of its transkinase activity for phosphorylation of other protein substrates *in vivo*.

Topology of DR2518 kinase was found to be similar to membrane kinases of mycobacterium. Except pknG all other Pkn kinases of mycobacterium having membrane localization and involved in various important cellular functions like cell division, cell wall synthesis, membrane transport and virulence (Av-Gay and Everett, 2000). The possibility of membrane localization of DR2518 kinase was ascertained by topology analysis software (TMpred server) and by detection of DR2518 protein in membrane fraction by DR2518 specific antibody. Bacterial eukaryotic Ser/Thr protein kinases showed conservation in catalytically important residues in their primary sequence, however phosphoacceptor sites in their activation loop may vary in numbers and phosphorylation of these amino acids required for activation of kinase function (Pereira *et al.*, 2011). Results showed that the lysine 42 amino acid of DR2518 kinase is important catalytic residues and T169 and S171 are two major phosphoacceptor sites present in activation loop of

DR2518 kinase. K42A mutation completely abolished the kinase function whereas T169 and S171 showed low level of kinase activity. Further no complementation of DR2518 function by K42A mutant and only partial complementation of DR2518 function by T169 and S171 mutant in *Δdr2518* suggested kinase function of DR2518 protein required for γ radiation resistance of *D. radiodurans*.

In eukaryotes the DNA damage induced protein kinases (ATM, ATR) transducer signal to effector molecules through series of protein phosphorylation event (Sancar *et al.,* 2004). In prokaryotes, some of the independent findings have emphasized the importance of protein phosphorylation in DNA repair and γ radiation resistance (Kota & Misra, 2008; Wang *et al.,* 2008; Mijakovic *et al.,* 2006). The complete arrest of DNA double strand break repair up to 24 hr PIR in *Δdr2518* mutant after receiving 6kGy gamma radiation, increased transcription and autophosphorylation of DR2518 kinase in response to gamma radiation and absence several phosphoproteins / or in hypophosphorylation status during post irradiation recovery time suggested the possible involvement of this eSTPK as a putative sensor kinase, having a role in gamma radiation resistance and regulation of DNA damage response in *D. radiodurans* perhaps through regulation of protein phosphorylation status of certain unknown phosphorproteins involved in DNA damage repair process was hypothesized and investigated. DR2518 kinase could phosphorylate PprA (pleotropic protein promoting DNA repair), RecA (Recombinase A), some of probable chromosomal and plasmid partitioning proteins *in solution*.

PprA protein is unique DNA repair protein present in deinococcaceae family and play important role in γ radiation resistance of *D. radiodurans* (Narumi *et al.,* 2004). As expected and known in other proteins like SSB of *Bacillus subtilis* (Mijakovic *et al.,* 2006), PprA protein showed both qualitative as well as quantitative improvement in its functions upon phosphorylation. Protein

phosphorylation mediated increase in the DNA binding activity of PprA by four fold and modulation of T4 DNA ligation activity from intramolecular to intermolecular ligation product by Phos-PprA, clearly indicated that Phos-PprA is efficient form of this protein which can perform its function with better efficiency. Phospho motif based bioinformatic analysis of primary sequence of PprA could identify four probable phosphoracceptor sites (S28, T48, T72 and S112). Further identification of T72 in PprA protein as a target phosphoacceptor site of DR2518 kinase and no complemtation of PprA function in γ radiation resistance of *D. radiodurans* by phosphormimitic T72D mutant of PprA conclusively conclude the importance of PprA phosphorylation in gamma radiation resistance of *D. radiodurans*. Protein phosphorylation is an important mechanism that cells use for communicating the changes in surroundings conditions to cellular and genetic levels is manifested by the coupled action of protein kinases and phosphatases (Kennelly *et al.,* 1996). In recent past importance of protein phosphorylation of DNA repair proteins has been discussed (Kota & Misra, 2008; Wang *et al.,* 2008; Mijakovic *et al.,* 2006) however the mechanistic nature was not explained substincially. This thesis work collectively showed the involvement of protein phosphorylation and roles of protein kinases in bacterial response to DNA damage. In addition to eukaryotes, the roles of eukaryotic serine/threonine protein kinases (eSTPKs) have also been reported in *Mycobacterium* and other pathogenic bacteria growing under stresses (Molle and Kremer, 2010; Park *et al.,* 2008; Cozzone, 2005). *D. radiodurans* does not confer the typical bacterial SOS response mechanism (Bonacossa de Almeida *et al.,* 2002), and therefore, the possibility of an alternate DNA damage response mechanism would be anticipated. This study has brought forth first time the involvement of PQQ, a protein kinase activity inducer in DSB repair and gamma radiation resistance and characterization of eukaryotic type serine / threonine protein kinase (DR2518),

and regulation DNA repair process through protein phosphorylation of DNA repair proteins (at least PprA) in this bacterium strongly suggest the existence of an alternative bacterial DNA damage response mechanism which is distinctly different from classical bacterial SOS response but close to eukaryotic type DNA damage responses. Our published findings of this study, are being viewed a logical shift in paradigm of DNA damage response mechanisms in prokaryotes, and the role of DR2518 in phosphorylation of DNA repair protein and modulation of their inherent activities might be a step forward to elucidate the alternate DNA damage response mechanism in this bacterium.

CHAPTER 5

SUMMARY

AND

CONCLUSION

Deinococcus radiodurans R1 exhibits an extraordinary tolerance to various abiotic stresses including radiations and desiccation. The amazing radioresistance of this bacterium is believed to be largely contributed by an efficient DSB repair and a strong oxidative stress tolerance mechanism. *D. radiodurans* exposed with γ radiation, shows gamma radiation mediated differential expression of a large number of genes at transcriptional levels and protein turnover with a unique process of protein recycling. The molecular mechanisms underlying the gamma radiation effects on transcription and protein turnover are not known in this bacterium. However, unlike *E. coli* and many other bacteria, where DNA damage induced genes expression have been implicated to SOS response, however existence of SOS repair in *Deinococcus radiodurans* has been ruled out. The possibility of an alternate mechanism to gamma radiation response could be envisaged in this bacterium. Interestingly, the genome of *D. radiodurans* R1 encodes a large number of hypothetical proteins, proteins known for recombination repair in other bacteria except RecBC enzymes. In addition, it also encodes numbers of eukaryotic type Ser/Thr protein kinases (eSTPK), stress response regulator and sensor proteins including histidine kinases and key regulatory enzyme named as PQQ synthase (*pqqE*) involved in biosynthesis of pyrroloquinoline quinone (PQQ), a well characterized antioxidant both in mammalian system and bacteria. Also PQQ was shown earlier, supporting the oxidative stress tolerance in *E. coli* and having role as an activity inducer of Ser/Thr protein kinase that has a role in radiation resistance in *E. coli*.

This study focused on identification of PQQ inducible putative Ser/Thr kinase from *D. radiodurans* and further understanding the mechanism underlying this kinase role in gamma radiation resistance and DSB repair of this bacterium. Since, PQQ was indicated as an inducer of STPK, could support the oxidative stress tolerance and the *E. coli* cells expressing *pqqE* from *D.*

radiodurans R1 became mineral phosphate solubilization positive, a phenotype regulated by PQQ in bacteria. These findings have provided enough indication on the functional status of *pqqE* albeit using *E. coli* as a heterologous system. In order to understand this gene role in *D. radiodurans*, the *pqqE* was disrupted with antibiotic marker in the genome of this bacterium and mutant was characterized for its response to different DNA damaging agents including gamma radiation. Mutant showed a severe loss of gamma radiation resistance and hydrogen peroxide but no effect was observed on UVC resistance, as compared to wild type cells. These cells also show impairment in DSB repair. These findings suggest a role of PQQ in gamma radiation resistance and DSB repair in this bacterium. In order to investigate the molecular basis of PQQ action in gamma resistance and DSB repair, the five ORFs (DR_0503, DR_0766, DR_1769, DR_2518 and DR_C0015) encoding polypeptides having multiple beta propeller motifs characterized for PQQ interaction in dehydrogenases, were individually deleted from *D. radiodurans* genome. Deletion mutants were checked for gamma radiation resistance. Interestingly, *dr2518* deletion (Δ*dr2518*) made *D. radiodurans* cells hypersensitive to the effects of different DNA damaging agents like gamma, UVC, hydrogen peroxide, mitomycin C and 5% desiccation. Other deletions did not show any change in wild type response to gamma radiation and effects of other agents were not checked on remaining mutants. This indicated that DR2518, which is also annotated as a putative STPK in the genome of *D. radiodurans*, has a role in DNA damage tolerance of this bacterium. The possibility that PQQ could have functioned through DR2518 was further strengthened as double mutant (Δ*dr2518 pqqE:cat*) did not show additive effect and levels of gamma resistance were similar to single Δ*dr2518* mutant. This might suggest that both *pqqE* and Δ*dr2518* function through common pathway in conferring radioresistance to *D. radiodurans.* The functional interaction of PQQ with DR2518 was further studied at protein levels.

Recombinant DR2518 was made and the purified protein showed autophosphorylation irrespective of PQQ presence but this activity of DR2518 was enhanced in presence of PQQ *in solution*. DR2518 was further checked for its *in vivo* phosphorylation both in wild type and *pqqE:cat* mutant cells. DR2518 autophosphorylation was although observed in both cells; its stimulation in response to gamma radiation was not observed in *pqqE* mutant. Transcriptome data showed the stimulation of both *dr2518* and *pqqE* transcription in response to gamma radiation. These results indicated that DR2518 is phosphoprotein irrespective of PQQ presence and provide evidence although indirectly to suggest that gamma radiation induces the levels of both DR2518 and PQQ, which in turned stimulate the activity of DR2518 kinase *in vivo*. Thus the roles of both PQQ and DR2518 kinase in gamma radiation resistance and DSB repair are suggested. Structure-function studies using bioinformatic tools, the catalytic cleft and activation loop of DR2518 were defined and using site directed mutagenesis approach the amino acids residues responsible for the kinase activity of DR2518 have been identified. The K42A mutant of DR2518 was nil in kinase activity also failed to complement the function of DR2518 in *dr2518* mutant suggesting that kinase activity of DR2518 is per se required for the contribution of DR2518 in radioresistance of this bacterium. Using bioinformatic approaches, the proteins having putative phosphorylation motifs were scanned in the proteomes of *D. radiodurans*. A large number of proteins were found having phospho-motifs characterized for proteins as substrate of eSTPK of mycobacterial kinases. Some of these proteins were checked for phosphorylation ability by wild type, different mutated derivative and only by kinase domain of DR2518. Interestingly, we observed that both full lengths DR2518 could phosphorylate many including PprA (a pleiotropic protein involved in DNA repair) but not K42A mutant. PprA phosphorylation by full-length DR2518 was stimulated but not by protein kinase domain missing

C-terminal sensory beta propeller repeats. These results suggest DR2518 could phosphorylate deinococcal-proteins *in vitro*; its ability to discriminate from the promiscuity is most like lying with C-terminal domain of DR2518 kinase. Nonetheless, these findings strongly supported the involvement of an eSTPK of *D. radiodurans* in its radiation resistance and DSB repair by phosphorylating the DNA metabolic proteins including PprA, a known DNA repair protein in this bacterium. Taking PprA as a representative candidate, we assessed the functional relevance of DNA metabolic proteins phosphorylation.

Recombinant PprA was purified and phosphorylation was achieved in presence of DR2518 incubated with ATP. PprA was known for its DNA binding activity, its role in stimulation of DNA end joining activity of DNA ligases and DNA protection from exonuclease III degradation. Phosphorylated PprA (P-PprA) was compared with unphosphorylated form of PprA (PprA), for these three well-characterized roles *in vitro* and significance of P-PprA in radiation resistance using functional complementation in *pprA* deletion mutant. P-PprA stimulated its dsDNA binding property by nearly 4 folds and DNA end joining activity of T4 DNA was switched from intramolecular ligation to intermolecular ligation in presence of P-PprA. The exonuclease III protection was however, reduced in presence of P-PprA. These results suggested that PprA phosphorylation alters its *in vitro* functions. The phosphoablative T72A mutant of PprA showed significantly lesser phosphorylation by DR2518 and accordingly low levels of gamma radiation resistance complementation by phosphomimitic T72D in *pprA* deletion mutant. This suggested that phosphorylation of PprA by DR2518 at least under this study, could change the functional characteristics of this protein both *in vitro* and *in vivo*. Although, the involvement of protein phosphorylation in regulation of gene expression is yet to be established, the findings from this study has laid down the possibility of eukaryotic type STPK roles in gamma radiation resistance

and modulation of DNA metabolic proteins functions e.g. PprA could be suggested. The phopshorylation mediated higher turnover of existing proteins in absence of their *de novo* synthesis during early post irradiation recover period may be required to cope up the greater demand of their functions under PIR and DR2518 or other possible kinases contribute to this requirement cannot be ruled out. Further the bacterium like *D. radiodurans*, which lacks classical DNA damage response mechanisms like SOS and extremely resistant to DNA damaging agents, the STPK mediated gamma radiation response mechanisms might provide the basis for further studies leading to the discovery of alternate DNA damage response in this bacterium.

Conclusions

1. PQQ roles in radiation resistance and DSB repair of *Deinococcus radiodurans* R1 have been demonstrated. Evidences were provided to suggest that PQQ functions as an inducer of a γ radiation responsive eSTPK protein kinase DR2518 in *D. radiodurans* and thus the role(s) of protein phosphorylation in radiation resistance and DSB repair in this prokaryote could be hypothesized.
2. DR2518 was characterized as eukaryotic type Serine / Threonine quinoprotein kinase (eSTPK). Stimulation of DR2518 protein kinase activity by PQQ and no stimulation of only N-terminal kinase domain of DR2518 by PQQ indicated the role of C-terminal domain containing beta propeller repeats (PQQ binding motifs) as a regulatory domain. Although, the molecular basis of DNA ends stimulation is not known, the possibility of this protein forming DNA interaction motifs upon dimerization may be speculated and required further investigation.

3. Lysine 42 (K42) is catalytically important residue for the kinase activity of DR2518, and Threonine-169 (T169) was found to be a phosphoacceptor site in activation loop of this kinase.
4. Protein kinase activity of DR2518 kinase is *per se* required for gamma radiation resistance in *D. radiodurans*.
5. DR2518 kinase could phosphorylate various proteins (RecA, PprA, DR0012 (putative chromosome I partitioning protein) and DRB0002 (plasmid partitioning protein). Phosphorylation of PprA has a role in its functional efficiency both *in vitro* and *in vivo*.
6. Thus, the present study reports for the first time the role of any eukaryotic type STPK in radiation resistance and DSB repair in a prokaryote, and provided a plausible basis to its function in radioresistance of *D. radiodurans*.

Future prospective:

This thesis is the first report studying the roles of STPKs in bacterial response to gamma radiation and oxidative stress. It has brought forth the direct or indirect answers to many interesting questions that are very closely related to gamma radiation response in *Deinococcus radiodurans.* On the other hand, this study has generated a number of intriguing facts required for investigation to complete the pathway (s) regulating DNA damage response in this bacterium. Some of the worth pursuing on priority would be as follows.

1. How does phopshorylation of DNA metabolic proteins associated with DNA repair and recombination contribute to gamma radiation resistance in *D. radiodurans* ?
2. Does DR2518 sit at crossroad of oxidative stress and gamma radiation induced DNA damage inducible signaling processes?

3. Why the gamma radiation inducible autophosphorylation of DR2518 was not observed in *pqqE* mutant would be worth investigating?
4. What are the roles of other putative quinoproteins in *D. radiodurans* ?

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LIST OF PUBLICATIONS

Papers in refereed Journals

1. Involvement of protein kinase activity inducer in DNA double strand break repair and radioresistance of *Deinococcus radiodurans*; **Rajpurohit, Y. S.,** Gopalakrishnan R., and Misra H. S., ***J. Bact.*** **2008, 190: 3948-3954.**

2. Characterization of a DNA damage inducible membrane protein kinase from *Deinococcus radiodurans* and its role in bacterial radioresistance and DNA strand break repair; **Rajpurohit Y.S.**, Misra H.S.; **Mol. Microbiol., 2010, 77 (6) : 1470-1482.**

3. A eukaryotic type Ser/Thr protein kinase activity is required for its role in gamma radiation resistance and phosphorylation of DNA binding proteins in *Deinococcus radiodurans.* **Yogendra S. Rajpurohit** and Hari S. Misra (**under revision at Journal of Biological Sciences**)

BARC NEWSLETTER Reports

1. An antioxidant from a radioresistant bacterium: its role *in radiation resistance b*eyond oxidative stress tolerance. H.S. Misra, **Y.S. Rajpurohit** and N.P. Khairnar, BARC NEWSLETTER, p1-9 May-June 2010.

Papers presented during national symposia / seminars/ conferences

1. **Rajpurohit Y.S.,** Misra H.S. A protein kinase activity inducer contributes to DNA double strand break repair and radioresistance in *Deinococcus radiodurans.* DAE-BRNS Life Science Symposium 2007 (LSS-2007), p-66, Dec.5-7, 2007, Mumbai.

2. Kamble V. A., Khairnar N. P., **Rajpurohit, Y. S.** and Misra H. S. Role of bacterial protein kinase autophosphorylation activity in DNA strand break repair. Proceedings of annual meetings of Indian Society of Cell Biologists, p-123, Dec. 14-16, 2007, Varanasi, India.

3. **Rajpurohit Y. S.** and Misra H. S. Involvement of a pyrroloquinoline –quinone binding protein kinase in DNA double strand break repair and radiation tolerance in *Deinococcus radiodurans.* Proceedings of international Conference on Radiation Biology, Abstract No. 159, Nov. 10-12, 2008, Jaipur, India.

4. **Rajpurohit Y.S.** and Misra H.S. PprA phosphorylation by STPK of Deinococcus radiodurans changes its in vitro functions. Poster presented at DAE-BRNS Life Science Symposium (LSS-2011), Anstract No. 12, p-54, Nov. 12-14, 2011, Mumbai, India.

Papers presented during international symposia / seminars/ conferences

1. **Rajpurohit Y.S.** and Misra H.S. Characterization of radiation inducible eukaryotic type serine / threonine protein kinase from *Deinococcus radiodurans.* 4th Congress of European Microbiologists, FEMS 2011, Abstract No. 258, p-144, June 26-30, 2011 Geneva, Switzerland.

2. **Rajpurohit Y.S.** and Misra H.S. DNA metabolic proteins phosphorylation by eukaryotic type Ser/Thr protein kinase impacts radiation resistance in *Deinococcus radiodurans* R1. 14th international workshop on ataxia-telangiectasia. P-97, February 7-11, 2012, New Delhi, India.

Gene Expression Omnibus submission:

GSM442568, GSE17722 (GSM442538, GSM442539, GSM442540, GSM442541)

Fellowship / Award/ other achievements

1. Received **Travel grant from DST**, for presenting my work entitled "Characterization of radiation inducible eukaryotic type serine / threonine protein kinase from *Deinococcus radiodurans"* authored as Rajpurohit Y.S. and Misra H.S. at 4th Congress of European Microbiologists, FEMS 2011, Geneva, Switzerland, June 26-30, 2011.

2. Received **FEMS young scientist travel award** for presenting my work "Characterization of radiation inducible eukaryotic type serine / threonine protein kinase from *Deinococcus*

radiodurans" authored as Rajpurohit Y.S. and Misra H.S. at 4th Congress of European Microbiologists, FEMS 2011, Geneva, Switzerland, June 26-30, 2011.

3. Received **Best poster award** for presenting my work "PprA phosphorylation by STPK of *Deinococcus radiodurans* changes its *in vitro* functions*"* authored as Rajpurohit Y.S. and Misra H.S. at Life Sciences Symposium, 12-14 Nov 2011, Mumbai.

APPENDICES

Table A- List of Primers in Chapter 3.1

Sl. No.	Name of primers	Nucleotide sequences of primers	Purpose
1	2518F	5-CGGGATCCATGCCGCTGACCCCTGGAAC-3	pET cloning
2	2518R	5-GGAATTCCTACCCTTCCTGCTCGCTG-3	pET cloning
3	2518-UF	5-GGC GGG CCC CGT GGC CGT TCG GGA AGT-3	*dr2518* knockout
4	2518-UR	5-CCG GAA TTC GCC GAG CAG GGC GAG CAA CT-3	*dr2518* knockout
5	2518-DF	5-CCC GGA TCC CCT TCA TGG ACG GTA CCC T-3	*dr2518* knockout
6	2518-DR	5-TCC CCG CGG CTG CGC CGC GAA CTG AAG AT-3	*dr2518* knockout
7	2518pqqF	5-AACTGCAGCTACGAGTTGCTGCCCCA-3	*pqqE*, dr2518 double knockout
8	2518pqqR	5-CGGGATCCTCAGGCGTAGCACCAGGC-3	*pqqE*, dr2518 double knockout
9	2518F-rad	5-AAAGGGCCCATGCCGCTGACCCCTGGA-3	pRADgro cloning
10	2518R-rad	5-CTAGTCTAGACTACCCTTCCTGCTCGCT-3	pRADgro cloning
11	C0015UF	5-GAG GGG CCC GGT GCA CCA TGA CTC TGC CGA-3	*drC0015* knockout
12	C0015UR	5-CGG AAT TCC GGG GTG GCG CTT CGC GTC A-3	*drC0015* knockout
13	C0015DF	5-CGC GGA TCC GCA TAC TCT CCT GGC CGT GA-3	*drC0015* knockout
14	C0015DR	5-TCC CCG CGG CCA GAG AAG TGT CGG TCA CCA-3	*drC0015* knockout
15	766-UF	5-GGC GGG CCC CCA AGG AAC CTA AAT TCA TCA-3	*dr0766* knockout
16	766-UR	5-CGGAAT TCC TCA ATC TCA AAT CAA GGT CA-3	*dr0766* knockout
17	766-DF	5-CGC GGA TCC CTG TGG TGC GCG AGC CCT GA-3	*dr0766* knockout
18	766-DR	5-GCT CTA GAA TGT GGT CGA TGC CCA TCT CCA-3	*dr0766* knockout
19	1769UF	5-GGC GGG CCC CAT CCC GAG GAA TTT GGT GTA-3	*dr1769* knockout
20	1769UR	5-CGG AAT TCG CGG GCA GCG GGG TCA GGC AT-3	*dr1769* knockout
21	1769DF	5-GCG GGA TCC AGC CGG AAT TCC CGG GCG T-3	*dr1769* knockout
22	1769DR	5-GTT GGA TCC TTG CCC TGC GCT TCG TCA CT-3	*dr1769* knockout
23	503UF	5-CGC GGG CCC ACG GAG GAG TAA CAG CCA GT-3	*dr0503* knockout
24	503UR	5-AAA ACT GCA GTG ACA CAC GCC CGC AAG GAC A-3	*dr0503* knockout
25	503DF	5-CGC GGA TCC CCA CGC CTA CCA GCC GCT-3	*dr0503* knockout
26	503DR	5-GCT CTA GAG CAT AAG CCT CAG TTC CCG GT-3	*dr0503* knockout
27	2518F	5-CGTTCACAGTCACGGCA-3	Screening of knockout clones
28	2518R	5-CAGTTCCTGCATGTCGGA-3	Screening of knockout clones

29	1769F	5-GACGACACGGTGTACGC-3	Screening of knockout clones
30	1769R	5- CCATTGGTGTTCAGGGC-3	Screening of knockout clones
31	C0015F	5- CTCGCGAAGTCTTCGCT-3	Screening of knockout clones
32	C0015R	5- CGGACGCAAACCTCCA-3	Screening of knockout clones
33	503F	5-GCACCAATTACGCCAC-3	Screening of knockout clones
34	503R	5-GGAACTTCTTCCAGGCT-3	Screening of knockout clones
35	766F	5-GCCGAACGTCAAGGTGGT-3	Screening of knockout clones
36	766R	5-CCGAGGCGGTAGAGCTGT-3	Screening of knockout clones

Table B- List of Primers used in chapter 3.2

Sl.No.	Name of primers	Nucleotide sequences of primers	Purpose
1	2518F	5-CGGGATCCATGCCGCTGACCCCTGGAAC-3	pET cloning
2	2518R	5-GGAATTCCTACCCTTCCTGCTCGCTG-3	pET cloning
3	2518KDR	5-GGAATTCCTACAGGTCGCGCCGGGCCAG-3	pET cloning
4	K42F	5' GAGGTGGCGCTCGCGGTGATGCACG 3'	K42A
5	K42R	5' CGTGCATCACCGCGAGCGCCACCTC 3'	K42A
6	S162F	5’ CTGGTGGCGCTGGCCGAACAGACCC 3’	S162A
7	S162R	5’ GGGTCTGTTCGGCCAGCGCCACCAG 3’	S162A
8	T169F	5' GACCCGGCACCTCGCCCGCAGTGGAGTG 3'	T169A
9	T169R	5' CACTCCACTGCGGGCGAGGTGCCGGGTC 3'	T169A
10	S171F	5' GCACCTCACCCGCGCAGGAGTGACGCTGG 3'	S171A
11	S171R	5' CCAGCGTCACTCCTGCGCGGGTGAGGTGC 3'	S171A

Table C- List of primers used in chapter 3.3

Sl. No.	Name of primers	Nucleotide sequences of primers	Purpose
1	PprA F	5′ GTGCTACCCCTGGCCT3′	pETpprA cloning
2	PprA R	5′ TCAGCTCTCGCGCAGGCCGT 3′	pETpprA cloning
3	33F	5’AGCACATCGGCTTCGCCGGGTGACACAACCGCT3’	EMSA PprA
4	33C	5'AGCGGTTGTGTCACCCGGCGAAGCCGATGTGCT3'	EMSA PprA
5	PprAF-11559	5’GGCCGCCCATATGGCAAGGGCTAAAGCAAAAGA3’	p11559, pprA
6	PprAR-11559	5’AAACTCGAGTCAGCTCTCGCGCAGGCCGT3’	p11559, pprA
7	S28AF	5’ CGGGCGTGGACGCCCAGATCGCCGC 3'	S28A
8	S28AR	5’CGCCCGCA CCTGCGGGTCTAGCGGC 3’	S28A
9	S112AF	5' GAACGCGGCCTGGCCCAGTGGGCGG 3'	S112A
10	S112AR	5' CCGCCCACTGGGCCAGGCCGCGTTC 3'	S112A
11	T48AF	5'GACGCGGCGCTCGCGCAGTCCTTGC 3’	T48A
12	T48AR	5'GCAAGGACTGCGCGAGCGCCGCGTC 3'	T48A
13	T72AF	5'GCCATGAGGCGCGGCTGGCCGACGACGGCCACATC 3’	T72A
14	T72AR	5'GATGTCGCCGTCGTCGGCCAGCCGCGCCTCATGGC 3'	T72A