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Characterization of a DNA damage-inducible membrane protein kinase from *Deinococcus radiodurans* and its role in bacterial radioresistance and DNA strand break repair

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Summary

***Deinococcus radiodurans* mutant lacking pyrroloquinoline–quinone (PQQ) synthesis shows sensitivity to γ-rays and impairment of DNA double strand break repair. The genome of this bacterium encodes five putative proteins having multiple PQQ binding motifs. The deletion mutants of corresponding genes were generated, and their response to DNA damage was monitored. Only the Δ*dr2518* mutant exhibited higher sensitivity to DNA damage. Survival of these cells decreased by 3-log cycle both at 6 kGy γ-rays and 1200 J m^{-2} UV (254 nm) radiation, and 2.5-log cycle upon 14 days desiccation at 5% humidity. The Δ*dr2518* mutant showed complete inhibition of DSB repair until 24 h PIR and disappearance of a few phosphoproteins. The Δ*dr2518pqqE:cat* double mutant showed γ-ray sensitivity similar to Δ*dr2518* indicating functional interaction of these genes in *D. radiodurans*. DR2518 contains a eukaryotic type Ser/Thr kinase domain and structural topology suggesting stress responsive transmembrane protein. Its autokinase activity *in solution* was stimulated by nearly threefold with PQQ and twofold with linear DNA, but not with circular plasmid DNA. More than 15-fold increase in *dr2518* transcription and several-fold enhanced *in vivo* phosphorylation of DR2518 were observed in response to γ irradiation. These results suggest that DR2518 as a DNA damage-responsive protein kinase plays an important role in radiation resistance and DNA strand break repair in *D. radiodurans*.**

Introduction

Cells exposed to different DNA-damaging agents survive by their ability to repair DNA damage. To achieve this goal, organisms have developed the efficient systems, which co-ordinate the DNA damage response with genome function and post-translation regulation of proteins turnover. In bacteria, the DNA damage response has been better studied in *Escherichia coli* cells exposed to DNA damage caused by either 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 by co-protease activity of single strand DNA-bound RecA leads to the derepression of a large number of SOS repair proteins including RecA and UmuD/C (Little *et al.*, 1980). 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 and Camerini-Otero, 2002). The molecular mechanisms underlying the DNA damage induced signalling processes through serine/threonine protein kinases (STPKs) are relatively better understood in eukaryotes (Zhou and Elledge, 2000; Sancar *et al.*, 2004). Although the bacterial genome encodes the eukaryotic-like STPKs (Magasanik, 1995; Leonard *et al.*, 1998), their involvement in DNA damage response has not been shown. 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 expression of RecA has been completely ruled out in *Deinococcus radiodurans* (Narumi *et al.*, 2001; Bonacossa de Almeida *et al.*, 2002; Sheng *et al.*, 2004).

Deinococcus radiodurans R1 is a bacterium, characterized for its extraordinary tolerance to the lethal and mutagenic effects of DNA-damaging agents, including γ-rays and UVC radiations and desiccation (Battista, 2000; Cox and Battista, 2005; Blasius *et al.*, 2008). These phenotypes are attributed to an efficient DNA strand break

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repair mechanism (Minton, 1994; Makarova *et al.*, 2001; Slade *et al.*, 2009) and a strong oxidative stress tolerance due to antioxidant enzymes (Markillie *et al.*, 1999) and by the non-enzymatic components like pyrroloquinoline–quinine (PQQ) (Khairnar *et al.*, 2003), carotenoids (Tian *et al.*, 2009) and accumulation of Mn (II) (Daly *et al.*, 2004; 2007). Initial phase of DNA strand break repair witnessed the reassembly of shattered genome by extended synthesis dependent strand annealing (ESDSA) process (Zahradka *et al.*, 2006), followed by exonuclase I sensitive pathway of homologous recombination (Misra *et al.*, 2006). Effects of DNA damage on gene expression (Liu *et al.*, 2003; Tanaka *et al.*, 2004) and both qualitative and quantitative changes in proteome in response to γ radiation (Joshi *et al.*, 2004; Zhang *et al.*, 2005) have been shown in *D. radiodurans.* This bacterium produces PQQ, a well-characterized antioxidant and role of this compound in radiation resistance and DSB repair beyond its oxidative stress tolerance, has been demonstrated in *D. radiodurans* (Rajpurohit *et al.*, 2008). PQQ has been classified as a member of B group vitamins (Kasahara and Kato, 2003) and functions as an antioxidant in mammalian system (He *et al.*, 2003). It stimulates the oxidative stress tolerance in *E. coli* (Khairnar *et al.*, 2003) *in vivo* and produces chemically inert adduct with artificially produced reactive oxygen species (Misra *et al.*, 2004) *in vitro.* Database search for the proteins containing PQQ binding motifs shows the presence of five uncharacterized open reading frames (ORFs) DR_0503, DR_0766, DR_1769, DR_2518 and DR_C0015 in the genome of this bacterium (http://smart.embl-heidelberg.de/). The molecular mechanism underlying PQQ function in radiation resistance and in DSB repair of this bacterium that contains proteins with multiple PQQ binding motifs (Makarova *et al.*, 2001) would be worth investigating.

Here we report the generation of deletion mutant of all five putative PQQ binding proteins separately, characterization of these mutants' responses to DNA damage and the possible mechanism underlying the role for one of these proteins i.e. DR2518, in radioresistance and DSB repair in *D. radiodurans*. The Δ*dr2518* mutant was the only mutant that showed DNA damage sensitivity. Cells having double mutation in *pqqE* (*drc0034*) and *dr2518* genes (hereafter referred to as Δ*dr2518pqqE:cat*) did not add further to the γ radiation sensitivity of Δ*dr2518* mutant, indicating the genetic interaction of these genes in this bacterium. DR2518 contains multiple PQQ binding motifs at the C-terminal, and a well-defined eukaryotic type serine/threonine protein kinase (STK) domain at its N-terminal. The *Δdr2518* mutant showed a significant change in phosphoproteins profile and a strong impairment in DSB repair. The recombinant protein showed autokinase activity, which was stimulated with both PQQ and DNA ends *in vitro*. This activity, however, was not stimulated with circular DNA. Nearly 15-fold higher synthesis of *dr2518* transcript and several-fold enhanced *in vivo* phosphorylation of DR2518 were observed in response to γ radiation. Structural topology of this protein matched with mycobacterial PknB sensor kinase with the well-defined transmembrane and sensor domains. These results suggest the regulatory role of DR2518 in radiation resistance and DNA strand break repair in *D. radiodurans* and characterized this protein as a DNA damage-responsive membrane protein kinase having eukaryotic STK domain, in a prokaryote.

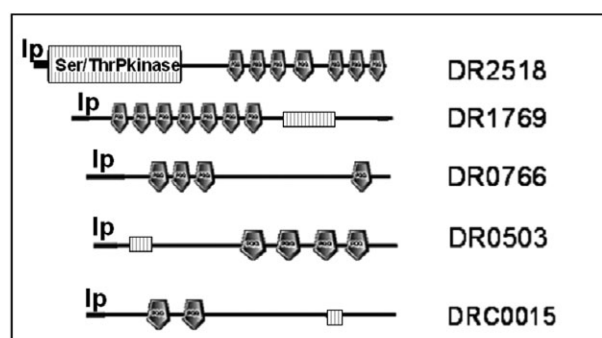

Fig. 1. Diagrammatic representation of individual open reading frames encoding putative quinoproteins in *Deinococcus radiodurans* R1. The DR0503, DR0766, DR1769, DR2518 and DRC0015 ORFs encoding uncharacterized proteins with multiple PQQ binding motifs and N-terminal leader peptides (lp) differ in protein kinase domains. DR2518 showed a well defined STKs type protein kinase domain (Ser/Thr Pkinase) at N-terminal while PQQ binding motifs at C terminal while DR1769 contains relatively less defined kinase domain at C-terminal and PQQ binding domains at N-terminal.

Results

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

Earlier we have demonstrated that *pqqE:cat* mutant of *D. radiodurans* lacking PQQ is sensitive to γ radiation and shows a strong impairment in DNA strand break repair (Rajpurohit *et al.*, 2008). These cells show a reduced level of total protein phosphorylation as compared with wild-type cells, indicating the possible role of protein phosphorylation in radioresistance and DSB repair in this bacterium. PQQ is known as a redox cofactor for dehydrogenases (Goodwin and Anthony, 1998). Since PQQ interacts with these enzymes through the well-conserved amino acid motifs, the presence of similar motifs in deinococcal- proteins was checked. Domain search analysis (http://smart.embl-heidelberg.de/) showed that there are five genes such as *dr0503*, *dr0766*, *dr1769*, *dr2518* and *drc0015* encoding uncharacterized proteins having multiple though different numbers of PQQ binding motifs (Fig. 1). DR2518 also contains a well-characterized eukaryotic type Ser/Thr kinase (STK) domain while

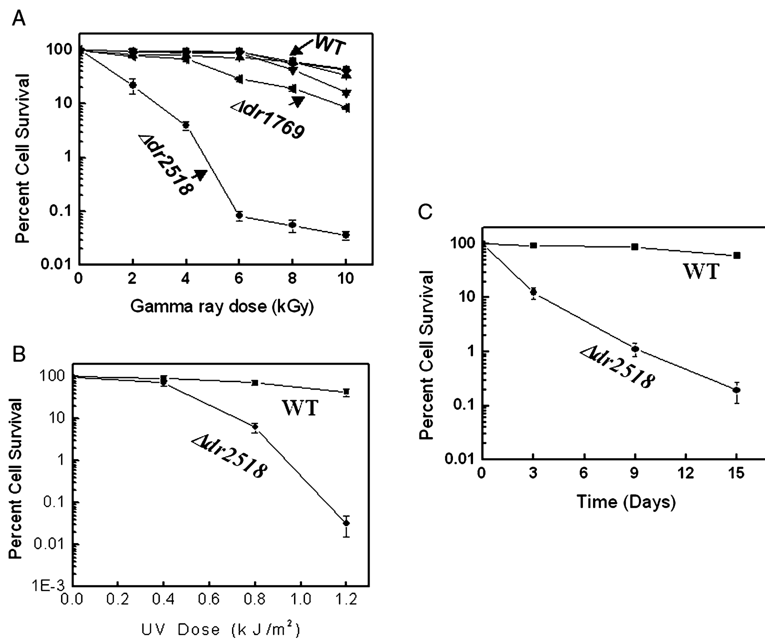

Fig. 2. DNA damage response of different mutants of *Deinococcus radiodurans* R1. Wild-type (-■-) and Δ*dr0503* (-►-), Δ*dr0766* (-▲-), Δ*dr1769* (-◄-), Δ*dr2518* (-●-) and Δ*drc0015* (-▼-) mutants were exposed to various doses of γ radiation (A). Data representing the Δ*dr2518,* Δ*dr1769* mutants and wild-type are indicated directly on figures while other mutants are represented by symbols. Similarly, the Δ*dr2518* deletion mutant (-●-) and wild-type cells (-■-) were treated with various doses of UVC (B) and for various period of desiccation at 5% humidity (C) and the effects of these mutations on cell survival were measured. All the experiments were repeated at least three times and variations in the results are calculated as standard deviations (SD) and shown as error bars.

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 genome and the homozygous replacement of wild-type alleles with *nptII* antibiotic marker was confirmed by PCR amplification (Fig. S1). These mutants were checked for γ radiation resistance.

Among these mutations, the Δ*dr2518* mutant showed comparatively higher sensitivity to different DNA-damaging agents (Fig. 2). This showed nearly 3-log cycle decreased cell survival at 6 kGy γ-rays (Fig. 2A) and at 1200 J m^{-2} of UVC radiation (Fig. 2B) and ~2.5 log cycle loss of viability upon 14 days desiccation at 5% humidity (Fig. 2C). The Δ*dr2518* mutant survival decreased by nearly 1.5-log cycles in presence of 20 μg ml^{-1} mitomycin C treated for 30 min and by nearly sevenfold at 30 mM hydrogen peroxide treatment (data not shown), as compared with wild-type. The levels of γ radiation tolerance in Δ*dr1769* mutant decreased to less than 1.0-log cycle at 10 kGy as compared with wild-type. Other mutants showed no effect of DNA damage and their tolerance to different DNA-damaging agents continued to be similar to wild-type (data not shown). These results suggested the significant role of DR2518 protein in extraordinary tolerance to DNA damages in *D. radiodurans.*

In order to ascertain that the loss of DNA damage resistance in Δ*dr2518* mutant was not due to the polar effect of this deletion and by rather the *in vivo* functional interaction of PQQ with DR2518, (i) the wild-type allele of *dr2518* was introduced into Δ*dr2518* mutant under P_{groESL} promoter in pRADgro (Misra *et al.*, 2006) and (ii) the *pqqE* (*drC0034*)*,* encoding PQQ synthase*,* was deleted from Δ*dr2518* mutant genome to obtain Δ*dr2518pqqE:cat* double mutant. The γ radiation resistance of transgenic Δ*dr2518* mutant cells expressing DR2518 *in trans* was recovered nearly full to wild-type levels (Fig. 3A), indicating that the γ radiation sensitivity of Δ*dr2518* mutant was due to absence of this protein *per se*. The Δ*dr2518pqqE:cat* mutant showed nearly similar response to γ radiation as that of Δ*dr2518* mutant (Fig. 3B). These results suggested that although *D. radiodurans* contains five putative PQQ binding proteins, DR2518 is most likely candidate through which PQQ might contribute to radiation resistance and DSB repair of this bacterium, as reported in our earlier study (Rajpurohit *et al.*, 2008).

DR2518 showed autokinase activity and functional interaction with PQQ in solution

The *dr2518* was cloned in pET28a+ and recombinant protein was purified using nickel affinity chromatography (Fig. 4A). The phospho-nature of DR2518 polypeptide and the autokinase activity of this protein were ascertained using phospho-Ser/Thr epitope polyclonal antibodies and by *in vitro* phosphorylation using [γ-^{32}P]-ATP. Recombinant DR2518 showed cross-reactivity with phospho-Ser/Thr epitope antibodies (Fig. 4A), suggesting that this protein is a phosphoprotein. The phosphorylation of recombinant protein can occur either by the phosphorylation of DR2518 by *E. coli* protein kinases or through its autokinase activity. Since the DR2518 contains putative PQQ binding motifs and a well-defined STK domain, the possibilities of this protein interacting with PQQ and having autokinase activity were checked. Recombinant

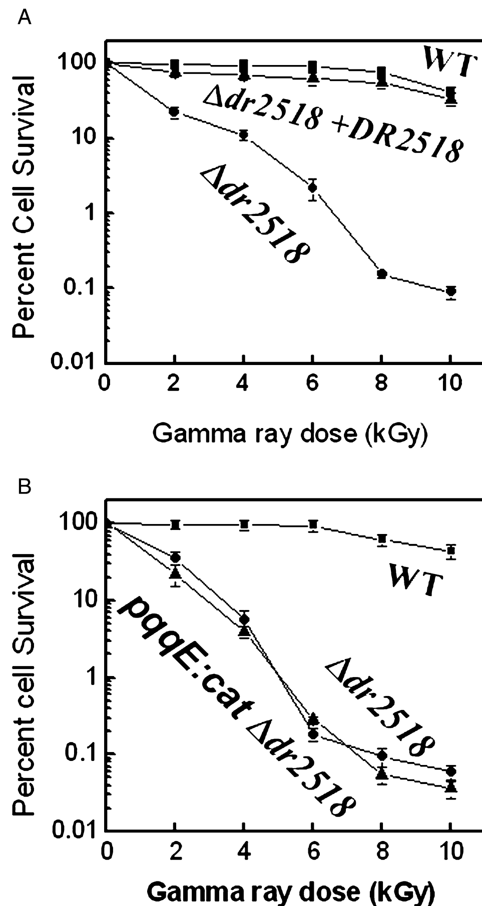

Fig. 3. Effect of *pqqE* disruption and wild-type DR2518 expression from plasmid on Δ*dr2518* mutant response to γ radiation. Wild-type (-■-), Δ*dr2518* mutant (-●-) and Δ*dr2518* mutant expressing DR2518 on plasmid (-▲-) were treated with different doses of γ radiation (A). Similarly, the Δ*dr2518* (-●-) and Δ*dr2518 pqqE:cat* double mutant (-▼-) cells were treated with different doses of γ radiation (B) and cell survival was measured. All the experiments were repeated three times and variations in the results are calculated as standard deviations (SD) and shown as error bars.

DR2518 was treated with alkaline phosphatase (AP) and phosphorylation was monitored in presence of both cold ATP and [γ-^{32}P]-ATP. AP-treated protein *per se* did not react with phospho-Ser/Thr epitope antibodies. However, when this protein was incubated with cold ATP, it showed cross-reactivity with antibodies, which increased further in presence of PQQ (Fig. 4A). Similarly, the AP-treated protein when incubated with [γ-^{32}P]-ATP showed phosphorylation, which was stimulated with PQQ by almost threefold as detected by autoradiography and quantified the phosphosignals by densitometric scanning using 'Gene Genius tools' (Syngene, UK). This indicated that DR2518 is a phosphoprotein having PQQ stimulated autokinase activity. These results also suggested the functional interaction of PQQ with DR2518 *in vitro*. 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 (Fig. 4B). Since PQQ alone did not show CD, the change in spectral characteristics as a function of PQQ concentration indicated the conformational change in protein upon PQQ interaction. This was in accordance with the typical characteristics of CD spectra generally obtained during ligand–protein interaction *in solution*. These results together suggested that DR2518 protein is a quinoprotein with autokinase activity.

The Δdr2518 *mutant showed altered phosphoprotein profile and impaired DSB repair*

The Δ*dr2518* mutant showed a major defect in DSB repair during post-irradiation recovery (PIR) and it did not recover its un-irradiated genome size even up to 24 h PIR growth (Fig. 5A). On the other hand, the wild-type cells gave normal pattern of DSB repair and full-length genome was recovered in as early as 4 h PIR. This indicated the role of DR2518 in DSB repair. Similarly, the γ-irradiated 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 were similar in un-irradiated cells. After γ irradiation, the levels of protein phosphorylation decreased significantly in 1 h PIR in both wild-type and mutant cells (Fig. 5B, lane 1). Subsequently, wild-type cells showed faster recovery of phosphoproteins and un-irradiated profile was recovered in 3 and 4 h PIR. Mutant on the other hand showed much slower recovery of phosphoproteins and the un-irradiated profile was not observed in these cells even up until 24 h PIR (compare lane 3 and 5 in Fig. 5B). The molecular weights of these phosphoproteins were in the range of 35–50 kDa and above 97 kDa, which were different from estimated size of DR2518 protein (~76 kDa). This might argue for the possibility that some of these proteins are phosphorylated by direct or indirect effect of autokinase 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 to radiation resistance and DSB repair of this bacterium. The identification of phosphoproteins that were either missing or downregulated in Δ*dr2518* mutant cells (Fig. 5B) and their possible involvement in radiation resistance and DSB repair would be investigated separately.

DR2518 showed increased synthesis and autokinase activity upon DNA damage

The effect of γ radiation induced DNA damage on both transcription of *dr2518* gene and phosphorylation of

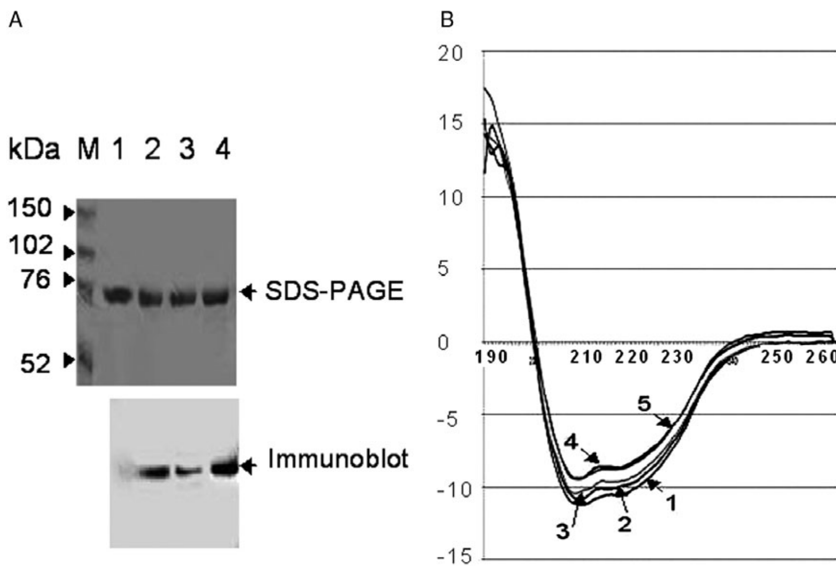

Fig. 4. PQQ interaction with DR2518 protein and its activity characterization *in solution*.
A. Purified recombinant protein was treated with alkaline phosphatase (1) and incubated with cold ATP in absence (3) and presence of (4) of PQQ. These samples were separated on SDS-PAGE along with untreated protein (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 1 nM (2), 10 nM (3), 100 nm (4) and 1000 nM (5) of PQQ was carried out. Data presented here are from a reproducible typical experiment.

DR2518 were studied. Post-irradiation expression kinetics of *dr2518* was compared by reverse transcription polymerase chain reaction (RT-PCR) using internal primers for *dr2518* and constitutively expressing glyceraldehydes 3-phosphate dehydrogenase (*gap*) gene as an internal control (Fig. 6A). The results showed nearly 15.355 ± 0.179-fold increase in levels of *dr2518* transcript within 30 min PIR; after that, *dr2518* expression was maintained closely to this level until 3 h PIR and then decreased to nearly un-irradiated level in 24 h PIR (Fig. 6B). This indicated the stimulation of *dr2518* transcription in response to γ radiation that also produces high density of DNA double strand breaks.

Earlier findings have also 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 its activity is stimulated with PQQ *in solution* (Fig. 4A). 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 the presence of γ radiation exposed PQQ (PQQ-ROS) *in solution*. PQQ exposed with γ radiation did not improve in the untreated PQQ-stimulated autokinase activity of DR2518. This suggested that the PQQ stimulation of 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 DNA strand break, the effect of double-stranded DNA (dsDNA) on autokinase activity of DR2518 was examined by *in vitro* phosphorylation with [γ-^{32}P]-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 1 kb linear dsDNA fragment irrespective of γ radiation exposure (Fig. 6C). This indicated that DR2518 is a dsDNA inducible kinase. Absence of the cumulative effect of γ radiation and linear dsDNA when compared with linear dsDNA alone, on stimulation of autokinase activity argued in favour of DNA ends mediated stimulation of DR2518 activity rather than oxidative damage of DNA. The stimulation of DR2518 activity (i) with total genomic DNA isolated from both un-irradiated and γ-irradiated cells, which presumably would have generated numerous open ends during preparation (Fig. 6D), and (ii) nearly twofold with linear plasmid DNA but not covalently closed circular (CCC) plasmid DNA (Fig. 6E) 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. 7). Since this protein does not contain any known DNA binding domain, the mode of its interaction and stimulation of its autokinase activity by linear dsDNA are unclear. These results, however, provided a strong evidence to suggest that DNA damage induces *dr2518* transcription *in vivo* and DNA ends stimulates DR2518 autokinase activity *in vitro*.

Gamma radiation treatment induced autophosphorylation of DR2518 in vivo

The DR2518 phosphorylation status in *D. radiodurans* and the effect of γ radiation on its *in vivo* phosphorylation

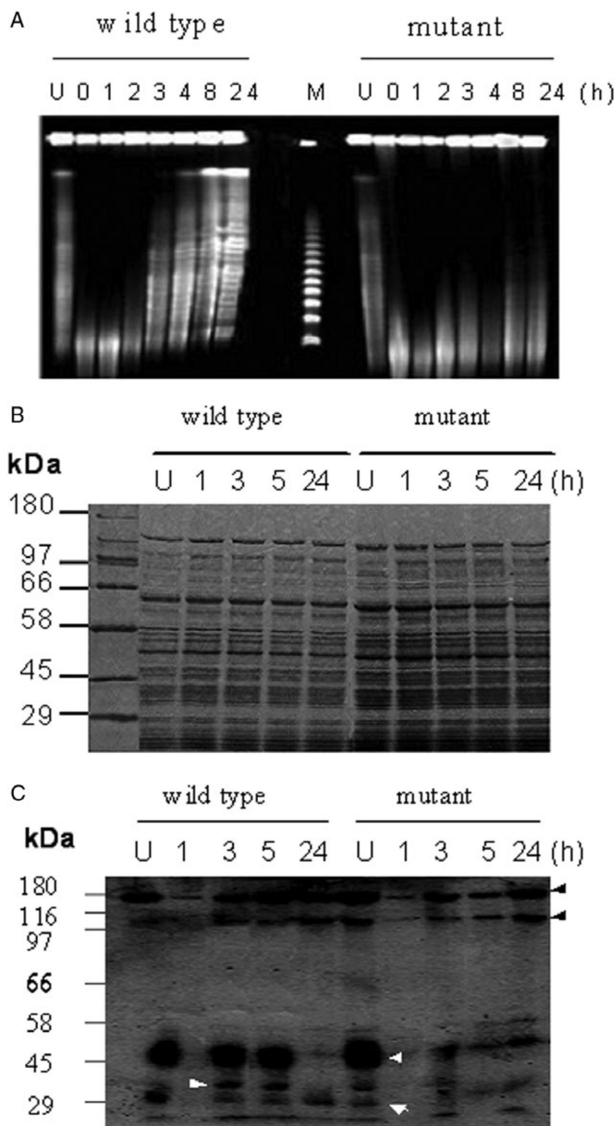

Fig. 5. Kinetics of DSB repair and phosphoproteins profiles change during post-irradiation recovery of *Deinococcus radiodurans* R1. Wild-type (wild-type) and Δ*dr2518* mutant (mutant) cells were irradiated with 6 kGy γ radiation dose and aliquots were collected at different post-irradiation recovery period (0, 1, 2, 3, 4, 8 and 24 h) and DSB repair pattern was compared with respective un-irradiated (U) controls by monitoring the recovery of wild-type NotI pattern of genomic DNA from mutant cells (A). Similarly, both wild-type (wild-type) and Δ*dr2518* mutant (mutant) cells pre-labelled 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 and 24 h post irradiation with 6 kGy γ radiation and un-irradiated (U) controls. Total proteins were analysed on SDS-PAGE (B), dried and autoradiograph (C) was developed. The phosphoproteins, which are missing in mutant cells, are indicated with arrow.

were determined by co-immunoprecipitation with antibodies raised against purified recombinant DR2518. The exponentially growing [^{32}P]-labelled cells were treated with 6.5 kGy γ radiation and aliquots were drawn at different PIR. The cell-free extracts of labelled cells and [^{32}P]-DR2518 were co-immunoprecipitated with DR2518 antibodies. The immunoprecipitates were purified through Protein-G affinity column chromatography and analysed on SDS-PAGE along with the proteins that did not bind to the column. The purified DR2518 could be immunoprecipitated with DR2518 antibodies (Fig. 8A, lane C). Un-irrradiated sample showed very low intensity of phosphosignal precipitated with DR2518 antibodies, while cell-free extract of 1–3 h PIR cells showed enhanced phosphorylation of DR2518 (Fig. 8A). Interestingly, the flow through fraction of immunoprecipitated mixture of PIR proteins, when passed through Protein G Sepharose column, showed higher levels of phosphoproteins that did not bind to column (data not shown). This indicated that the higher level of protein phosphorylation occurs in response to γ irradiation. This was further ascertained by immunoprecipitating the cell-free extract of 1 h PIR, its corresponding un-irradiated control and purified [^{32}P]-DR2518 with antibodies. Phosphoproteins profiles of immunoprecipitate and unbound proteins as eluted in flow through were analysed. Results showed immunoprecipitation with purified DR2518 (Fig. 8B, lane C) and higher levels of DR2518 phosphorylation upon γ irradiation (Fig. 8B, compare lane PU and PI). Interestingly, Protein-G flow through fraction from γ radiation treated 1 h PIR cells showed an extensive phosphorylation of an additional protein of around 97 kDa (Fig. 8B, lane SI). The corresponding signal was also seen though low levels, in un-irradiated control (Fig. 8B, lane SU). These results suggested the phosphorylation of DR2518 *in vivo* and stimulation of this protein phosphorylation in response to γ irradiation. The higher size phosphoprotein band seems to be at least one substrate for this kinase getting extensively phosphorylated in this bacterium in response to DNA damage, and would be identified and studied independently.

Discussion

Deinococcus radiodurans tolerates extensive DNA damage and regulates gene expression upon γ irradiation (Liu *et al.*, 2003; Tanaka *et al.*, 2004) by still unidentified mechanisms of DNA damage response. The signal transduction mechanisms in response to nutritional stress and other abiotic stresses except DNA damage have been reported in bacteria (Parkinson, 1993; Dutta *et al.*, 1999; Hengge-Aronis, 2002). Also, the involvement of a two-component system in the expression of recombination and DNA repair genes in response to environment changes has been reported in *E. coli* (Oshima *et al.*, 2002; Zhou *et al.*, 2003). Enhanced sensitivity of PQQ mutant to γ radiation and the defect in DNA double strand break repair (Rajpurohit *et al.*, 2008) led us to investigate the

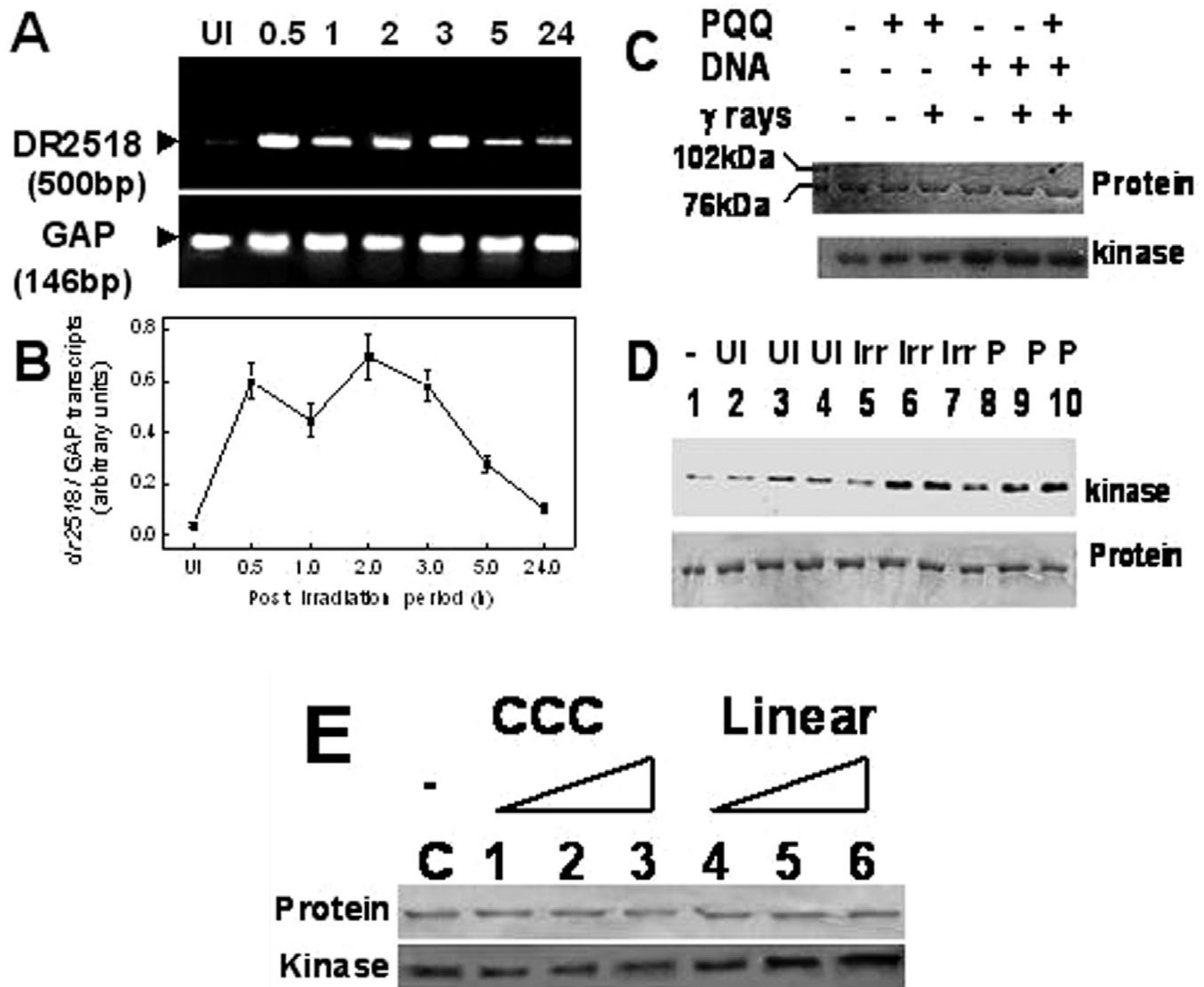

Fig. 6. Effect of DNA damage on *dr2518* gene expression and autokinase activity *in vitro*. The RT-PCR analysis of total RNA of wild-type cells collected at various time of postirradiation (0.5, 1, 2, 3, 5 and 24 h) and un-irradiated (UI) control (A) was carried out using internal primers of *dr2518* 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. Recombinant DR2518 was incubated with PQQ and PCR product in different combinations and the autokinase activity was detected by immunoblotting (C). Similarly, purified protein (1) was incubated with 10 ng (2, 5, 8), 100 ng (3, 6, 9) and 1000 ng (4, 7, 10) of genomic DNA prepared from un-irradiated (UI), 6 kGy γ-irradiated (Irr) cells and PCR amplified (P) DNA, and the autokinase activity was detected by autoradiography (D). DR2518 was also incubated with 100 ng of each superhelical form (1, 2, 3) and linear form (4, 5, 6) of plasmid DNA and autokinase activity was detected by immunoblotting (E). All the experiments repeated at least three times and results without error bars (standard deviation) are from a reproducible typical experiment.

possible mechanisms of PQQ action in DNA damage tolerance of this bacterium. Characterization of deletion mutants of all five genes encoding proteins having PQQ binding motifs and *Δdr2518 pqqE:cat* mutant for their response to γ radiation suggested the regulatory role of DR2518 in radiation resistance and DSB repair of *D. radiodurans*. Further studies on this protein led to the identification and characterization of DR2518 a DNA ends inducible eukaryotic STK type protein kinase in this prokaryote.

Although the precise mechanisms of DR2518 function in radiation resistance and DSB repair are not clear, this protein showed close similarities with two protein kinases: (i) YfgL, a periplasmic protein kinase of *E. coli*, which contains multiple PQQ binding motifs and a defined STK domain (Khairnar *et al.*, 2007); and (ii) PknB, an oxygen and nutritional stress sensor kinase of mycobacterial two-component system that is associated with cell wall synthesis and cell division in mycobacterium (Kang *et al.*, 2005). DR2518 and PknB showed highest identity with

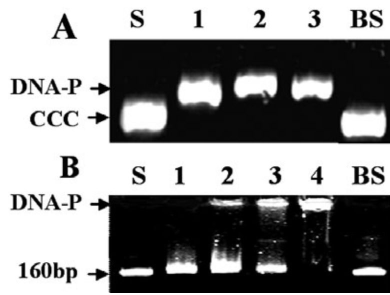

Fig. 7. DNA binding activity of recombinant DR2518 protein *in vitro*. The 500 ng of superhelical form of plasmid DNA (S) was incubated with 300 ng (1), 600 ng (2) and 1000 ng (3) recombinant DR2518 for 20 min (A). Similarly, the 200 ng of 160 bp linear double-stranded DNA was incubated with 100 ng (1), 300 ng (2), 600 ng (3) and 1000 ng (4) purified DR2518 for 20 min (B). Both types of DNA substrates were incubated with 500 ng BSA (BS) in place of DR2518, as controls under identical conditions. Products were analysed on 0.8% agarose. Data provided were from a reproducible representative experiment. Data presented here are from a reproducible typical experiment.

eukaryotic STKs, in N-terminal domain (Fig. S2). The PASTA motifs in sensory domains located at C-terminal of PknB were replaced with PQQ binding motifs in DR2518 (Fig. 9A). PknB is a member of Pkn series protein kinase, has a well-defined membrane localization signal (Av-Gay and Everett, 2000; Greenstein *et al.*, 2007). *In silico* membrane topology analysis (http://www.ch.embnet.org/software/TMPRED_form.html) showed that DR2518 has membrane organization similar to PknB. DR2518 seem to be an 'N-in-middle-N-out-C-in' membrane protein having N-terminal catalytic kinase domain and C-terminal PQQ binding domain lying in the cytoplasm (Fig. 9B and C). Thus, the structural topology of DR2518 protein mimics the domain organization of two-component system protein kinases. Since PASTA domain in PknB is a signal-receiving domain, the similar function of PQQ binding domain in DR2518 might be speculated. These results indicated DR2518 a membrane protein. Initial studies on localization of this protein in different sub-cellular fractions of γ-irradiated cells indicated that DR2518 gets localized maximally in the membrane, but is also seen in the cytoplasmic and periplasmic fractions (data not shown). Although the mechanism of periplasmic proteins phosphorylation in periplasmic space is still intriguing, the evidence of such proteins undergoing phosphorylation has been reported including a nutritional stress responsive signalling protein of *E. coli* (Celis *et al.*, 1998) and YfgL, in our earlier studies (Khairnar *et al.*, 2007). Detailed studies on cellular localization of DR2518, its activity and their functional significance in DNA damage response will be published separately.

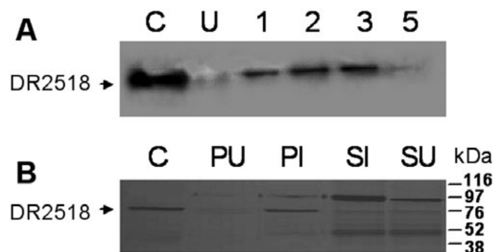

Fig. 8. Detection of *in vivo* phosphorylation of DR2518 in *Deinococcus radiodurans*. Polyclonal antibodies raised against purified recombinant DR2518 were incubated with purified [^{32}P]-phosphorylated DR2518 (C) and with cell-free extract of exponentially growing un-irradiated cells (U) and 1, 2, 3 and 5 h post-irradiation recovery period (A) at 4°C overnight. Similarly, the purified [^{32}P]-phosphorylated DR2518 (C) and cell-free extract of un-irradiated (PU, SU) as well as 1 h post-irradiation recovery (PI, SI) cells were incubated with DR2518 antibodies at 4°C overnight (B). Reaction mixtures were passed through Protein-G column. Both immunoprecipitates [C, U, 1, 2, 3, 5 as in (A) and C, PU, PI as in (B)] 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 autoradiograph was developed. Data from a typical reproducible experiment have been presented.

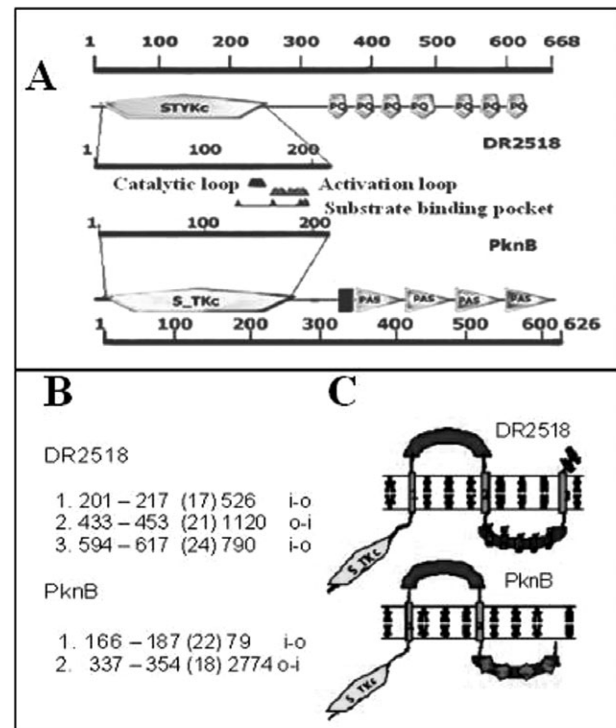

Fig. 9. Structural similarities of DR2518 with PknB sensor kinase. DR2518 amino acids were compared with PknB for functional domains alignment (A). Topology search showed three transmembrane domains distributed from amino acids 201–217 (1), 433–453 (2) and 594–617 (3) (B) distinctly dividing this protein into intracellular (STKc and five of seven PQQ binding motifs), transmembrane (1, 2, 3) and extracellular (217–433 an uncharacterized domain, and two of seven PQQ binding motifs) domains (C), which resemble closely to the corresponding regions of amino acids and ligand binding motifs of mycobacterial PknB sensor kinase.

The role of PQQ that is synthesized in *D. radiodurans* under normal growth conditions (Rajpurohit *et al*., 2008) as a signalling molecule is yet to be established. However, the level of expression of *pqqE* (PQQ synthase) gene was induced by approximately fourfold within 1 h PIR (Liu *et al*., 2003; H.S. Misra and colleagues, unpubl. data). PQQ reactivity with ROS (Misra *et al*., 2004) and PQQ as an inducer of membrane protein kinase activity in *E. coli* (Khairnar *et al*., 2007) have been demonstrated. The roles of other antioxidants as singling molecules (Williams *et al*., 2004) and the DNA damage induced signal transduction mechanisms (Kitagawa and Kastan, 2005) are well studied in eukaryotes. In prokaryotes however, the DNA damage-induced signal transduction processes and involvement of protein kinase/phosphoproteins in DNA metabolism have gained significant importance recently. Some of the recent findings, such as (i) the role of a response regulator DrRRA (DR_2418), in regulation of DNA repair genes like *recA, pprA, katA*, etc. (Wang *et al*., 2008), (ii) the interaction of DNA repair proteins with protein kinases and phosphoproteins in a multi-protein complex (Kota and Misra, 2008) and (iii) the phosphorylation of single strand DNA binding protein (SSB) in *Bacillus subtilis* (Mijakovic *et al*., 2006), have indicated the possible involvement of signal transduction/protein phosphorylation processes in DNA metabolism and radiation tolerance in prokaryotes.

Through this study we report that a bacterial membrane protein kinase with eukaryotic type STK domain, was stimulated several folds both at synthesis and at activity levels in response to DNA damage *in vivo* and by PQQ and DNA ends *in vitro*. Interestingly, DR2518 showed productive interaction with dsDNA substrate but its autokinase activity was stimulated by DNA ends and not by covalently closed circular DNA (Fig. 6E). These results indicated that DR2518 was a protein kinase with DNA binding activity and possibly interacts with DNA ends. The mode of its interaction with DNA without having any defined DNA binding motifs is not clear. Since this protein has seven putative PQQ binding motifs at C-terminal of protein and its autokinase activity is stimulated with both PQQ and DNA ends, the possibility of DNA interaction through the amino acid regions located between highly conserved seven PQQ binding motifs and STK domain can be speculated. *In silico* fold prediction studies using FUGUE server package, also revealed that the 273–401 amino acids region located between STK and PQQ binding domain of DR2518 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). Stimulation of DR2518 activity by PQQ and DNA ends, which also increases upon γ irradiation, suggested that both PQQ and DNA fragments act as inducers of this membrane protein kinase. Taken together, the results on the enhanced expression of *dr2518* in response to γ radiation, the stimulation of DR2518 kinase activity by PQQ and DNA ends, and the disappearance of certain phosphoproteins in Δ*dr2518* mutant, collectively suggested this protein as a possible DNA damage sensor kinase, having a role in radiation resistance and DSB repair in *D. radiodurans.*

Experimental procedures

Bacterial strains and materials

The wild-type *D. radiodurans* R1 (ATCC13939) was a generous gift from Dr M. Schaefer, Germany (Schaefer *et al*., 2000). Wild-type and its derivatives were grown aerobically in TGY (0.5% Bacto Tryptone, 0.3% Bacto Yeast Extract, 0.1% Glucose) broth or on agar plate at 32°C in presence of antibiotics as required. Shuttle expression vector pRADgro and its derivatives were maintained in *E. coli* strain HB101 as described earlier (Misra *et al*., 2006).

Construction of recombinant plasmids

Genomic DNA of *D. radiodurans* R1 was prepared as published previously (Battista *et al*., 2001). The coding sequences of DR2518 were PCR amplified from genomic DNA of *D. radiodurans* using gene specific primer 2518F and 2518R (Table S1) and confirmed by nucleotide sequencing. PCR product was ligated at ApaI and XbaI sites in pRADgro (Misra *et al*., 2006) to yield pGro2518 and at NdeI and BamHI to yield pET2518. The pGro2518 plasmid was transformed into Δ*dr2518* mutant cells as described earlier (Meima *et al*., 2001), and the recombinant clones were scored in presence of chloramphenicol (5 μg ml^{-1}). Similarly, pET2518 plasmid was transferred to *E. coli* BL21 (DE3) pLysS and transgenic cells were induced with IPTG for the synthesis of recombinant DR2518 protein.

Generation of deletion mutant's derivative of D. radiodurans

The plasmid constructs for generation of deletion mutants of *dr2518*, *dr1769*, *dr0766*, *dr0503* and *drc0015* were made in pNOKOUT using strategies as described earlier (Khairnar *et al*., 2008). In brief, the 1 kb upstream and downstream to the coding sequences of each ORF were PCR amplified using sequence specific primers (Table S1) and cloned, respectively, at upstream and downstream to *nptII* cassette in pNOKOUT to yield pNOK2518, pNOK1769, pNOK0766, pNOK0503 and pNOK0015 plasmids (Fig. S3). These plasmids were linearized and used for generation of *dr2518*, *dr1769*, *dr0766*, *dr0503* and *drC0015* deletions respectively. The transformants harbouring respective plasmids were grown several generations in presence of Kan (10 μg ml^{-1}) 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 S1). 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 neighbouring genes in pNOKpqqE (Rajpurohit *et al.*, 2008), were replaced with chloramphenicol acetyl transferase (*cat*) cassette to yield pPQCAT. The linearized 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*).

Cell survival studies and DNA strand break repair studies

Deinococcus radiodurans cells were treated with different doses of UV and γ radiations as described earlier (Misra *et al.*, 2006). MMC (20 μg ml^{-1}) treatment was given as described in Keller *et al.* (2001) and hydrogen peroxide treatment as described in Kota and Misra (2006). In brief, the bacteria grown in TGY medium at 32°C were washed and suspended in sterile phosphate-buffered saline and treated with different doses of γ radiation at dose rate 6.82 kGy h^{-1} (Gamma 5000, ^{60}CO, Board of Radiation and Isotopes Technology, DAE, India). For UVC, the different dilutions of these cells were plated and exposed with different doses of UV (254 nm) radiation. Treated cells were plated on TGY agar plate supplemented with kanamycin (10 μg ml^{-1}) if required, and colony-forming units were recorded after 48 h of incubation at 32°C.

The kinetics of DNA strand break was monitored on pulsed field gel electrophoresis as described in Mattimore and Battista (1996). In brief, the cells were treated with 6.5 kGy γ radiation (6.82 kGy h^{-1}) and aliquots were collected at different time intervals during PIR. Cell lysis and NotI restriction digestion were carried out in gel and DNA fragments were separated by pulsed field gel electrophoresis using the modified protocol as described earlier (Rajpurohit *et al.*, 2008).

Purification and characterization of DR2518 for PQQ binding and autokinase activity

Transgenic *E. coli* BL21 (DE3) pLysS cells harbouring pET2518 plasmid were induced with 0.5 mM IPTG and recombinant protein containing hexa-histidine tag at N-terminal was purified using nickel affinity chromatography as described earlier (Misra *et al.*, 1998). The PQQ–protein interaction studies were carried out using modified protocols described earlier (Khairnar *et al.*, 2007). The purified recombinant protein was incubated with 70 molar excess ratio of PQQ in sample buffer containing 4 M Urea and dialysed overnight at 10°C against similar buffer minus urea. Free PQQ was removed using Sephadex G-25 spin columns. Free PQQ, DR2518 and DR2518 and PQQ mixture purified through G-25 spin columns were used for recording the CD spectra (JASCO, J815, Japan).

The autokinase activity of purified DR2518 was assayed in presence of both cold ATP and [γ-^{32}P]-ATP in solution and protein phosphorylation was detected by immunoblotting with phospho-ser/thr epitops antibodies in case of cold ATP and by autoradiography in case of [γ-^{32}P]-ATP. The AP-treated protein was incubated with or without PQQ (1 μM) at 37°C for 1 h in a reaction mixture containing 10 mM Tris-HCl, pH, 7.6, 20 mM KCl, 0.5 mM DTT, 2% Glycerol, 1.5 mM $MgCl_2$ and 5 mM ATP/50 μci [γ-^{32}P]-ATP with 10 mM sodium fluoride (Mishra and Parnaik, 1995). Samples were heated with equal volume of 2× Laemmli sample buffer at 95°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) as described earlier (Khairnar *et al.*, 2007). 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 autoradiograph. For checking the effect of damaged DNA on autokinase activity, the protein was pre-incubated with required DNA before either ATP or [γ-^{32}P]-ATP was added in reaction mixture. The reaction was incubated for 1 h at 37°C and levels of DR2518 phosphorylation were detected by autoradiography followed by densitometric scanning of phosphosignals using Gene Genius tools (Syngene, UK).

DNA protein interaction and in vivo *protein phosphorylation studies*

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

In vivo phosphorylation of proteins in wild-type and mutant cells was studied by growing the γ-irradiated cells in presence of [^{32}P] phosphoric acid overnight as earlier (Mann *et al.*, 1991). Cells were treated with 6 kGy γ radiation, allowed to revise in presence of [^{32}P] phosphoric acid and aliquots were drawn at regular intervals. For the determination of [^{32}P] incorporation in total proteins, the labelled cells were treated with lysozyme (10 mg ml^{-1}) for 1 h at 37°C followed by 1% NP40 in cell lysis buffer (20 mM Tris-HCl, pH8.0, 50 mM NaCl, 1 mM PMSF, 1 mM DTT. Treated cells were disrupted by repeated cycles of freezing at liquid nitrogen temperature and thawing at room temperature. The cell-free extracts was obtained by centrifugation at 20 000 *g* and were treated with DNaseI (50 μg ml^{-1}) and RNaseI (50 μg ml^{-1}) for 2 h at 37°C. The incorporation of [^{32}P] was monitored by TCA precipitation as described earlier (Misra *et al.*, 1998). Equal amount of total proteins from both the cells was analysed on SDS-PAGE and [^{32}P]-labelled phosphoproteins were detected by autoradiography.

Determination of in vivo *phosphorylation of DR2518*

Immunoprecipitation of DR2518 protein was carried out with [^{32}P]-labelled protein from un-irradiated and 6 kGy γ-irradiated cells collected at different time interval of PIR, using Seize X protein-G Immunoprecipitation Kit (Pierce, IL, USA). The cell-free extract of un-irradiated cells was prepared as described earlier (Kota and Misra, 2008). In brief,

the cells were treated with lysozyme (10 mg ml^{-1}) 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 12 000 *g* for 30 min. Approximately 500 µg total proteins in cell-free extract and 500 ng equivalent purified DR2518 were incubated with DR2518 antibodies raised in rabbit in binding buffer (140 mM NaCl, 8 mM sodium phosphate, 2 mM potassium phosphate and 10 mM KCL, pH 7.4). Mixture was incubated overnight at 4°C and to this the Protein G agarose beads were added. Content was passed through Econopack column (Bio-Rad, USA) and washed thrice with binding buffer and eluted with 500 mM 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 2× Laemmli buffer for SDS-polyacrylamide gel electrophoresis. Proteins were separated and autoradiograph was developed.

Quantification of dr2518 *expression during post-irradiation recovery*

Total RNA was prepared from un-irradiated and γ-irradiated cells of *D. radiodurans* in three replicates, using the modified protocols as described in Chen *et al.* (2008). The 14 µg total RNA was annealed with 10 µg random hexamer primers in 20 µl at 70°C for 10 min followed by 2 min incubation on ice. The cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (Invitrogen) and 0.5 mM deoxynucleotide triphosphates (dNTP) at 42°C overnight. The reaction was terminated by adding 20 µl of 0.5 M EDTA followed by 20 µl of 1 M NaOH and heating at 65°C for 20 min. The reaction mixture was neutralized with 50 µl 1 M HEPES buffer pH 7.0 and unincorporated free dNTPs were removed by ultra filtration with G-25 columns (GE, Piscataway, NJ, USA). PCR amplification was carried out using standard protocols as described in Sambrook and Russell (2001), using 18F (5′-CTGGGGCCGCTGGAAGACGC-3′) and 18R (5′-GCCCGCTTGGCGAGCAGCCA-3′) primers for *dr2518* gene and gapF (5′-GAAGGGGCCTCCAAGCACAT-3′) and gapR (5′-TTGTACTTGCCGTTCGCGGCT-3′) primers for *dr1343* gene and total cDNA mixture as templates.

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