MOLECULAR MECHANISMS UNDERLYING GAMMA RADIATION RESISTANCE IN DEINOCOCCUS RADIODURANS

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Abstract

Gamma radiation damages biomolecules by direct energy deposition and breaking of covalent bonds, and also indirectly by producing reactive oxygen species through water radiolysis. An organism that survives the higher doses of $\gamma$ radiation would have to have the efficient mechanisms for combating the deleterious effects of ionizing radiations. Deinococcus radiodurans is a bacterium known for its extreme resistance to $\gamma$ radiation. We have been studying the molecular basis to its extreme phenotypes by understanding both DNA damage and repair, and the mechanisms that protect biomolecules from oxidative damage in this bacterium. Using the technological and intellectual capabilities developed in house, we demonstrated that there is no single but several protective and repair mechanisms that work together for providing radiation resistance in this bacterium. A summary of our significant findings related to radiation resistance in D. radiodurans has been presented.

Introduction

DNA damage occurs in all organisms as a consequence of ultraviolet (UV) light emanating from the sun and ionizing radiation ($\gamma$ rays & X-rays) from natural sources, medical treatment and contaminated land. The repair of DNA damage is crucial for life and defects in DNA repair pathways can lead to genetic disease and cancer in mammals. Unlike eukaryotes, the cellular and molecular responses to DNA damage have not been fully characterized in prokaryotes except UV induced SOS response in certain bacteria, possibly because bacteria differ from higher organisms into both cellular complexity and tolerance to DNA damage. Deinococcus radiodurans is a Gram-positive bacterium primarily known for its extraordinary resistance to both ionizing and non-ionizing radiations [1, 2]. It can tolerate up to 6.5kGy $\gamma$ radiation without a measurable loss of cell viability [3, 4]. The extraordinary tolerance of D. radiodurans (hereafter referred as Deinococcus), to other abiotic stresses including radiation and desiccation, has attracted both radiation biotechnologist and molecular geneticists for using it (i) for bioremediation of radioactive waste and, (ii) as a model organism for understanding the molecular basis of its extreme radioresistance. The radiobiotechnological use of this bacterium however, suffers a major setback because this microorganism is yet to be known for any
desirable traits for its use in bioremediation of radioactive waste, except that it is resistant to both chronic as well as acute exposures of $\gamma$ radiation, and has a number of proteins that needs to be characterized for their usefulness. On contrary, there are microorganisms that do not confer such a magnitude of radiation resistance but have numerous features that are useful in bioremediation of hazardous waste mixed with metals and aromatic compounds. Therefore, the basic understanding of mechanisms underlying the radiation resistance and at least the identification of molecules required for the usefulness of this bacterium in radio-biotechnological applications would come under the core areas of basic research and would be worth pursuing. Further, both basic and applied research using this bacterium would have required the development of technology for its genetic modification and for the introduction of desirable characters in this bacterium. Our laboratory has been focusing on both of these aspects i.e. the development of both technological and intellectual capabilities for carrying out advanced research in this bacterium and the basic research on understanding the molecular basis of the extraordinary radiation resistance. Using in house expertise in these areas, we have significantly contributed in the global efforts on the subject where our findings have been used for discussing the findings from the renowned laboratories working in this area of research. Some of our works are summarized below.

**Development of technological expertise for easy manipulation of Deinococcus radiodurans**

Genetic manipulation of any organism requires the organism specific genetic tools. These are developed depending upon the need of researchers. We were in need of technology for genetic manipulations of *Deinococcus* mainly on two aspects (i) the synthesis of proteins from other bacteria as well as over production of this bacterial proteins itself, and (ii) the inactivation of any gene of interest for studying their contributions in radiation resistance of *Deinococcus*. We developed many constructs for this work using the resources available in our laboratory. The notable ones were pRADgro (Fig 1a) and pNOKOUT (Fig 1b). For the development of pRADgro, a 261bp DNA fragment containing regulatory sequences of *groESL* genes of *Deinococcus* (*Pgro*), Shine-Delgarno (SD) sequences for ribosome binding and five codons of GroE along with four new unique restriction enzyme sites for providing correct translation frame for recombinant protein synthesis, was cloned in pRAD1 [5] cloning vector. The pRADgro was checked for its nature as a shuttle expression vector between *Deinococcus* and *Escherichia coli*, another model organism mostly used for gene cloning and recombinant proteins synthesis and the expression of transgenes on this vector was ascertained in both the hosts [6, 7]. Similarly, pNOKOUT was constructed by cloning the neomycin phosphotransferase II (*nptII*) selection marker cassette into pBluescript SK+ an *E. coli* cloning vector. The *nptII* cassette was earlier known for the expression of kanamycin resistance in *Deinococcus* and pBluescript SK+ should not be surviving in *Deinococcus*. Thus pNOKOUT was anticipated to carry an expressing *nptII* cassette into *Deinococcus* but would not be maintaining in it. This will make pNOKOUT a suicidal vector and the replacement of desired gene with *nptII* would take place through genetic recombination. These features of pNOKOUT were confirmed again in both *E. coli* and *Deinococcus*. The results showed the integration of *nptII* into *Deinococcus* R1 genome and expression of kanamycin resistance in these cells. So far, we were expressing recombinant proteins in *Deinococcus* under constitutive and a relatively weaker promoter, pGroES. For many experiments, both high levels and controlled expression of recombinant proteins is required. For
Fig. 1: Partial restriction map of pRadgro and pNOKOUT constructs. The 261bp BglII-XbaI fragment containing promoter (Pgro) was cloned in pRAD1 to get pRadgro (A) and 937bp an expressing cassette of nptII (nptII (DR) was cloned at SmaI site of pBluescript SK+ to get pNOKOUT (B). Similarly, another deinococcal shuttle expression vector pVHS559, for the controlled expression of desired proteins in Deinococcus was constructed (C).

that, a new vector pVHS559, having IPTG inducible promoter backbone was modified for providing additional cloning sites and inducible expression of recombinant in Deinococcus (Fig 1c) and being used for localization of deinococcal proteins fused with fluorescent tag. Using these constructs a large number of proteins from both E. coli and Deinococcus have been expressed and a number of genetic knockout strains have been generated, localization of both host as well as external proteins, and the roles of various proteins in radiation resistance have been ascertained in this bacterium. These vectors have also been provided several laboratories abroad on material transfer agreement (MTA) and being used for basic research in Deinococcus. This allowed us to have the complete autonomy in any kind of genetic manipulation required for making Deinococcus suitable for both basic and applied work.

Functional significance of macromolecular interactions in radioresistance of D. radiodurans

Proteins are ultimate functional units that contribute to different characteristics in a living system. The protein’s functions are intimately regulated by the microenvironment around these molecules and therefore, the different organisms surviving in different environment would be expected to have different microenvironment inside the cells. Although, different organisms having similar array of proteins, might exhibit different characteristics due to change in microenvironment in which these proteins are functioning in a particular living system. With these existing paradigm, we hypothesized a possibly of DNA metabolic proteins required for extreme phenotypes in this bacterium might be interacting in a different microenvironment and thus functioning differently in this bacterium. This assumption got further support when we observed that Deinococcus with E. coli, which exhibit just opposite response to γ radiation, have nearly similar protein complements for DNA recombination and repair pathways. Therefore, to test our hypothesis, the possible existence of these proteins in form of macromolecular complex was first checked. The multiprotein complexes were isolated from stationary phase cells of Deinococcus and E. coli and compared for different DNA metabolic activities and the presence
of recombination repair proteins as detected by immune blotting [8]. Multiprotein complexes from both these organisms although have several of the DNA metabolic activities common, they differ grossly on some of the crucial functions. For example unlike *E. coli*, the multiprotein complex from *Deinococcus* did not contain RecA but it had some of the other important DNA repair function like ATP sensitive nuclease (Fig 2a) and ATP stimulated DNA ligase (Fig 2b). The protein components of this complex were identified by mass spectrometry and 24 different polypeptides were detected that include PprA, ATP type DNA repair ligase (DRB0100) and a 5’ nucleotidase (DR0505).

Fig. 2: Regulation of two incompatible functions in multiprotein complex. Multiprotein complex isolated from *D. radiodurans* was checked for nuclease activity on dsDNA in presence of Mg2+ (1-5) and Mn2+ (6-10) with increasing concentration of ATP (A). The DNA ligase activity of complex (Cm) was compared with T4 DNA ligase (T4) in presence of different concentration of ATP (B).

The functional significance of some of these proteins like PprA, DRB0100 and DR0505 in γ radiation resistance of this bacterium was further studied. We deleted *dr0505*, *pprA* and *drb0100* genes from *Deinococcus* genome as well as the recombinant proteins were made in *E. coli* and purified. By checking the γ radiation effect on the survival of these mutants and activity characterization of recombinant proteins, we demonstrated the roles of these proteins in γ radiation resistance of *Deinococcus* and the possible mechanisms of action were shown.

Interestingly, the activity of recombinant DRB0100 ligase compulsorily required PprA and DRB0098, another protein of this bacterium for its enhanced activity (Fig. 3). Further DRB0100 supported radiation resistance in *Deinococcus* only when PprA, DRB0098 and DRB0099 were present [9]. These results suggested the functional significance of protein-protein interaction in macromolecular complex for its efficient functioning in γ radiation resistance in *Deinococcus*. Similarly, the recombinant DR0505 is characterized as an ATP sensitive nuclease with a novel thermostable phosphoesterase activity, which could withstand up to 65°C *in vitro* [10] and implicated for its role in nucleotide recycling.
Fig. 3: DNA end joining activity assay of DRB0100 identified from multiprotein complex. Purified proteins were incubated with PCR amplified linear DNA and checked on agarose gel (A) and linearised plasmid DNA ligated samples were checked for transformation in *E. coli* (B). Ligation efficiency directly correlates with the number of transformants obtained with each samples.

Fig 4. Protein-protein interaction studies leading to reveal hitherto unknown function of a DNA repair protein. A. The wild type (WT) and *pprA* mutant (Mutant) cells were treated with nalidixic acid (Nal) and gamma radiation (Gamma) separately. These cells were compared with untreated controls (Control) for the loss of nucleoid as estimated microscopically. B. The recombinant TopoIB was assayed for relaxation activity in the presence and absence of its complex partner PprA (Top panel) and in vivo interaction using bacterial two hybrid system (Bottom panel).

*In vivo* localization of PprA and other components of multiprotein complex were proposed in *D. radiodirans*. PprA-GFP cellular localization studies showed that PprA binds to septum trapped nucleoid. Further studies on this observation resulted in deciphering a hitherto unknown function of this protein in genome maintenance and cell division [11, 12]. When we generated *pprA*
mutant of *D. radiodurans* and measured the genome stability of mutant and wild type cells treated with nalidixic acid and gamma radiation (Fig. 4). We provided evidence on the mechanism of action of PprA and showed that PprA interacts with both TopoIB and DNA gyrase *in vivo* and modulates deinococcal TopoIB and DNA Gyrase activities *in vitro* (Fig. 4).

Functional significance of known DNA recombination / repair proteins in radioresistance of *Deinococcus*

There are two major DNA recombination pathways like RecF and RecBC, which have been implicated in the repair of radiation-induced DNA damage in most of the bacteria. RecBC enzyme complex has been characterized for their roles in DNA double strand break repair in many radiation sensitive bacteria as well. Surprisingly, the genome of *Deinococcus* encodes all the components of RecF recombination pathway but lacks the components of classical RecBC pathway. How does this bacterium mend its shattered genome in the absence of RecBC enzymes was intriguing. First the possibility of RecF contributing to efficient DNA strand break repair was checked by *in-trans* expression of SbcB, an exonuclease I, a known inhibitor of RecF pathway, and then by over expression of RecBC from *E. coli* into *Deinococcus*. *Deinococcus* cells expressing these proteins separately become sensitive to radiation but the levels of radiation sensitivity was more in SbcB expressing cells than RecBC suggesting the involvement of RecF pathway in radiation resistance of this bacterium [6] and less significance of RecBC to these phenotypes at least in *Deinococcus* [13]. The role of RecF recombination pathway in radiation resistance of this bacterium was further supported from the observation where the functions of other suppressors like SbcCD of RecBC pathway is required for higher radioresistance [14]. Similarly, a protein (PolX) of unknown function was predicted for its DNA polymerase activity and characterized *in vitro* as a short patch base excision repair (SPBER) polymerase [15] (Fig 5).

![Fig 5. Functional characterization of PolX from *D. radiodurans*. Purified recombinant PolX (P) was assayed for primer extension activity using normal template – primer and compared with a replicative DNA polymerase Klenow fragment (K) and products were analysed on UREA-PAGE gel (A). Base excision repair activity was assayed on oxidative damage DNA template in the presence of DNA glycosylase and AP endonuclease (B).](image-url)
Characterization of a novel oxidative stress and DNA damage response mechanism in *Deinococcus*

Living cells exposed to various stress exhibit a rapid change in cellular and molecular processes to counteract the deleterious effect of stressors. For example, the cells exposed to $\gamma$ radiation respond to both oxidative stress and DNA damage effects leading to in genome functions both at the levels of gene expression and functional modulation of proteins associated with DNA repair and cell division. The DNA damage response in prokaryotes has been termed as SOS response. *Deinococcus* does not exhibit classical SOS response [16]. Despite, it shows DNA damage induced gene expression and protein turnover. The molecular mechanisms underlying the regulation of the synthesis and activity modulation of various proteins by $\gamma$ radiation induced DNA damage are not known. The systematic studies on the changes occurring in *Deinococcus* cells exposed to $\gamma$ radiation were carried out at both cellular and molecular levels. It showed that cells exposed to $\gamma$ radiation, rapidly synthesized very high levels of signaling nucleotides including ATP, AMP and cyclic AMP etc. and stress responsive enzymes like adenylyle cyclase, phosphodiesterase, protein kinases [17]. These molecules are well known for their roles in regulation of various cellular and molecular processes, suggesting that *Deinococcus* does respond to $\gamma$ radiation induced DNA damage. If it does not confer classical SOS response mechanism, the possibility of some alternate mechanisms could be hypothesized.

Since gamma radiation kills living cells by directly damaging biomolecules and indirectly through oxidative stress, we studied both these components for understanding the gamma radiation response in *Deinococcus*. We searched the genome of this bacterium for metabolic pathways associated with antioxidant synthesis in bacterial system. We observed that the genome of this bacterium confers a gene (we hereby designate as *pqqE*) that encodes an enzyme responsible for pyrroloquinoline quinone (PQQ) biosynthesis in bacterial system. This gene was cloned and expressed in *E. coli*. Transgenic *E. coli* producing PQQ showed several fold higher tolerance to oxidative stress in *vivo* and protected biomolecules from $\gamma$ radiation damage in *vitro* [18]. PQQ was subsequently shown as strong antioxidant as vitamin C and Trolox [19] and could neutralize superoxide, hydroxyl and oxygen free radicals in solution without any indication of its consequential effect [19]. PQQ was subsequently shown as pro-oxidant in mammalian cells by depleting the redox equivalents that acts as antioxidants in mammalian cells. It showed higher rates of apoptotic cell death in tumor cells as compared to normal cells [20].

The role of PQQ in radiation resistance phenotypes of *Deinococcus* was investigated. For that, *pqqE* gene was deleted from genome of *Deinococcus* and the effect of different DNA damaging agents including $\gamma$ radiation, on cell survival and DSB repair ability of mutant cells were investigated. Results showed that the absence of PQQ makes this bacterium incompetent to withstand higher doses of DNA damage and $\gamma$ radiation in particular (Fig 6A). Molecular mechanisms contributing to this phenotype of PQQ was checked and found that PQQ has a role in DNA strand break repair (Fig 6B) [21]. Subsequently, a PQQ inducible putative protein kinase encoded from *dr2518* gene was identified from this bacterium and its role in $\gamma$ radiation resistance of *Deinococcus* through DNA strand break repair was demonstrated [22].
Fig. 6: Involvement of PQQ in radiation resistance of *D. radiodurans* through its role in DSB repair. *D. radiodurans* cells devoid of PQQ were generated and the effect of γ radiation on survival of these cells (mutant) was compared with wild type (wild type) (A). The cells recovering from γ radiation effects were collected at different time interval (1-24 h) and analyzed for shattered genome reassembly on pulsed field gel electrophoresis (B) and compared with unirradiated (U) controls.

DR2518 was earlier shown as a PQQ inducible protein kinase having a role in radiation resistance in *Deinococcus*. The effect of γ radiation induced DNA damage on expression and activity of DR2518 protein kinase was checked. Results showed that both synthesis and activity of this kinase are regulated by γ radiation induced changes in the cells, which indicated that this kinase could be a typical candidate of an alternate DNA damage response mechanism in this bacterium. The ability of this kinase phosphorylating several important DNA repair proteins of *Deinococcus* and regulating the differential expression of genes in response to γ radiation induced DNA damage (Fig. 7) strengthened its role in radioresistance. The results obtained from this particular study suggested that unlike other bacteria that confer SOS response, *Deinococcus* might have an alternate mechanism of DNA damage response and DR2518 kinase seems to be a candidate protein kinase playing crucial role in this process. Recombinant DR2518 has been characterized as a radiation and quinone responsive protein kinase and named as RqkA [22]. Further we showed it contribution in radioresistance of this bacterium through phosphorylation of important DNA repair proteins like PprA and RecA and those involved in bacterial cell division. Phosphorylation sites in PprA and RecA of *D. radiodurans* were mapped mass spectrometrically and confirmed by site directed mutagenesis. Both in vivo and in vitro phosphorylation of PprA and RecA by RqkA kinase have been demonstrated. Importance of PprA and RecA phosphorylation in the regulation of these proteins functions in vivo and their respective roles in radioresistance have been demonstrated [23]. For instance, the phosphorylation of PprA by RqkA kinase had increased the DNA binding activity of PprA and its ability to stimulate intermolecular ligation activity of T4 DNA ligase was stimulated by ~ 6 fold. Similarly, RecA phosphorylation by RqkA kinase showed enhanced strand exchange.
reaction activity of RecA in the presence of dATP as compared to ATP and non-phosphorylated RecA in vitro (Fig. 8). The phosphorylation also affected RecA roles in radioresistance in *D. radiodurans*.

![Diagram](image)

Downregulated- 113 genes by >1.5 fold

Upregulated- 41 genes by >1.5 fold

Fig. 7: Involvement of a radiation responsive serine/threonine protein kinase (STPK) in regulation of gene expression in *D. radiodurans*. *Deinococcus* cells devoid of STPK were exposed with 6.5kGy radiation and global change in gene expression was measured by microarray analysis (Microarrayer) in collaboration with Prof. Yeujin Hua, Zhejiang University, Hangzhou China. Different numbers of genes showing reduced expression (downregulated) and increased expression (upregulated) by 15 fold or more were taken into consideration for making conclusions.

![Diagram](image)

Fig 8. Effect of phosphorylation on strand exchange reaction of RecA from *D. radiodurans*. Purified recombinant RecA was phosphorylated by RqkA kinase and assayed for strand exchange activity in the presence of ATP and dATP, respectively.
Multipartite genome system and cell division regulation in *D. radiodurans*

Apart from the extreme radioresistance, the cytogenetic features of *Deinococcus* are equally interesting. It has multipartite genome system and ploidy. All genome elements are held together producing a toroidal ring shaped nucleoid structure. Genome maintenance and cell division regulation in cells recovering from gamma radiation effects would be worth investigating. Here, we demonstrated that chromosome I encodes proteins that regulate its partitioning by following the Type IB mechanism (Pulling mechanism) of genome segregation [24].

For the first time, we identified centromeric sequences in chromosome I and showed that ParB1 binds to these centromeric elements both in *vivo* and in *vitro*. ParA1 is a non-specific DNA binding protein, it showed oscillation on nucleoid of the bacterium from one pole to other, and once ParA1 encountered the ParB1 bound to centromere, the depolymerization of ParA1 is triggered leading to the pulling of two daughter chromosomes in opposite direction followed by cytokinesis.

In order to ascertain the involvement of ParB proteins of *D. radiodurans* in genome maintenance and in the radioresistance, the genes encoding ParBs on different genome elements were deleted. These deletion mutants were checked for various stressors response and anucleation phenotype. Interestingly, it is observed that parB1 deletion produces the high frequency of anucleate cells, growth retardation and sensitivity to nalidixic acid. It showed a little less effect on gamma radiation resistance as compared to parB2 and parB3 deletion mutants.

The parB2 deletion also showed effect on normal growth while parB3 mutant grew similar to wild type. Both parB2 and parB3 mutants showed the high frequency of anucleate cells and higher sensitivity to DNA damaging agents but not to nalidixic acid. Thus we provided evidence that the ‘ParB’ proteins encoded on different genome elements are not functionally redundant and these are specifically responsible for maintenance of respective genome elements during cell division. Further, the chromosome II and megaplasmid seem encoding complements responsible for gamma radiation resistance of this bacterium.

Further in order to understand the effect of gamma radiation cell growth, the molecular mechanisms underlying cell division regulation is investigated. FtsZ, a protein centre to the entire bacterial cell division complex was characterized both *in vitro* and *in vivo*. This protein is found to be different from its homologues known in other bacteria in terms of its kinetics, rate of polymerization/ depolymerization.

These characteristics determine the growth rate, and the requirements of other divisome components for a productive cellular localization and FtsZ ring formation in *Deinococcus* [25]. FtsZ-GFP expressing in *Deinococcus* produced typical FtsZ ring perpendicular to first plane of cell division (Fig.9).
Fig. 9. In vivo localization of FtsZ in the dividing cells of *D. radiodurans*. FtsZ-GFP was expressed in *D. radiodurans* on plasmid and the localization of FtsZ tagged with GFP was carried out using confocal fluorescence microscope. FtsZ ring was constructed using Z stacks of images generated in three dimensional planes.

Molecular mechanism underlying extraordinary radioresistance of *Deinococcus* is being progressively unfurled. Several laboratories globally, working on various aspects leading to a single question on how this bacterium confers unthinkable and unbelievable tolerance to radiation effect. Our work from Bhabha Atomic Research Centre has contributed significantly to the global efforts on this aspect. Some of our notable contributions that are being used for explaining and in the discussion of the work from other laboratories are (i) the RecF recombination pathway in absence of RecBC enzymes, contributes to efficient DSB repair and radiation resistance (ii) an antioxidant metabolite PQQ has a role in both oxidative stress tolerance and DSB repair, (iii) the multiple proteins having greater significance in DSB repair and radiation resistance, are present and function together in a multiprotein complex, (iv) the bacteria do not confer classical SOS response but have eukaryotic type serine/threonine protein kinase might have a different DNA damage response mechanism as an alternate to SOS response and (vi) the involvement of a eukaryotic type Ser/Thr protein kinase in DSB repair and radiation resistance was reported first time in any bacteria.

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