COPING UP WITH HIGH RADIATION STRESS: MICROBES *NOSTOC* SP. STRAIN PCC 7120 AND *DEINOCOCCUS RADIODURANS* SHOW THE WAY

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Abstract:

The ability to efficiently and correctly repair the damage in DNA caused by exposure to γ-radiation governs the proficiency of a microbe to withstand high doses of radiation. Through the intricacies of the network of different DNA repair proteins, their interactions and regulation, the cyanobacterium *Nostoc* PCC 7120 and the polyextremophile *Deinococcus radiodurans* have shown the bacterial world how to survive exposure to high radiations. This chapter also provides an overview/glimpse on the work carried out in Molecular Biology Division, Bio Science Group towards deciphering the key DNA repair mechanisms in *Deinococcus radiodurans* and *Nostoc* PCC 7120.

Introduction:

Microbes exhibit varied response upon exposure to γ-radiation in terms of their ability to tolerate it, with the LD_{50} ranging from 0.1 kGy to over 10 kGy. Exposure to radiation can cause extensive damage to the membranes, proteins and DNA, all of which need to be mitigated. For the sake of brevity, in this chapter we are focussing on important DNA repair mechanisms and regulation of key components/candidates. The major known pathways of DNA Double Strand Break (DSB) repair employed in bacteria are briefly described here. **(1) Homologous Recombination** (HR) requires large stretches of homologous regions, is an error-free mechanism mediated by either the RecBCD or the RecF pathway[1]. **(2) Non-**

Homologous End Joining (NHEJ) pathway, a fast, but error-prone mechanism, which is independent of the requirement of homologous regions, and is mediated by Ku and LigD proteins[2]. **(3) Microhomology-mediated End Joining** (MMEJ) pathway requires short (5-25 bp) homologous sequence stretches and is independent of Ku proteins[3]. **(4)** Strand annealing pathways such as (a) **Single Strand Annealing** (SSA) which repair DSBs in direct repeat regions and is mediated by SbcD and independent of RecA and (b) **Extended Synthesis Dependent Strand Annealing** (ESDSA) has been reported to contribute significantly to radioresistance in *Deinococcus*[4]. **(5) Break Induced Replication** (BIR) pathway mediated by Primases and other DNA replication and repair proteins is activated when DSBs occur at replication fork[5].

When one talks about radioresistant microbes, the first name that crops up in the mind is that of *Deinococcus*, wherein this phenomenon has been extensively studied. Studies in the past two decades also identified few species of cyanobacteria, which are the oldest inhabitants on Earth, such as *Chroococdiopsis* and *Nostoc* (*Anabaena*) sp. which are equally radioresistant and exhibit similar ability to repair their damaged DNA. However, the knowledge on their radioresistance mechanisms and DNA repair proteins is relatively unexplored in cyanobacteria. This chapter will take you through the nuances of radioresistance mechanisms, DNA repair genes and their regulation in *Nostoc* and *Deinococcus*. The extent of DNA damage upon exposure to ionising radiations is comparable across microbes, but it is their ability to rectify them with minimal loss in information, which is the key to radioresistance.

1. Negotiating the harsh environment on Earth by the ancient cyanobacterium, *Nostoc:*

Cyanobacteria, as they are known today, were earlier classified as Blue Green Algae (BGA) due to abundance of blue (phycocyanin) and green (chlorophyll *a*) pigments[6]. Cyanobacteria are considered the oldest organisms as their roots can be traced back to nearly 3 billion years ago on this earth[7]. Due to their ability to perform oxygenic photosynthesis, they have been considered to have contributed to the oxygenation of the environment during Precambian period[8]. In addition to being photosynthetic, some of them are also capable of fixing atmospheric nitrogen (N_2) into ammonia (NH_3) with the help of nitrogenase enzyme complex[9]. Having inhabited the earth since time immemorial, they have been able to adapt to several extreme environmental stresses and thus are present in many ecological niches like soil, air, hot spring and aquatic systems.

1.1 Response of cyanobacteria to radiation:

Some of the cyanobacteria exhibit high tolerance to γ-radiation and desiccation[10-13], which has been attributed to genome redundancy, morphological modifications, and the possibility of robust DNA repair mechanisms. Of the various cyanobacteria, studies on radiation resistance and DNA repair proteins have been mainly carried out in the filamentous nitrogen-fixing cyanobacterium, *Nostoc* (*Anabaena*) sp. PCC 7120 (hereafter referred to as *Nostoc* 7120). It exhibits high γ-radiation resistance with ~50% survival upon exposure to 6 kGy and ~10% survival after 12 kGy[12]. When subjected to 6 kGy, *Nostoc* cells exhibited complete recovery within 7 days (**Fig. 1A**). At this dose, its genomic DNA was shattered into smaller

DNA (~few kilobases). However, when the cells were allowed to recover, the stitching back of DNA was observed within a day and subsequently the cells exhibited DNA profile similar to that of unstressed cultures by the end of the 3rd day of post-irradiation recovery (PIR)[12] (**Fig. 1B**). This indicated that *Nostoc*, which exhibits a doubling period of 18-24 h, is able to rectify its damaged DNA within 3-4 generations, suggestive of the presence of an efficient DNA repair system. Comparative proteomic analysis of unirradiated and irradiated *Nostoc* 7120 cultures during PIR indicated increased abundance of several proteins belonging to important functional categories like oxidative stress, C-metabolism, chaperones and proteases but none of the protein from DNA repair categories could be identified[14], unlike *Deinococcus* where abundance of DNA repair proteins was reportedly evident[15]. Recent studies on the DNA repair proteins of *Nostoc*, revealed that these proteins are expressed at very low levels in the cells even during the PIR phase, which could account for the inability to detect these proteins during proteomic analysis.

1.2 DNA repair proteins and probable mechanisms of DNA repair:

Use of bioinformatic analysis, primarily sequence/domain homology, helped in the identification of at least 38 DNA repair genes across cyanobacteria[16,17]. Among the prominent bacterial DNA repair proteins, the RecC, AddA, AddB and Ku proteins were absent while the RecB protein was truncated in *Nostoc* 7120, which rendered the RecBCD-mediated HR and NHEJ pathways non-functional in this organism[17]. *Nostoc* 7120 exhibited certain intriguing features such as, presence of multiple DNA repair genes encoding for proteins with similar structural and functional domains, e.g., three genes for Single Stranded DNA binding (SSB) proteins, three for RecQ helicases, and two for RecJ endonuclease[17]. SSB along with RecA is considered to be central to any DNA repair pathway. Of the three SSB proteins in *Nostoc*, two were truncated and harboured only the N-terminal region. Of them, SSB1 (Alr0088) was found to be capable of binding short stretches of single-stranded DNA, while SSB2 (Alr7579) bound ssDNA regions of DNA with secondary structures. Enhancing the levels of SSB1 decreased the radiotolerance of *Nostoc* suggesting interference in the DNA repair process[18,19]. The 3rd SSB protein (All4779) is a full length SSB having all the domain characteristics of bacterial SSBs, exhibited dual mode of ssDNA binding and enhanced the radioresistance of *Nostoc* when overexpressed[19]. It has been speculated that the truncated SSBs (also present across cyanobacteria) could be involved in DNA replication rather than DNA repair and might be a precursor to the PriA/PriB, which evolved as a DNA replication protein in modern day bacteria. The RecA protein was found be expressed at very low levels in *Nostoc* and was not found to be involved in the LexA-mediated regulation of DNA repair genes[20], typically observed in other bacteria. Enhanced expression of RecA had a negative impact on the radioresistance of *Nostoc*.

Nostoc 7120 and several species of cyanobacteria lack the canonical *recB, recC*, *sbcA* and *sbcB* genes, but possess *sbcC* and *sbcD* genes. However, unlike other bacteria wherein SbcCD is fully functional only as a complex, the SbcC and SbcD of *Nostoc* could independently and individually contribute towards its radioresistance[21]. These proteins have been speculated to be involved in SSA and MMEJ pathways of DNA repair in *Nostoc*[17] (**Fig. 1C**). The other DNA repair proteins of *Nostoc* which are being currently explored include RecN, RecF, RecO, RecR and RecQ proteins. *Nostoc* RecN exhibited DNA binding activity

and was found to be associated with DNA when the cells were exposed to DNA damaging agents[22] suggesting its probable involvement in binding to SSBs/DSBs[17] (Single Strand Breaks / Double Strand Breaks) as has been demonstrated for *Deinococcus*[23]. Preliminary investigations have shown that *recF*, *recO* and *recR* genes are expressed and functional in *Nostoc*[24] and exhibit multi-faceted regulation[25] as discussed in the section below. Among the helicases, which form an essential component of any DNA repair pathway, bacteria are known to possess only one or at the most two RecQ proteins unlike eukaryotes, which have multiple RecQ helicases[26,27]. Cyanobacteria defy this logic and several species possess two RecQ helicases, with a few including *Nostoc* 7120 possessing three RecQ helicases. In case of *Nostoc* 7120, the three RecQ helicases differ in their domains in the C-terminal region and were also found to be differentially expressed in response to DNA damaging stresses, suggesting probably distinct roles in DNA repair/replication/recombination processes[28], which is being currently explored.

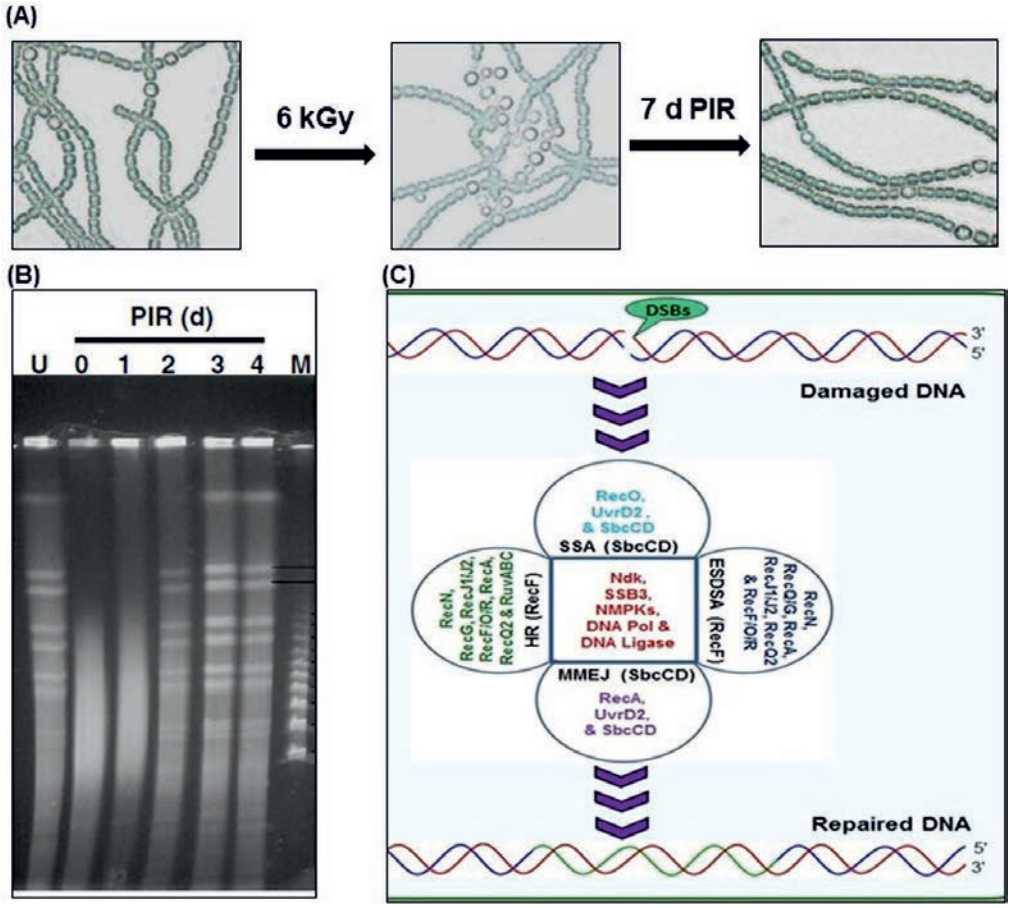

Fig. 1. Story of *Nostoc* 7120: DNA damage at 6 kGy and its repair during post-irradiation recovery (PIR)-Probable pathways of efficient DNA repair. (A) Micrograph of *Nostoc* 7120 upon exposure to 6 kGy of γ-radiation and after 7 days of PIR (B) Genomic DNA during PIR observed on Pulse Field Electrophoresis Gels, (C) Proposed pathways of DSB repair in *Nostoc* (Adapted from Singh et al 2013; Rajaram et al 2020)

Based on wet-lab experiments and *in silico* analysis of the various DNA repair proteins, it has been speculated that the RecF pathway of DNA repair either through HR or ESDSA could be the major DNA repair pathway in *Nostoc*[17] (**Fig. 1C**). Since, these are synthesis-dependent pathways, maintenance of dNTP pools would be essential for an efficient DNA repair process and hence, investigations were initiated into the proteins involved in dNTP synthesis. The only protein in *Nostoc* to have been characterised among these is TMK/TMPK (Thymidylate kinase), which is involved in the synthesis of (d)TDP. *Nostoc* TMK was shown to be structurally different from other known bacterial TMKs, in having larger pocket for TMP binding resulting in lower stability. It was also found to have an additional function in regulating photosynthesis through photoinhibition[29], which had not been reported earlier for any of the photosynthetic organisms or plants, where function of this protein has been studied. It is possible that these proteins had multiple functions in the ancient cyanobacteria and during the course of evolution, structural changes also resulted in certain functional changes restricting them to single/fewer specific activities.

1.3 What regulates the DNA repair genes in *Nostoc* :

Regulation of DNA repair genes in bacteria is synonymous with the SOS response regulator LexA, which has been extensively studied in *E. coli*[30]. Among cyanobacteria, the function of LexA protein was first explored in unicellular cyanobacterium *Synechocystis* PCC 6803, wherein it was shown to be involved in the regulation of Carbon-metabolism genes, but not the DNA repair genes[31]. This was attributed to the LexA being non-cleavable in *Synechocystis*[32]. However, in most other cyanobacteria LexA was found to have the cleavable form[32] as is known in *E. coli*. Studies in *Nostoc* 7120 revealed that the cleavage mechanism of LexA was pH-dependent but RecA-independent[20], rendering it different from the observed activated RecA-dependent proteolysis of LexA in *E. coli*[30]. However, the decreased radiotolerance of *Nostoc* upon overexpression of LexA[33], suggested the involvement of LexA in the regulation of DNA repair genes. Earlier studies had shown the negative regulation of two of the *ssb* genes[34], *sbcC* and *sbcD*[21]. Detailed study showed its involvement in the regulation of over 10 DNA repair genes, which was through the binding of LexA to AnLexA Box (AGT-Nx-ACT), present in the promoter region[31]. The AnLexA Box was distinct from the LexA Box of *E. coli* not only in the sequence *per se*, but also in the flexibility of the length of the spacer region between the left and right arms[31]. Further *in silico* analysis showed the presence of the AnLexA-Box upstream of several DNA repair genes of *Nostoc*[17], of which the negative regulation of *tmk*[29] and *recF, recO and recR*[25] was confirmed by *in vitro* and *in vivo* studies. However, unlike in *E. coli*, the regulatory role of LexA was not restricted to DNA repair genes, but covered a gamut of proteins involved in C-metabolism, metal tolerance, oxidative stress alleviation and photosynthesis[33,35]. The other regulators of DNA repair genes identified in *Nostoc* 7120 were (i) Fe^{2+}-FurA which negatively regulated the full length *ssb* gene (*all4779*), (ii) nitrogen-regulator NtcA which positively regulated the *recF*, *recO* and *recR* genes, (iii) heptameric DNA repeats upstream of *recF* and *recO* genes and (iv) rare initiation codon as observed for *recR* and non-canonical Shine Dalgarno sequence upstream of these genes[25]. Thus, the DNA repair genes in *Nostoc* are tightly regulated both at transcriptional and post-transcriptional levels through a gamut of proteins and *cis*-acting elements to maintain the levels of the proteins as per physiological requirement.

2. The polyextremophile *Deinococcus*: How it deals with high doses of radiation:

Bacteria belonging to *Deinococcaceae* family are some of the most radiation-resistant organisms discovered. The most studied species of this family is *Deinococcus radiodurans*, a red-pigmented, vegetative, non-sporulating, non-pathogenic, gram positive and easily culturable bacterium[36]. *Deinococcus radiodurans* strain R1 was the first of the deino-bacteria to be discovered and was isolated in Oregon in 1956 from irradiated canned meat that had spoiled[37]. This bacterium is frequently found thriving in extreme habitats such as irradiated environments, and arid deserts[36]. So far, more than 60 species have been isolated from diverse environmental niches. One of the Deinococcal species, *Deinococcus mumbaiensis* was isolated from contaminated agar plate at Bhabha Atomic Research Centre (BARC), Mumbai, India in 2006[38]. *D. radiodurans* is a remarkable polyextremophile, often referred to as "Conan the Bacterium" due to its legendary toughness, has the ability to withstand extreme radiation, desiccation, many chemical mutagens and high levels of oxidative stress[39]. *Deinococcus* achieves this remarkable feat due to presence of an inherent robust and efficient DNA repair mechanism, enabling it to mend its genetic material even after extensive damage from ionizing radiation and other environmental insults[36,39]. *D. radiodurans* is about 30-fold higher resistant to gamma radiation than common bacteria like *E. coli* and 1000-fold more resistant than humans[39]. *D. radiodurans* can tolerate 5 kGy of radiation without any detectable mutation, and can also grow in continuous radiation exposure of 60 Gy/hr[40]. During stress conditions *D. radiodurans* protects its DNA repair proteins from oxidative damage through the non-enzymatic Mn^{2+} complex, other anti-oxidants like deinoxanthin and enzymes like SOD, catalases, thereby enabling the shredded DNA to get repaired within 2-4 h of radiation exposure[41]. Its unparalleled resilience has made it a subject of extensive scientific research, offering valuable insights into the limits of life's adaptability to extreme conditions and its potential applications in biotechnology and environmental remediation.

2.1 Efficient DNA repair in *D. radiodurans*: Role of different proteins:

D. radiodurans has learnt to cope with extensive DNA damage by evolving several specialized DNA repair proteins and mechanisms, with the observed genetic redundancy also contributing to it[39]. This is an extensively explored field across the world leading to the identification of newer functions to DNA repair proteins and new proteins as well as mechanisms, and is ever unfolding to reveal more on the mystery of its high radioresistance. The key radiation responsive proteins mapped during post-irradiation recovery belonged to the functional categories of DNA repair (SSB, DdrA, DdrB, RecA and PprA), oxidative stress alleviation (SodA, KatA) and protein synthesis/transport (DnaK, GroEL and Ef-Tu)[15]. The SSB protein was twice the size of the bacterial SSB proteins with twice the number of each domain. Of the two OB-fold domains, the one at N-terminus was shown to be involved in dimerization and that at C-terminus in ssDNA binding[42]. DdrA protects 3′ DNA ends generated immediately after exposure to ionizing radiation and other genotoxic stresses, and is essential for radioresistance [43]. Recent studies revealed that DR0041 (a Rad52 family protein) also bound to various forms of DNA including broken ends and protected it from nucleases[44]. *D. radiodurans* lacks the RecBCD repair pathway as *recB* and *recC* genes are absent and employs the alternate RecFOR pathway for double strand DNA break (DSB)

repair through ESDSA pathway[45]. The DNA repair proteins, RecA, RecF, RecO and RecR have been shown to play important role in DSB repair pathway in *Deinococcus*[46]. RecJ nuclease plays an important role in broken DNA end processing, and its deletion made this organism radiation sensitive[47]. The RecD and RecQ helicases seem to have redundant helicase activities since only their simultaneous gene deletions affect radiation resistance, but not that of the individual genes[45]. In addition to these proteins, other DNA repair proteins such as UvrA, UvrB, of NER pathway, glycosylases of BER pathway, methyl transferases of direct damage reversal have also been shown to contribute to the radioresistance of *D. radiodurans*[39]. It is not the individual proteins *per se*, but the protein interactome which plays a significant role in conferring radioresistance to *Deinococcus*[48]. *D. radiodurans* lacked the error-prone translesion DNA polymerase, which could be responsible for the observed error-free repair of the massive DNA damage it undergoes upon exposure to radiation[49].

2.2 Radiation responsiveness in *Deinococcus*: Multiple regulatory mechanisms:

The levels of DNA repair proteins need to be tightly regulated during the entire repair process to prevent any kind of unwanted protein-protein interactions so as to maintain the high efficiency and fidelity of the repair process. Both SOS-dependent and SOS-independent regulatory mechanisms are present in *Deinococcus*, but it is the SOS-independent IrrE-DdrO-dependent mechanism (**Fig. 2A**), which is essential for efficient regulation of DNA repair proteins[49]. Additionally, role of DNA secondary structures such G-quadruplexes (G4 motifs) in the promoter regions of certain DNA repair genes (**Fig. 2B**) and serine-threonine protein kinases involved in the phosphorylation of few DNA repair proteins (**Fig. 2C**) also contribute towards the regulation of radioresistance of *Deinococcus*.

RDRM/DdrO/PprI regulatory pathway: Radiation/Desiccation Response (RDR) regulon of *D. radiodurans* consists of several DNA repair genes with a palindrome like sequence called radiation desiccation response motif (RDRM) in their promoter sequences[50-52]. RDR regulon is operated through cis-acting sequence RDRM [53], and a unique system well-conserved across *Deinococcus* sp., comprised of trans-acting repressor DdrO and protease IrrE (also called PprI)[52]. Cleavage of the DdrO repressor protein by metalloprotease PprI under DNA damage[54], dislodges it from the RDRM sequence allowing for the transcription of the DNA repair genes under this regulon. The PprI protein is a constitutively expressed protein but its protease activity is activated only under DNA damaging conditions through an unknown pathway. It was hypothesised that release of Zn^{2+} from the Zn-peptidase like domain, which was shown for PprI of *D. deserti*[55], or the high Mn^{2+} accumulated during radiation stress could be responsible for activation of the PprI protease[56]. However, our recent study showed that any kind of structural distortion in the DNA induces the PprI proteolytic activity, while no increase in activity was observed in the presence of $ZnCl_2$ and $MnCl_2$[50]. Thus, it is possible that the activation of PprI is triggered by changes in DNA structure, rather than the mere presence or absence of divalent cations. Unearthing of the exact mechanism may shed more light into the regulation of DNA repair genes in *Deinococcus*.

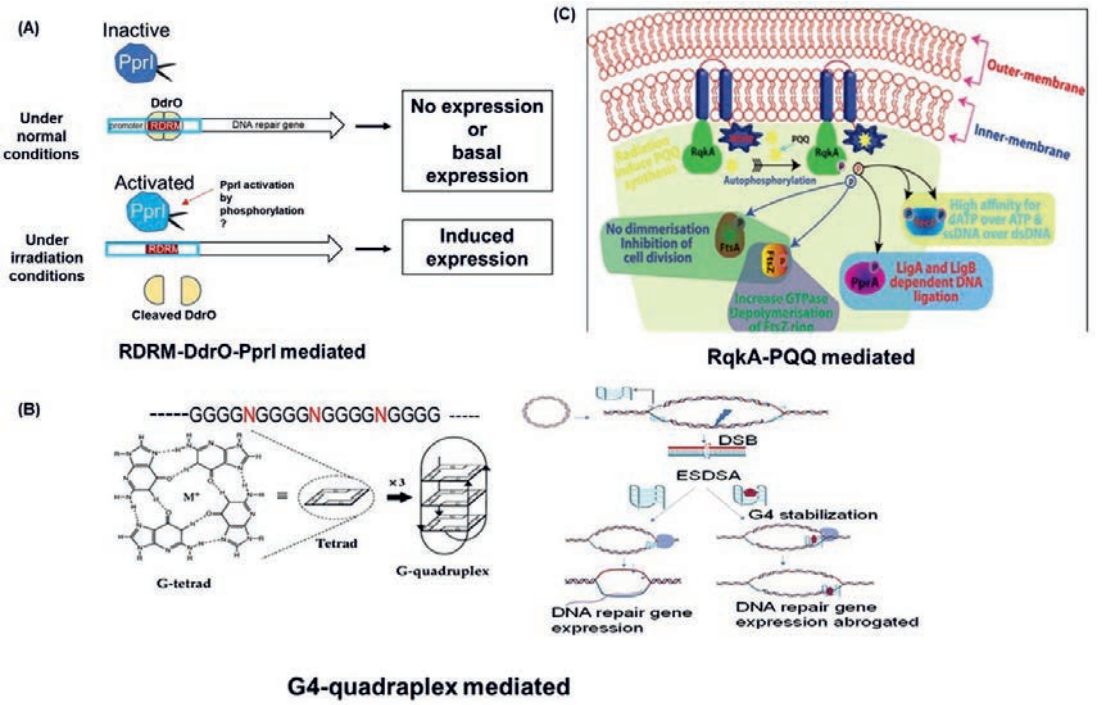

Fig. 2. Different modes of regulation of DNA repair genes in *Deinococcus radiodurans* (A) Ddr-PprI mediated, (B) G-quadraplexus (C) RqkA-PQQ mediated

Dynamics of guanine quadruplex structures: G-quadruplex (G4) structures are stable secondary structures in DNA and RNA formed by four guanine base pairing through Hoogsteen hydrogen bonding resulting in a stacked structure[57]. Depending on the directionality of strand folding the G4 motifs may be parallel, anti-parallel or mixed structures. Due to high GC content (66.6%) several putative G4 motifs are present across the *Deinococcal* genome[57], which are spread across different regions such as promoters, intergenic regions etc. In *D. radiodurans*, the G-quadruplex present in DNA repair gene promoters supress the gene expression under normal growth conditions, upon radiation stress these structures get relaxed allowing the RNA Pol and transcription factors to bind the promoter thereby triggering gene expression. This was also proven with the use of G-quadruplex stabilizing ligands like N-methyl mesoporphyrin IX (NMM)[58]. Using transcriptomics and promoter assay-based analysis, it was shown that G-quadruplex dynamics not only regulates several DNA repair genes such as *recA*, *recQ*, *recF*, *mutL* but also other genes involved in DNA metabolism, DNA synthesis (PolA), transcription[58]. One of the DNA repair proteins, DrRecQ was shown to play a role in G4 dynamics[58,59]. Thus, DNA secondary structures such as G4 motifs also play an important role in regulation of genome functions and radioresistance in *D. radiodurans.*

Serine-Threonine Protein Kinase (STPK)-mediated regulation: Serine-threonine protein kinases (STPKs), extensively studied in eukaryotes, function as reversible molecular switches in the regulation of various cellular responses to DNA damage[60]. Though not extensively investigated in bacteria, presence of STPKs fused with the diverse regulatory domains have been identified indicating a crucial role for STPKS in post-translational regulation even in bacterial physiology[61]. *D. radiodurans* harbours one of STPK family protein RqkA (Radiation quino-kinase A), which along with its inducer and a known antioxidant pyrroloquinoline quinone (PQQ) facilitates DSB repair[62,63]. Synthesis of both PQQ and RqkA has been shown to be inducible by gamma radiation, with PQQ playing a vital role in scavenging Reactive Oxygen Species (ROS)[64]. Through deletion and complementation analysis, it was shown that the C-terminal PQQ-interacting domain of RqkA protein is crucial to the radioresistance of *D. radiodurans*[65]. RqkA phosphorylates key DNA repair proteins, such as PprA and RecA[66]. These collective findings strongly suggest that PQQ may function as a sensor of oxidative stress and potentially link to the radiation stress-induced activation of Serine-Threonine phosphorylation in *D. radiodurans.*

Conclusion:

Mechanistic insights into the high radioresistance of the cyanobacterium *Nostoc* and the polyextremophile *Deinococcus* revealed exemplary abilities to repair damaged DNA within 2-4 h in case of *Deinococcus* and 2-3 days for *Nostoc*. The time taken corresponds to two-three doublings under normal growth conditions as the doubling time for *Deinococcus* and *Nostoc* is around 1.5 h and 20 h, respectively. Certain features with respect to the DNA repair proteins were found to be similar in both the organisms, which included the absence of RecB, RecC, SbcA, SbcB, as a result of which the RecF pathway is expected to be predominant in both the organisms. They also showed genome redundancy in having multiple genes encoding similar protein functions, which could be a contributary factor to the observed radioresistance. The regulation of the DNA repair proteins was also found to be multi-faceted in both the organisms, though the regulatory mechanisms involved were different. This allows for a tight regulation of expression of the DNA repair proteins ensuring fruitful interactions for an efficient DNA repair. Being the most ancient bacterium, multiple DNA repair proteins and pathways would have been generated in *Nostoc* to enable it to counter the harsh environment, and these evolved into specific functions and pathways in modern day bacteria, with some such as *Deinococcus* retaining the high radioresistance and acquiring new features, while some other such as *E. coli* lost some of the features rendering them radiosensitive.

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