Characterization of selected radiation induced gene promoters in *Deinococcus radiodurans* R1

By

Narasimha Anaganti

LIFE01201104002

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2.	Dr. S.K Apte	Guide & Convener		23/11/16
3.	Dr.(Smt) Bhakti Basu	Co-Guide		23/11/16
4.	Prof. Anand K. Bachhawat	External Examiner		
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Place: Molecular Laboratories, BARC

Dr. Bhakti Basu
Co-guide

Dr. Shree Kumar Apte
Guide

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(Narasimha Anaganti)

List of publications

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Proximity of Radiation Desiccation Response Motif (RDRM) to the core promoter is essential for gamma radiation induced gene expression in *Deinococcus radiodurans*, **Anaganti Narasimha**, Bhakti Basu, Rita Mukhopadhyaya and S.K. Apte*. *(Communicated)*

Papers presented in symposia/conference/seminars

1. **Ananganti Narasimha**, Bhakti Basu and S. K. Apte. "The construction of gfp based promoter probe shuttle vector pKG for *Deinococcus radiodurans* R1", 79th Annual Meeting of the Society of Biological Chemists (India) held at IISc Bangalore, during 13 - 15th Dec, 2010. Page: 264 - 265.

2. **Ananganti Narasimha**, Bhakti Basu and Shree Kumar Apte, "Radiation and Desiccation Response Motif mediates radiation induced gene expression in *D. radiodurans"*, in Life Science Symposium during 3rd-5th Feb 2015. **(Received Best poster Award)**

3. **Anaganti Narasimha**, Bhakti Basu and Shree Kumar Apte"Radiation responsive gene promoters in *Deinococcus radiodurans* are regulated by radiation and desiccation response motif (RDRM)", International Conference on Bacterial Expression-II held at NCBS Bengalore, India, during 1-5th Dec 2015, P.42.

4. **Anaganti Narasimha**, Bhakti Basu and Shree Kumar Apte "Radiation responsive promoters of *Deinococcus radiodurans*", International Conference on Radiation Research: Impact on Human Health and Environment (ICRR-HHE 2016), held at Anushaktinagar, Mumbai, India, during Feb 11-13th 2016.

Gene bank submission

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Dedicated to my father
Late Shri Anaganti
Eadaiah

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Narasimha Anaganti,

MBD, BARC

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PH.D. PROGRAMME

1. **Name of the Student: Narasimha Anaganti**
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SYNOPSIS

Introduction

Deinococcus radiodurans strain R1 is a reddish-pink pigmented Gram positive, nonpathogenic bacterium capable of recovering from high exposures to ionizing and ultraviolet radiations, desiccation, and diverse genotoxic chemicals [1]. Both radiation and desiccation cause single and double strand breaks in the DNA. *D. radiodurans* can stitch back its shattered DNA without incorporating detectable mutations [2], due to a highly efficient DNA repair system. Single strand breaks (SSB), and base and nucleotide damage are repaired by excision repair systems. Double strand breaks (DSB) in *D. radiodurans* are repaired by an early phase of a RecA independent extended synthesis-dependent strand annealing (ESDSA) and non-homologous end joining (NHEJ), followed by RecA dependent homologous recombination (HR) [3] to complete the task. *D. radiodurans* encodes novel proteins PprA, PprM, DrRRA etc which have been shown to be essential for its radiation resistance. In addition, physical factors like genome condensation and toroid ring-like nucleoids also contribute to Deinococcal radiation resistance [4]. The accumulation of Mn^{2+} ions during post-irradiation recovery (PIR) protect proteins involved in repair of damaged DNA from oxidation in *D. radiodurans* [5].

Distinct radiation responsive gene expression has been demonstrated in *D. radiodurans* [6-8]. Many Deinococcal genes do not have typical *E. coli* like -10 (TATAAT) and -35 (TTGACA) consensus promoter sequences in their upstream region, though several of them do harbor *E. coli* -10 like AT rich motif upstream [9]. Genome wide search for regulatory motifs in *D. radiodurans* and *D. geothermalis* strains found a 17bp palindrome like sequence, termed radiation and desiccation response motif (RDRM), upstream of radiation-responsive genes [10]. However, among the several radiation inducible genes only 24 genes carry RDRM like sequences. Our laboratory was the first to provide experimental evidence that RDRM is indeed involved in radiation induction of Deinococcal *ssb* gene [11]**.** Recent work in other laboratories, carried out in parallel to this work, shows that RDRM is also found in other deinococcal species, a repressor protein DdrO binds to it *in vitro* and the DdrO dimer is cleaved by the protease activity of PprI [12, 13]. However, the underlying regulatory mechanisms are not completely known. A study of Deinococcal radiation-induced promoters is crucial to understand structural features responsible for regulation of gene expression and for their biotechnological exploitation in high radiation environment. The present work was undertaken with this prime objective.

A promoter is a regulatory sequence of DNA located upstream of a gene (a *cis*-element) and provides a control point for regulated gene expression. Promoters control binding of RNA polymerase and transcription factors to DNA and are directly responsible for the amount of transcript generated [14]. These factors, which are responsible for activation/repression of gene expression, bind to the promoter sequences and recruit RNA polymerase which synthesizes RNA from the coding region of the gene. There are few reports on Deinococcal promoters in literature, for example PgroESL [15], PpprA [16] and Pssb [11]. To study the structure/function relationships in promoter activity a good shuttle vector, which can work in both *D. radiodurans* R1 and in *E. coli*, with a suitable reporter gene is essential. A few shuttle vectors available for *D. radiodurans* [17, 18] are large in size (6.3-17kb). A few of them have *lacZ* or *phoN* as reporter genes [15, 19]. However, these require expensive substrates and are not suitable for detection of *in situ* gene expression in individual cells. In comparison, the green fluorescent protein (GFP) encoding *gfp* gene [20] has been successfully used as robust reporter that allows visual (microscopic) and quantitative (Fluorescence spectrophotometric) assessment of gene expression. No GFP-based promoter probe vector is currently available for *D. radiodurans*. It was, therefore, proposed to construct a GFP-based promoter probe vector for obtaining cell-based information and to provide

in situ, real time quantitative assessment of radiation inducible and other promoters in *D. radiodurans*.

The present study was undertaken with the following objectives:

1. Design and construction of a new promoter probe shuttle vector, with the GFP gene (*gfp*) as a reporter, and its validation using known *D. radiodurans* promoters.
2. Selection of radiation responsive genes based on bioinformatics, microarray and proteomic data, for analysis of their promoters.
3. Cloning of selected promoters (up to 300bp upstream DNA sequences), containing/lacking RDRM motif, in promoter probe shuttle vector.
4. Assessment of promoter activity under ambient and radiation stress conditions by visualization and quantitation of reporter gene expression and activity.
5. Selective mutagenesis, swapping and reorientation of RDRM to elucidate its effect on corresponding gene expression.

The work carried out is presented in the thesis as following 6 chapters:

Chapter 1: General introduction.

Chapter 2: Materials and Methods.

Chapter 3: Construction of promoter probe shuttle vector and its validation.

Chapter 4: Cloning of selected putative *Deinococcal* promoters and assessment of their activity under normal and radiation stress conditions.

Chapter 5: Regulation of promoter activity under normal and radiation stress conditions in *D. radiodurans*.

Chapter 6: Summary and Conclusions

Chapter 1: General introduction

This chapter contains a general review on the extremely radiation resistant bacterium *Deinococcus radiodurans* strain R1 which includes its taxonomy, characteristics, genome structure and response to DNA damaging stresses, such as gamma and UV radiation, prolonged desiccation and chemical mutagens. Mechanisms adopted by this bacterium to repair DNA damage and survive are elaborated. An account of structure and function of different types of bacterial promoters and a brief description of regulatory mechanisms of gene expression in prokaryotes, including *D. radiodurans* is presented. The need for a suitable promoter probe shuttle vector to investigate *D. radiodurans* promoters is emphasized and the specific objectives of the study are specified.

Chapter 2: Materials and Methods

The chapter details various bacterial strains and plasmids used or constructed, chemicals, enzymes, various molecular biology kits, their sources and experimental techniques used in this study. Different microbiological techniques involved, including culture medium and growth conditions, protocol for irradiation, GFP fluorescence quantification and fluorescence microscopy of bacterial cells are described. Protocols for genomic DNA/RNA/plasmid isolation, gel extraction, DNA/RNA/protein quantitation, electrophoretic resolution of DNA, RNA or proteins, western blotting and immuno-detection of GFP protein are specified. Various molecular biology techniques including PCR amplification, purification of PCR product, cloning, generation of point mutations and deletion/addition mutations, swapping of RDRM sequence among the different promoters, and primer extension for transcription start site mapping (TSS) are elaborated. Methods for over-expression of PprI and DdrO proteins in *E. coli* and their purification, and construction of *pprI* knockout mutant of *D. radiodurans* are detailed. Bioinformatic tools used to find -10, -35 like consensus sequences or AT rich motifs, and ribosome binding sites (RBS) in *D. radiodurans* genes are described.

Chapter 3: Construction of promoter probe shuttle vector and its validation

No promoter probe shuttle vector that can provide *in situ* real time quantification of promoter activity currently exists for *D. radiodurans*. GFP is a sensitive, robust reporter whose activity can be quantitated without requiring cell lysis or a substrate. The *gfp-mut2* gene (hereafter called as *gfp* gene) when cloned in pRAD1 plasmid yielded strong GFP fluorescence in *E. coli* and weak fluorescence in *D. radiodurans*, even in the absence of any cloned promoter. This leaky expression of reporter may be due to a read-through from upstream *amp*^{r} gene and is undesirable for promoter analysis. Therefore, a leak proof, no background promoter probe shuttle vector pKG was constructed, which comprised of (i) kanamycin resistance *aph* gene with its promoter (derived from pUK4K plasmid), that expresses both in *E. coli* and *D. radiodruans*, (ii) a 44bp transcription terminator term116 from *D. radiodurans* [21], which was synthesized as two oligos, annealed and cloned upstream of *gfp* reporter gene, (iii) a new multiple cloning site (MCS) with eight unique restriction sites for cloning of desired promoter sequences, which was derived by annealing of two synthetic primers (iv) the *gfp* gene derived from pAM1956 vector [22] encoding the GFP protein as a qualitative/quantitative reporter for *in situ* real time assessment of promoter activity, and (v) *E. coli* and *D. radiodurans* origins of replication, low GC region and *repU* gene encoding putative Deinococcal replication protein (all derived from pRAD1), for maintenance of the plasmid in both the strains. The vector pKGX, thus generated, still displayed leaky GFP fluorescence in *E. coli*. A 386 bp DNA fragment present in pKGX was suspected to contribute promoter activity, probably since the Term116 did not function in *E. coli.* Bioinformatic analysis of the 386 bp DNA sequence indeed revealed presence of an *E. coli* like promoter sequence (TATGTT at -10, TTTACA at -35). Therefore, the 386bp DNA fragment immediately upstream of Term 116 and downstream to pUC19 origin of replication, and a 305bp DNA fragment downstream to *aph* gene in pKGX, were deleted in the final construct pKG. Deletion of the aforesaid sequences resulted in complete abolition of GFP expression from pKG in both *E. coli* and *D. radiodurans* hosts, in the absence of a cloned promoter sequence. The vector pKG is, thus, a promising zero background vector to study Deinococcal promoters both in *E. coli* and *D. radiodurans* strains. The pKG vector was completely sequenced and the sequence was deposited in public database (GenBank^{TM} Accession number: KF975402**)**.

The functionality of pKG vector was validated with two known *D. radiodurans* promoters, namely P*groESL* (P0606) - a strong non-RDRM promoter [19] and P*ssb* (P0099) - a RDRM-based radiation inducible promoter [11]. From P*groESL* promoter the GFP was expressed strongly in both *E. coli* and *D. radiodurans* and no radiation induction of its expression was observed in *D. radiodurans*. The Pssb promoter showed basal GFP expression in both organisms and displayed six fold induction of expression in *D. radiodurans* during PIR, as was also reported earlier [11]. These data confirmed the utility of pKG vector for promoter screening in *E.* coli and *D. radiodurans*. The vector pKG adds a new tool to the currently existing battery of few Deinococcal vectors by offering sensitive, qualitative as well as quantitative determination of real time promoter activity *in situ*, without the need for cell lysis or expensive reagents.

Chapter 4: Cloning of selected putative Deinococcal promoters and assessment of their activity under normal and radiation stress conditions.

Based on published transcriptome, proteomic data and bioinformatics analyses, 20 *D. radiodurans* genes were selected for promoter analysis. The selected genes were divided into two groups (i) promoters having RDRM, and (ii) promoters without RDRM. About 300-500bp DNA sequences upstream of these genes were PCR amplified using specific primers designed based on available *D. radiodurans* chromosomal genome sequence (http://www.genome.jp/dbget-bin/www_bget?dra). The PCR amplified fragments were cloned in pKG promoter probe shuttle vector upstream of *gfp* reporter gene, to obtain promoter-reporter translational fusions in which Shine-Dalgarno sequence (SD) was derived from individual promoter fragments. *E. coli* (DH5α) and *D. radiodurans* strains harboring pKG vector with different promoters were assessed for GFP expression based promoter activity. The GFP fluorescence intensity was a reflection of promoter activity and the corresponding SD sequence. The promoter sequences of P_{0099}, P_{0219}, P_{0423}, P_{0596}, P_{0606}, P_{0906}, P_{1143}, P_{1913} and P_{2338}, which have *E. coli* σ^{70} like consensus promoter sequences, showed bright green fluorescence in *E. coli*, while the promoters of P_{0053}, P_{0070}, P_{0694}, P_{1262}, P_{1314}, P_{1720}, P_{2220} and P_{2275}, which do not have *E. coli* like promoter sequence, showed either no or very weak GFP fluorescence in *E. coli*, irrespective of the presence/absence of RDRM. All promoters carrying RDRM motif showed very weak/no GFP fluorescence in *D. radiodurans* while all the non-RDRM promoters showed a strong fluorescence, irrespective of presence/absence of

conserved *E. coli* like promoter sequences. Some of the Deinococcal gene promoters (P_{0070}, P_{0596}, P_{1143} and P_{1314}) harbor SD sequences downstream of annotated start codon of ORFs. GFP fluorescence from these promoters was observed only when the SD sequence was included in the promoter sequence. Thus, gene expression in *E. coli* required -10, -35 consensus sequences but was not influenced by RDRM. The *E. coli* like promoter sequences were dispensable in *D. radiodurans* wherein RDRM negatively regulated gene expression

The recombinant *D. radiodurans* promoter clones were exposed to 6kGy gamma radiation and promoter activity was monitored as GFP fluorescence during post irradiation recovery (PIR). The promoters which exhibited greater than 2 fold enhancement of fluorescence after irradiation were considered as radiation induced. All promoters (P_{0070}, P_{0099}, P_{0219}, P_{0423}, P_{0596}, P_{0906}, P_{1143}, P_{1262}, P_{1913}, P_{2275} and P_{2338}) which harbored RDRM sequence exhibited radiation induction. Highest induction of 25 and 11 fold was observed for P_{DR0070} and P_{DR1143} respectively at 4h of PIR. The RDRM lacking promoters P_{0053}, P_{0606}, P_{0694}, P_{1314}, P_{1720} and P_{2220} showed no change in expression during PIR. These results clearly show that RDRM also plays a regulatory role in radiation stress induced gene expression. Although DR0053, DR0694, DR1314 and DR2220 were earlier reported to be induced at transcriptional level during PIR, their induction was not observed in the present study probably because of different dose and dose rate used. When *E. coli* cells carrying Deinococcal P_{DR0070}, P_{DR0906} and P_{DR1143} promoters were subjected to irradiation no induction was observed, indicating that *Deinococcus* specific factors are needed, both for repression under normal condition as well as radiation induction of promoters.

To further elucidate the role of RDRM in radiation stress, the RDRM from *ddrB* (P_{DR0070}), *gyrB* (P_{DR0906}), and P_{DR1143} (hypothetical) genes and *cinA* (P_{DR2338}) operon were deleted. The basal level of GFP expression was significantly enhanced in all RDRM deleted mutants (except P_{DR0070} in which TSS was disrupted upon deletion of RDRM), compared to wild type promoters with RDRM. This clearly established a negative repressive role for RDRM under ambient growth conditions. The RDRM deletion mutants of *D. radiodurans* when subjected to 6 kGy gamma irradiation showed no gamma radiation responsive induction in GFP expression. The result clearly demonstrated that RDRM plays a negative role under ambient growth conditions and is also essential for gamma radiation responsive enhancement in promoter activity during PIR.

Chapter 5: Mutagenesis, reorientation and swapping of RDRM and analysis of corresponding promoter activity under normal and radiation stress conditions in *D. radiodurans*.

To elucidate the role of RDRM in radiation induction of promoters, several mutants were constructed in two selected promoters, P_{DR0070} and P_{DR0906}. The promoter P_{DR0070} shows very weak basal expression under normal conditions but highest induction in PIR (25fold). Its transcription start site (TSS) was mapped within the 3' region of RDRM. The P_{DR0906} harbors -10, -35 like sequences positioned respectively at -11 and -34 bases upstream of its TSS. It shows high basal activity and 6 fold induction during PIR. A G at 5^{th} and C at 13^{th} positions are two highly conserved bases in RDRM. When these nucleotides were replaced by G5A and C13T bases in both $P_{DR0070,}$ P_{DR0906} promoters, the radiation inducibility of P_{DR0070} promoter came down to 15 fold from 25 fold in wild type whereas radiation induction was totally lost in P_{DR0906} promoter, suggesting that these two bases are very important for radiation induction. When five bases from 5'end of RDRM were deleted in both promoters, the basal level activity increased by 7 fold in P_{DR0070} but only slightly in case of P_{DR0906} promoter. If complete RDRM was replaced with equal length of non-RDRM random DNA sequence, the P_{DR0070} promoter showed neither the basal promoter activity nor any radiation induction due to disruption of its TSS which lies within the 3' end of the RDRM. Since the TSS is part of RDRM in P_{DR0070} promoter, no further mutations were made in this promoter. When five bases were deleted from the 3' end of RDRM in $P_{DR0906,}$ a 5-fold increase in basal expression was observed but only minor radiation induction was seen during PIR. The result suggests that the 5 bases from 3' end of RDRM are critical for the negative regulation as well as radiation induction from RDRM.

As mentioned earlier, the RDRM is a degenerate palindrome. Generally palindrome sequenceswork in both orientations, with dimeric regulator proteins. To assess this possibility, the 17bp RDRM was cloned in reverse orientation, keeping all the other P_{DR0906} promoter components intact. The basal activity was reduced by 4 fold, but it retained radiation induction property suggesting that in reverse orientation, RDRM is a stringent negative regulatory *cis*-element. To examine possible additive effect of RDRM on promoter activity, one more RDRM was cloned in P_{DR0906} at -179 in addition to the existing one at -257**.** As expected, it showed additive repression

of basal promoter activity and also retained its radiation induction. Deletion of RDRM from P_{DR0906} promoter increased basal promoter activity marginally but radiation induction was lost. Instead, if the whole upstream region including -10, -35 sequences and RDRM were deleted, basal promoter activity was reduced by 4-fold under normal growth conditions and no radiation induction was observed during PIR. The result indicates that for basal promoter activity of $P_{DR0906,}$ -10, -35 sequences are required. A recent study has shown that many of the transcripts harbor AT rich motifs upstream of TSS in *D. deserti* [9].

The promoters P_{DR0053} and P_{DR0694} of *D. radiodurans* do not harbor RDRM sequence and are not induced during PIR. To evaluate the ability of RDRM to (a) introduce negative regulation under normal conditions, and (b) enhanced expression following irradiation, the 17bp RDRM sequence from the highly radiation inducible promoter P_{DR0070} was introduced into P_{DR0053} at two locations, first at -14 bases from start codon (same location as in P_{DR0070}) and the second at -215 bp upstream of the start codon. In P_{DR0694} promoter, RDRM was introduced at 153 bp upstream of start codon. The basal level of promoter activity of P_{DR0053} and P_{DR0694} with addition of RDRM came down to near zero, as against its normal activity without RDRM in *D. radiodurans*. However, during PIR following 6kGy gamma irradiation, no radiation induction of these RDRM conferred promoters was observed. The results corroborate the role of RDRM as a negative regulator, irrespective of its position. Since the precise location of the promoter is not known, the lack of radiation induction upon RDRM swapping can be explained as follows: (a) introduction of RDRM does interrupt either the binding to or movement of RNA polymerase from P_{DR0053} promoter, or (b) possible involvement of additional sequence(s) for repressor release/degradation, leading to de-repression following irradiation, or (c) alternatively, the swapping may have destroyed the native promoter.

The PprI protein is a general switch responsible for activation of several genes during PIR [23]. A recent study revealed that PprI is a metalloprotease which cleaves DdrO repressor protein (which binds to RDRM *in vitro*), during PIR [13]. To assess the effect of PprI and DdrO proteins on the activity of Deinococcal promoters, the corresponding genes were cloned in pET21a expression vector and overexpressed in the *E. coli* strain [BL21(DE3)pLysS] by induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG). The pKG plasmids carrying promoters P_{DR0070}, $P_{DR0099,}$ $P_{DR0219,}$ $P_{DR0423,}$ $P_{DR0596,}$ $P_{DR0906,}$ $P_{DR1143,}$ $P_{DR1913,}$ P_{DR2275} and P_{DR2338}, which harbor RDRM,

or harbouring $P_{DR0606,}$ P_{DR0694}, which do not carry RDRM, were transformed into BL21 cells carrying plasmids pET21-*pprI* or pET21-*ddrO*. Successful coexistence of the two plasmids pET21a and pKG, having different antibiotics ampicillin and kanamycin markers respectively, was carefully ascertained in *E. coli* beforehand. The pET21a or pKG empty vectors served as controls.

Effect of overexpression of Deinococcal PprI or DdrO proteins on promoters with/without RDRM was evaluated in *E. coli*, which does not possess any of these regulatory proteins. No significant change in promoter activity was observed with PprI alone for any of the promoters or even for empty vector. However, overexpression of DdrO protein substantially reduced activity of all RDRM-based promoters in *E. coli*, while no effect was observed on non-RDRM promoters. The results suggest that binding of DdrO protein to RDRM represses promoter activity *in vivo*, in conformity with the earlier reports [13, 24]. To observe the effect of PprI protein on DdrO repression of RDRM promoters, RDRM containing ($P_{DR0070,}$ P_{DR0906} and P_{DR1143}) or non-RDRM (P_{DR0606}) promoters were transformed into *pprI* knockout mutant of *D. radiodurans*. As expected, no change in basal promoter activity was observed but the radiation induction was abolished. These data substantiate the model of radiation regulation proposed earlier based on *in vitro* studies [13], *and demonstrate it in vivo*.

Chapter. 6. Summary

A GFP-based promoter probe shuttle vector pKG was successfully constructed for *in situ* real time promoter analysis, both in *E. coli* and *D. radiodurans*. The functionality of pKG vector was validated with two known Deinococcal promoters, P_{DR0606} - a strong promoter and P_{DR0099} - a radiation inducible promoter. A total of 20 promoters were successfully cloned in pKG vector. Deinococcal promoters that were active in *E. coli* contained conserved -10 and -35 like sequences, but these were dispensable for gene expression in *D. radiodurans*. The RDRM containing promoters showed low basal GFP expression in *D. radiodurans*, compared to non- RDRM promoters, but gene expression in *E. coli* was indifferent to the presence/absence of RDRM. RDRM was necessary for radiation induction of all RDRM-based promoters, but RDRM repressed the promoter activity when it was swapped into non-RDRM promoters at different locations (with reference to the start codon) but did not induce promoter activity in PIR. Thus, RDRM is necessary

but may not be solely responsible for radiation induction. DdrO repressed the activity of RDRM containing promoters, but not of non RDRM promoters, in *E. coli*. PprI, which is known to cleave DdrO protein *in vitro,* was shown to be essential for radiation induction of RDRM promoters *in vivo,* in *D. radiodurans*.

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S. No.	Name	Designation	Signature	Date
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Chapter 1

Introduction

Deinococcus radiodurans strain R1 is a reddish-pink pigmented Gram positive, nonsporulating, nonmotile, nonpathogenic bacterium [25], capable of recovering from high exposures to ionizing and ultraviolet radiation, prolonged desiccation, and diverse genotoxic chemicals [1]. It is spherical in shape, with size ranging from 1.5 to 3.5 µm in diameter and occurs as diads or tetrads (Fig 1.1). Although it is a Gram positive bacterium it has a complex cell envelope similar to that of Gram negative organisms, comprising of a thick peptidoglycan layer, outer and inner membranes and two surface layers [26]. *D. radiodurans* is a mesophile with optimal growth temperature of 32^0C and is typically grown with aeration in TGY (1% tryptone, 0.1% glucose, 0.5% yeast extract) rich medium. Under optimal growth conditions, the cell doubling takes approximately 90-100 min and the microbe takes 48-72h after plating to appear as clear visible colonies on agar plate.

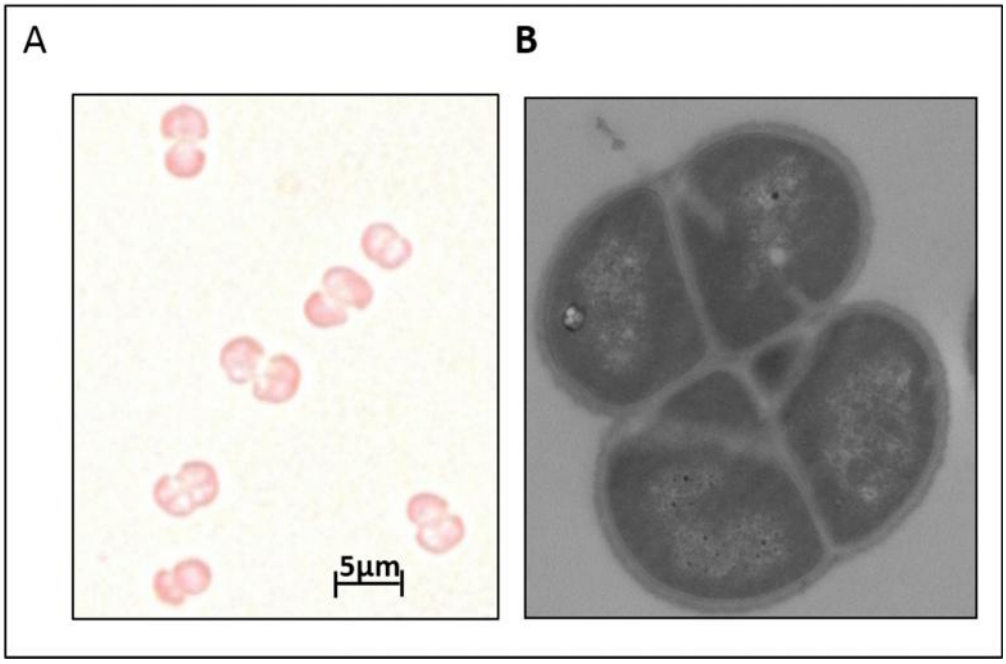

Fig. 1.1. Microphotographs of *D. radiodurans*. (A) Photographs taken with light microscope with 100X objective, and (B) transmission electron microscope (TEM) (magnification: 100K).

1.1 History, habitat and classification

D. radiodurans was first isolated from irradiated (4kGy) canned meat at Oregon, USA by Andersson *et al* in the year 1956 [27]. Its name derives from the Greek “deinos” meaning strange or unusual, and “coccus” meaning a grain or berry while “radiodurans” refers to its extreme radio resistance. Initially it was named as *Micrococcus radiodurans* based on its morphological and physiological characteristics [28]. Later chemotaxonomic studies and phylogenetic analysis of Deinococcal 5S and 16S rRNA analysis revealed that *Deinococcus* is not related to *Micrococcus* and forms a separate phylogenetic group of bacteria [29]. Subsequently, therefore, it was included in a new family of Deinococcaceae, and renamed as *Deinococcus radiodurans* [30] (Table 1.1). Around fifty *Deinococcus* species have been isolated to date, by radiation or desiccation survival based selection [31]. There is no defined habitat for this organism. The *Deinococcus* genus comprises of mesophilic, thermophilic, and psychrophilic representatives. This organism is distributed all over the world, with various species isolated form a wide variety of habitats, such as animal gut, deserts, hot springs, sewage, processed meat, alpine environments, and Antarctica [4, 25, 32].

Table 1.1 Scientific classification of *D. radiodurans*

Kingdom	Eubacteria
Phylum	Deinococcus- Thermus
Class	Deinococci
Order	Deinococcales
Family	*Deinococcaaceae*
Genus	*Deinococcus*
Species	*radiodurans*

All Deinococcus species are highly resistant to lethal effects of DNA-damaging agents, particularly those of ionizing radiation and UV radiation [4, 25] but the desiccated *D. radiodurans* is sensitive to high humidity [33].

1.2 Cell structure and cell division

Deinococcus radiodurans commonly exists as a unit of two cells (diads) or four cells (tetrads) (Fig.1.1). Although it is a Gram-positive bacterium it has an unusual composition of cell wall which has 150nm thickness [4] and comprises of at least six layers. It's envelop consists of plasma membrane and outer membrane with peptideglycan (14-20nm) layer and a compartmentalized layer sandwitched in between the two membranes [34]. The first innermost layer is plasma membrane, followed by a peptidoglycan containing cell wall (holey layer). The third layer is a compartmentalized layer and the fourth is the outer membrane, while the fifth is a distinct electrolucent layer and sixth is the S-layer consisting of regularly packed hexagonal protein subunits. The S-layer consists of carotenoids, lipids, proteins and polysaccharides [35, 36]. The holey layer is composed of a mucopeptide, muramic acid, and rarely occurring amino acid L-ornithine. Its membrane lipids are composed mainly of phosphoglycolipids which contain alkylamines as structural components unique to *D. radiodurans* [37]. Unsaturated fatty acids, which help in fluidity of membrane, play an important role of varying cell volume during desiccation [4]. Out of six layers of *D. radiodurans* only the cytoplasmic membrane and the peptidoglycan layer are involved in septum formation during cell division. Other layers act as sheath which surrounds groups of cells and forms on the surface of daughter cells as they separate [34, 35, 38]. The *D. radiodurans* cells divide in two planes, with septa which sweep across the cell from opposite sides [39].

1.3 Genome structure of *D. radiodurans*

The genome (3.28 Mbp) of naturally transformable *D. radiodurans* is smaller than the well-studied bacterium *E. coli* (4.6 Mbp). The *D. radiodurans* genome was completely sequenced in 1999 by

White *et al* [40]. It consists of (a) two chromosomes, chromosome-I with a size of 2.6 Mbp and chromosome-II of 0.41 Mbp, and (b) two plasmids, of which one is a mega plasmid of 0.17 Mbp while the other plasmid is 45 kbp in size. The base composition of its genome is 66% GC rich. The *D. radiodurans* genome contains a total of 3,187 annotated open reading frames (ORFs) with an average size of ~900 bp each [40]. More than 1000 ORFs have no matches in the database and more than 500 ORFs match with hypothetical genes. In total, nearly 50% of its genome has unknown functions. Exponentially growing cells of *D. radiodurans* contain 8-10 copies of the genome with an average DNA content of 3×10^7 bp [41]. Earlier it was considered that *D. radiodurans* genome is methylation deficient (DAM^- and DCM^-) [42] but later adenine and cytosine methyltransferase activites have been demonstrated [43] in this organism. *D. radiodurans* genome also has mobile genetic elements such as insertion sequences (IS) and small noncoding repeats (SNR) [4] and seems to have gained novel prokaryotic and eukaryotic genes by horizontal gene transfer [44].

1.4 Metabolism of *D. radiodurans*

D. radiodurans is an organotrophic bacterium with minimum requirements of a carbon source, a nitrogen source, a sulfur source, nicotinic acid, and a source of manganese (Mn) [4]. This proteolytic bacterium obtains its main carbon source from amino acids which it generates from protein hydrolysis [4, 45]. Energy derived from sugars is marginal and they are imported mainly *via* phoenolpyruvate phosphotransferase system [4, 40]. As a proteolytic bacterium *D. radiodurans* encodes several protein degradation and amino acid catabolism related enzymes which were mostly obtained from horizontal gene transfer [44]. Following radiation stress, intracellular proteolytic activity increases [46] which expedites utilization of proteins degraded by radiation stress and proteins derived from dead cells. During the initial stages of post irradiation recovery

(PIR) the microbe resynthesizes all the depleted protein [4]. Such *protein recycling* minimizes the biosynthetic demand and actively contributes to the antioxidant complexes of amino acids and peptides with manganese [46]. During post irradiation recovery several secreted subtilisin-like proteases and hemolysin encoding genes, peptide and amino acid transporters and ABC transporters are highly induced in expression [47] and help in recovery from stress. In *D. radiodurans*, carbohydrates are stored as granules. It has glycolysis, gluconeogenesis and Pentose phosphate pathway (PPP) to utilize glucose. Pentose phosphate pathway (PPP) is highly active in this organism with 4 fold higher glucose-6-phosphate dehydrogenase (G6PDH) activity compared to *E. coli*. G6PDH mutation resulted in UV, H_2O_2 and MMC sensitive phenotypes [48, 49]. The PPP pathway generates ribose-5-phosphate, NADPH and glyceroldehyde-3-phospahte which are precursors for dNTPs. NADPH replenishes antioxidants glutathione and thioredoxin which provides protection against ROS. Glucose depletion renders *D. radiodurans* susceptible to UV, ionizing radiation and MMC, all of which trigger ROS generation. *D. radiodursans* encodes fewer proteins with iron-sulfur cluster [4Fe-4S] and iron chelating and transport enzymes [47]. Upon degradation of these enzymes free Fe is released and generates free radicals through Fenton's reaction which exacerbates oxidative stress. To combat oxidative stress *D. radiodurans* possesses abundant ROS scavenging enzymatic and non-enzymatic mechanisms [46, 50, 51].

1.5 Tolerance of *Deinococcus* to DNA damage stress

D. radiodurans is well known for its extraordinary resistance to various DNA damaging agents (Fig. 1.2). Gamma radiation and desiccation cause double strand and single strand breaks in DNA, ultraviolet (UV) light generates pyrimidine dimers, mitomycin-C (MMC) forms DNA interstrand cross links (adducts), alkylating agents like methyl methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and oxidizing agents hydrogen peroxide, hydroxylamine and nitrous

acid alter nucleotides bases of DNA [4, 25, 52, 53]. *D. radiodurans* cultures can survive up to 15kGy γ-radiation and show a shoulder up to 5 kGy. The D_{37} dose for γ-radiation in *D. radiodurans* is ~6kGy whereas D_{37} of *E. coli* is just 30Gy [25]. *D. radiodurans* can also grow continuously under chronic low level radiation (60 Gy/h) stress [54]. *D. radiodurans* can also survive 1000 J/m2 of UV radiation. The D_{37} dose of UV radiation for *D. radiodurans* is ~600 J/m^2 as compared to ~30 J/m^2 in *E. coli* [25]. *D. radiodurans* can withstand 20 μg/ml MMC for 10 min and years of desiccation. *D. radiodurans* can survive 6 weeks desiccation at <5% relative humidity, with 85% viability which causes around 60 double strand breaks (DSBs) [55]. Radiation resistant and sensitive species have remarkably similar numbers of DSBs generated per Gy per genome (0.002 to 0.006 DSBs/Gy/Mbp) [4]. The amount of DSBs were found to be same in both *E. coli* and *D. radiodurans* cells when they were irradiated under identical conditions [56]. While *E. coli* lost viability at such doses *D. radiodurans* survived because it can proficiently repair its damaged DNA. In general *D. radiodurans* is 30 times more resistant to ionizing radiation than *E. coli* and 1,000 times more than humans [4]. *D. radiodurans* can reconstitute its genome from 1,000 to 2,000 DSB fragments compared to the maximum capability of *E. coli* of restoring its genome from 10 to 15 DSB fragments [34]. *E. coli* cannot survive even a dozen of DSBs caused by radiation [4, 57] however it can survive more than a dozen of DSBs caused by transient expression of EcoRI restriction endonuclease [58]. During meiotic phase of cell division yeast and human cells can repair upto 200 and 400 DSBs respectively [4, 59], but do not survive >40 DSBs caused by radiation stress [4, 60]. These results indicate that DNA is not the only important primary target of radiation damage, as was believed earlier, but proteins and membranes are also important targets for ionizing radiation induced damage. Radiation generates various reactive oxygen species (ROS) which cause indirect damage to macromolecules like DNA, proteins, lipids etc.

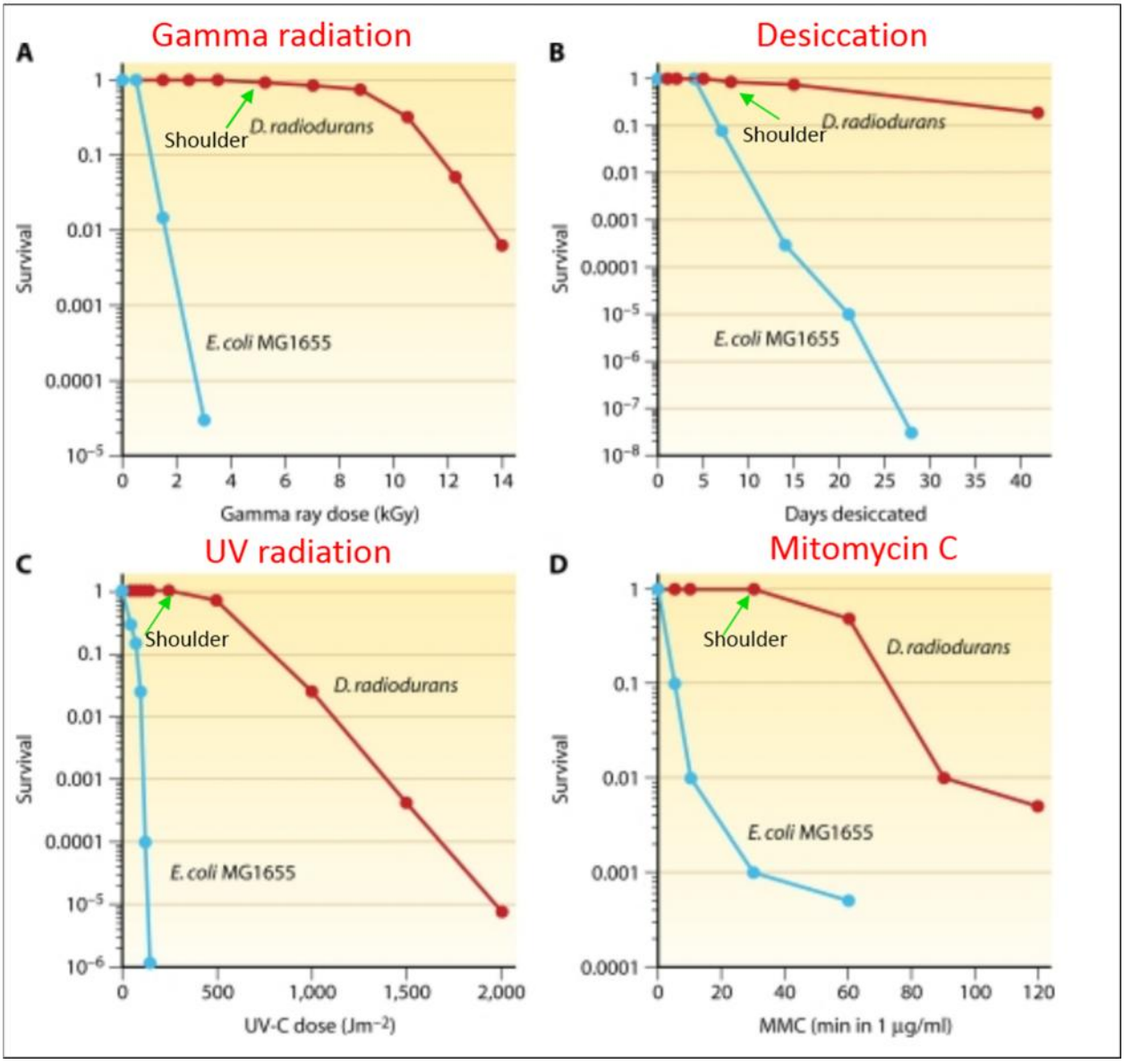

Fig. 1.2. Survival of *D. radiodurans* and *E. coli* from various DNA damaging stresses (A) gamma rays, (B) desiccation, (C) UV-C radiation, and (D) mitomycin C. (Source: Slade D and Radman M, 2011[4])

In *D. radiodurans*, enzymatic ROS scavenging is mediated by three catalases, four superoxide dismutases, two peroxidases, and two Dps proteins, whereas nonenzymatic scavengers include divalent manganese complexes (MnII) and carotenoids [46, 51]. Accumulation of high

intracellular Mn (II) during post-irradiation recovery (PIR) protects against protein oxidation in *D. radiodurans* [5]. Divalent manganese ions (Mn^{2+}) can scavenge superoxide and peroxide radicals [4]. It appears that the presence of the orange/pink (carotenoids) pigment may not contribute to radiation resistance, since even pigment-less mutants of *D. radiodurans* show equal radiation resistance as the wild type *D. radiodurans* [4].

1.6 Factors affecting Deinococcal radiation resistance

The radiation resistance of *D. radiodurans* depends on several physiological conditions, such as the age of the culture, the growth medium, the cell concentration, the pH, the irradiation medium, the irradiation temperature etc. [61]. Earlier studies showed that stationary phase cells were more resistant than the log phase cells [62, 63]. But recent studies have revealed that exponentially growing cells are more resistant than the stationary phase cells [64]. The cells growing in rich media are more resistant than those growing in minimal media [65]. Nutrient rich media are required for complete recovery of irradiated *D. radiodurans* cells. The survival was lowered when *D. radiodurans* was irradiated in media with higher pH [66]. The multicellularity of *D. radiodurans* (diads and tetrads) does not contribute to its radiation resistance. Single cell cultures of *D. radiodurans* also display resistance to radiation [67]. The dry cells of *D. radiodurans* are expectedly more radiation resistant compared to aqueous cultures, probably because of reduced water radiolysis and consequently lowering of indirect damage [4, 68]. Cells showed more resistance to radiation when they were irradiated on dry ice than on ice or at room temperature [4].

1.7 DNA repair pathways in *D. radiodurans*

Compared to other organisms *Deinococcus* is endowed with a distinct survial advantage. Exposure of any organism to 5 kGy of IR generates hundreds of DSBs in its genomic DNA and *Deinococcus*

is no exception. However, *Deinococcus* repairs them quickly in 3-4 hours, overlapping fragments are spliced together into complete chromosomes, and the cells soon resume normal growth [4, 8, 69]. There are several DNA repair mechanism for direct damage reversal in *D. radiodurans*, such as base and nucleotide excision repair, mismatch repair, and recombinational repair. DNA repair in irradiated *D. radiodurans* is highly dependent on DNA synthesis [3]. Several reports have described DNA double strand break repair mechanisms that involved little DNA synthesis, such as non-homologous end joining (NHEJ) of DNA fragments, homologous recombination via crossovers, and single strand annealing (SSA). These mechanisms were proposed as the major DNA repair pathways for the reconstitution of shattered chromosomes in this bacterium [3]. The SSA model was refuted by UV photolysis which degraded *D. radiodurans* DNA repaired in BrdU by double-strand breakage instead of SSBs. The UV proteolysis experiment also supports a novel synthesis-dependent strand annealing (SDSA) [3, 4]. NHEJ in which the fragmented DNA ends are joined without strand invasion or homology search in eukaryotes [70], has never been observed in *D. radiodurans* [4].

There are two types of recombinational repair pathways in *D. radiodurans* for DSBs repair (a) extensive synthesis dependent strand annealing (ESDSA) and (b) homologous recombination (HR) by crossovers, both of which depend on the RecA recombinase [3, 4, 69, 71] (Fig. 1.3). The SDSA repair process in *D. radiodurans* was later termed as ESDSA due to involvement of extensive DNA synthesis between DNA fragments [3]. Unlike in *E. coli* RecBCD pathway is absent in *D. radiodurans* which also lacks genes encoding for RceBC enzymes. Therefore DSB repair is initiated by the RecFOR pathway (49). The ends of DSBs are presumably processed by UvrD and RecJ into 3’ single-stranded DNA substrates onto which the RecFOR complex loads RecA (49). RecA is essential for RecA-mediated DNA synthesis priming and its homolog RadA

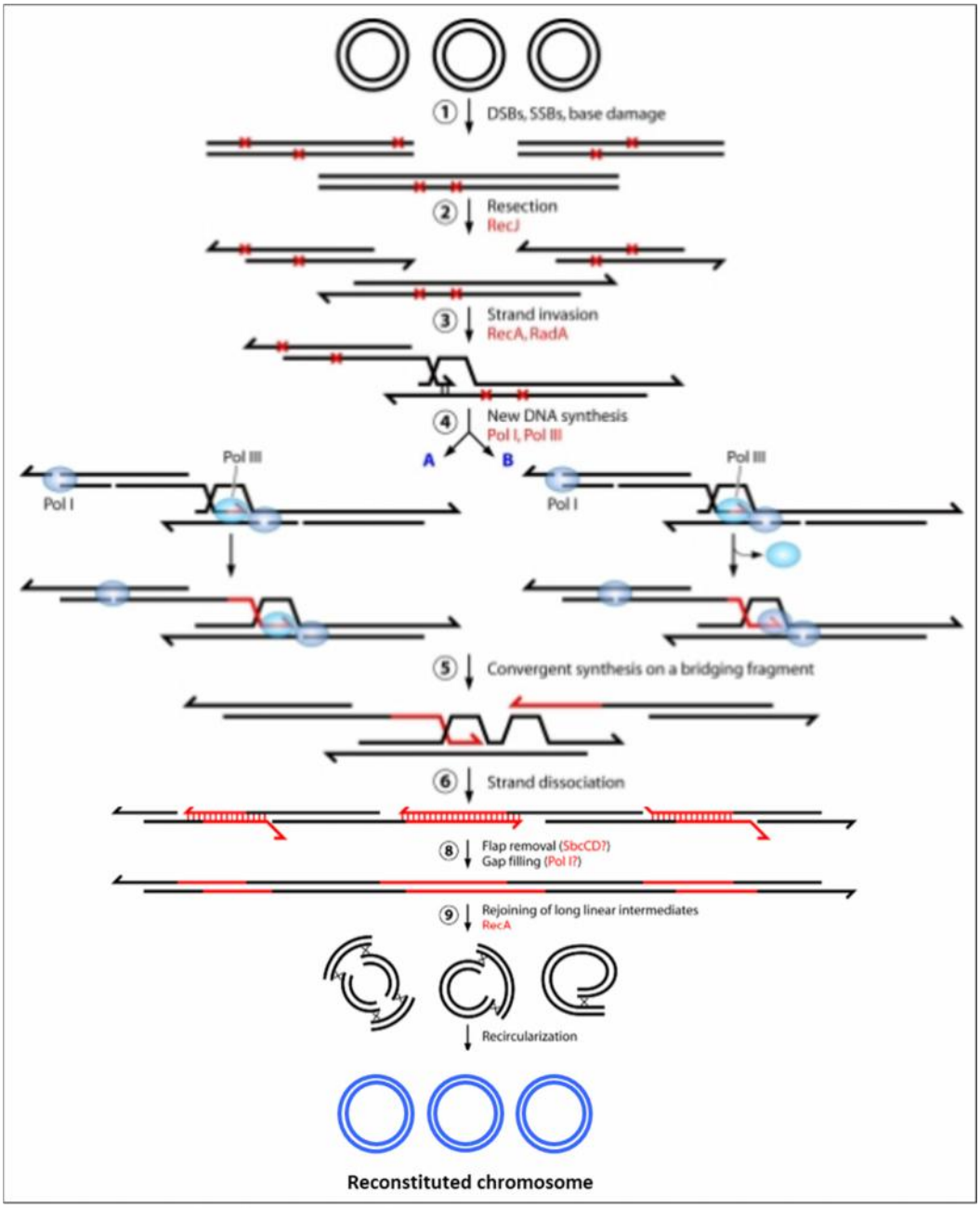

Fig. 1.3. DNA repair in *D. radiodurans*. Extensive synthesis dependent strand annealing (ESDSA) pathway of DNA repair in *D. radiodurans*. The different steps involved in ESDSA pathway shown in the schematic diagram. (Source: Slade D and Radman M, 2011[4]).

cannot replace it. Following RecA-RadA-catalyzed priming, DNA Pol III initiates DNA repair synthesis, whereas the elongation step is performed by either (i) Pol III alone or (ii) Pol I [4, 69].

Some structural features of *D. radiodurans* such as (i) genome condensation, (ii) ring-like nucleoid morphology, (iii) DNA-membrane association, and (iv) chromosome alignment are also attributed to rapid and accurate RecA mediated homology search and recombination repair of irradiated *D. radiodurans* chromosomes [4, 72]. Genes facilitating the operation of these pathways are part of a DNA damage regulon (*ddr* genes) and are coordinately induced in *D. radiodurans* upon DNA damage, by a fascinating but inadequately understood gene regulation.

1.8 General characteristics of gene promoters

In living organisms some genes express highly, while some express at basal level and some do not express at all in normal growth conditions [73]. Such variation in gene expression depends on the strength of the promoter and availability and interaction of sigma factors and RNA polymerase (RNAP), because the level of these factors are limited in a cell, and there is an intense competition among the promoters for RNAP holoenzyme [73]. A promoter is a regulatory segment of DNA located upstream of a gene, providing a control point for regulated gene transcription (Fig. 1.4). Promoters are adjacent and typically upstream (5') of the sense strand of the regulated gene. Promoters are a vital component of genes because they control binding of RNA polymerase to DNA, transcription initiation and are directly responsible for the amount of transcript generated. The promoter contains specific DNA sequences that are recognized by proteins known as transcription factors. These factors bind to the promoter sequences, recruiting RNA polymerase, the enzyme that synthesizes the RNA from the coding region of the gene (Fig.1.4). DNA sequences within promoters can be identified as binding sites for trans-acting factors, which may cause either activation or repression of transcription.

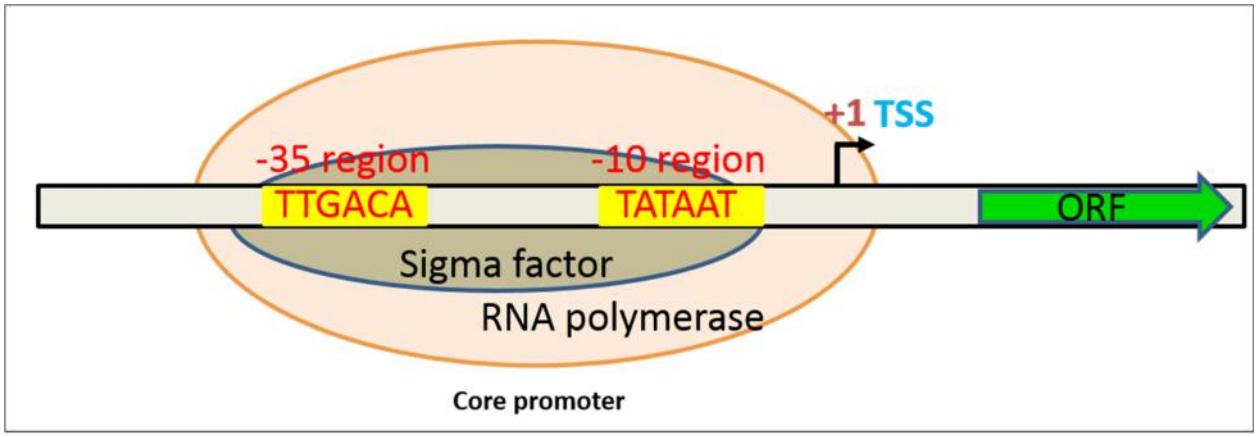

Fig. 1.4. Typical *E. coli*-like promoter structure. The schematic diagram represents a typical *E. coli*-like promoter. TSS: transcription start site.

The core promoter is generally defined to be the DNA region that directs the accurate initiation of transcription by RNA polymerase [74]. The core promoter region is located most proximally and contains the RNA polymerase binding site, -10 (TATAAT) and -35 (TTGACA) consensus sequences and transcription start site (TSS). RNA polymerase binds to this core promoter region stably aided by appropriate σ factor and initiate transcription of the template strand. TSS is present on the 3' side of the core promoter, which is where transcription is initiated. There are two main strategies for transcription initiation, focused and dispersed initiation. In focused initiation, transcription starts from a single nucleotide or within a cluster of few nucleotides, whereas in dispersed initiation, there are several weak transcription start sites over a broad region of about 50 to 100 nucleotides. Tightly regulated genes tend to have focused promoters, while constitutively expressed genes typically have dispersed promoters [74].

1.8.1 Promoters in Prokaryotes and Eukaryotes

Prokaryotic promoters are simpler than in eukaryotes. In prokaryotic organisms there are hexameic sequences at -10 (Pribnow box) and -35 positions with respect to the transcription start site (TSS).

In addition to *E. coli*-like -10, -35 sequences many Gram positive bacteria such as *Bacillus subtilis* promoters contain a conserved motif with TRTG sequence at -16 position, mutations in this sequence significantly reduced the promoter strength [75, 76]. *B. subtilis* has extended promoter structures with conserved A and T repeats on upstream of -35 region and upto -70 region from TSS which have contact with RNA polymerase [77]. The equivalents of prokaryotic -10, -35 sequences in the eukaryotes are TATA box and GC box at varying positions. Prokaryotic cells contain sigma factors which assist the RNA polymerase in binding to the promoter region. Each sigma factor recognizes different core promoter sequences. In prokaryotes a single promoter can serve to initiate transcription of multiple structural genes organized in operons, for example the *lac* operon. In prokaryotes the promoters are adjacent, almost juxtaposed to genes. Eukaryotic promoters are more complex and diverse than prokaryotic promoters. In eukaryotic promoters, the regulatory elements are spread out over a far distance from TSS. Eukaryotic promoters are so complex in structure that the DNA often tends to fold back on itself to initiate transcription. Promoters in eukaryotic organisms such as plants, animals comprise multiple elements TATA box, GC box, CAAT box GC box etc. [78], in addition to gene/tissue/developmental stage specific enhancer elements.

1.8.2 Types of promoters

There are different types of promoters present in the living organisms or created by rDNA technology such as (a) constitutive promoters which drive the gene expression constantly. These promoters control the housekeeping genes. These are common to all living organisms. (b) Stress-inducible promoters, which drive the expression of specific genes in response to various stress conditions like heat, cold, salt, radiation etc. (c) tissue specific promoters present in multicellular eukaryotes. Which express only in specific tissue but not active in other tissues, (d)

developmentally regulated promoters, and (e) synthetic promoters (man-made), which are derived from combination of various elements of a promoter region from diverse origin. The role of Tat protein in regulation of HIV gene expression was studied using synthetic promoters [79]. Operons of prokaryotes are regulated by two modes: positive regulation and negative regulation.

1.8.3 Gene regulation at transcriptional level

The gene regulation in prokaryotes majorly occurs during transcription in which RNA is synthesized from DNA template by RNA polymerase (RNAP) [80, 81]. RNAP consists of core enzyme ($\alpha_2\beta\beta'$) and sigma (σ) factor. Sigma factor(s) are needed for recognition of promoter by RNAP during transcription initiation [82]. Transcription factors (TF) are very essential for activation or repression of gene expression. Regulation of gene expression in prokaryotes is a complex network controlled by *cis*-elements and DNA-binding *trans*-acting factors as key component. Transcription factors (TFs) regulate gene expression by binding to *cis*-regulatory sequence, within the promoters or interacting with each other, or by binding to DNA as well as to other TFs [82]. Transcription factors bind to a specific promoter at operator sequence and either activate or repress transcription initiation [83]. An activator stimulates its target gene expression by acting on a promoter to stimulate RNA polymerase. In the opposite case the TFs repress the transcription by blocking the RNAP binding, or transcription initiation and progression (elongation). Some TFs can function both as activators or repressors. The LacI (repressor) allolactose (inducer) of the lac operon are an example of negative control. The LacI repressor, represses the expression of downstream genes (*lac* operon) in presence of glucose in the growth media (Fig1.5). The CAP-cAMP system is an example of positive control. A positive signal (c-AMP) triggers the expression of lac operon by binding to catabolite activating protein (CAP) (Fig.1.5).

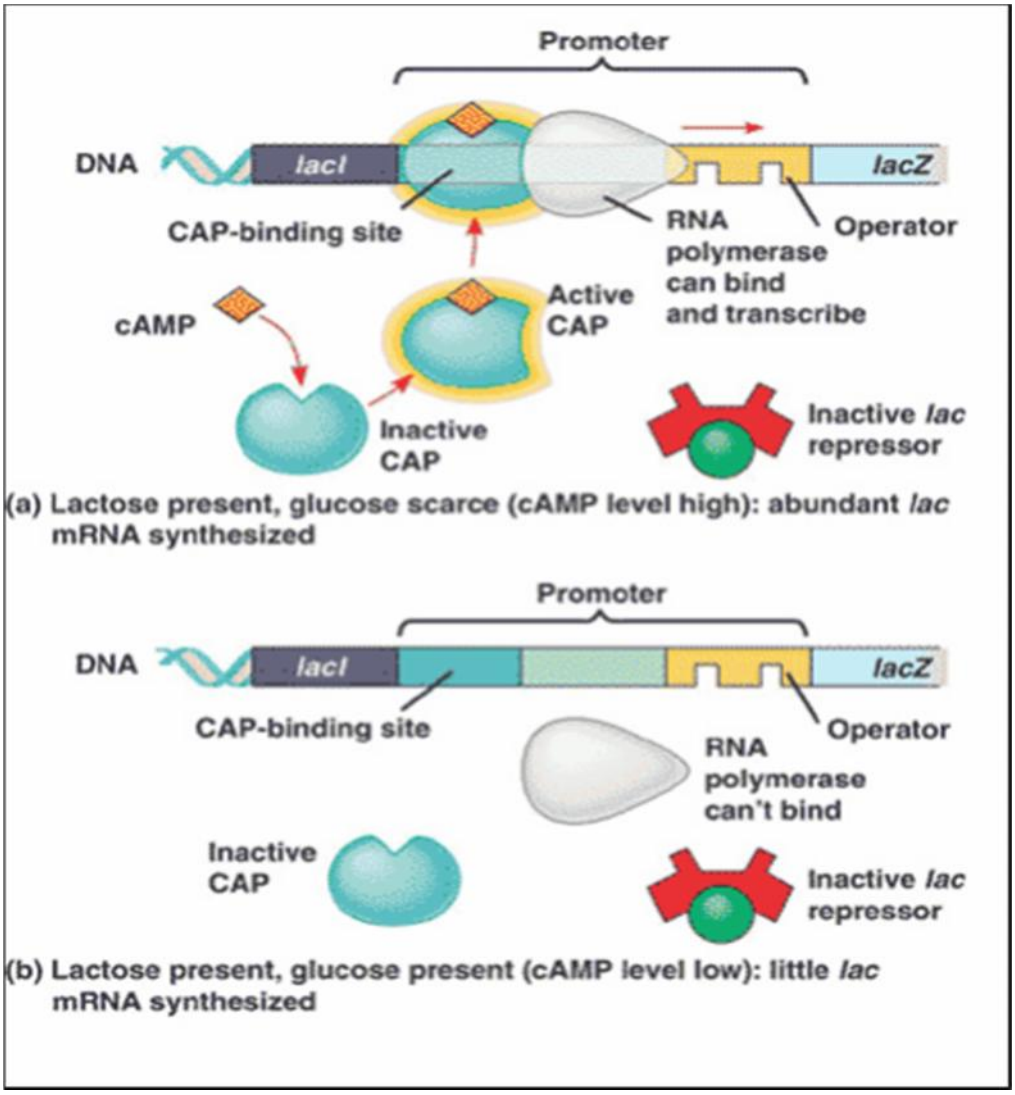

Fig. 1.5. Regulation of *lac* operon. The schematic representation of regulation of *lac* operon in *E. coli* (Source: https://www.quia.com/jg/1274005list.html).

Attenuation is another type of proposed mechanism of gene regulation in some bacterial operons which works on the fact that in bacteria transcription and translation proceed simultaneously. The best example of this regulation is *trp* operon (Fig. 1.6). Attenuators are generally located upstream of about 50bp untranslated regions of genes or operons with rho-independent terminator [84]. The leader sequence is rich in Trp codons and transcription is continued or terminated depending upon

whether Trp is available in the medium or not. In bacteria, expression is also regulated at the level of translation by some small RNA regulators. They act through base pairing with RNAs in the cell and modulating the translation and stability of mRNA [85].

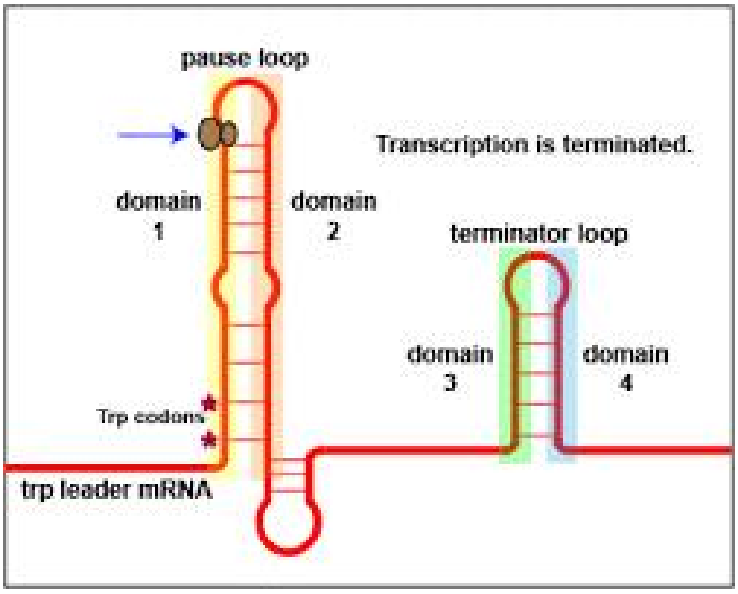

Fig. 1.6. Regulation of *trp* operon by attenuation. Schematic representation of tryptophan operon regulation by attenuation.
(Source:https://www.google.co.in/search?q=regulation+of+trp+operon+by+attenuation+reviews)

1.9 Deinococcal genes and promoters

The genome of *D. radiodurans* contains a large number (nearly 30%) of uncharacterized genes [40], many of which responds to radiation stress [6]. However the radiation-responsive mechanism of gene regulation in this organism has not been studied adequately. Promoters are very important *cis* regulatory elements which control the gene expression under given growth condition. In general prokaryotic promoters harbor σ^{70} interacting hexameric consensus sequences at -10, -35 position from the transcription start site (TSS) [78, 86]. But many of Deinococcal genes lack these sequences and their promoters and TSS are not known precisely [87]. Earlier studies showed that

Deinococcus promoters are poorly recognized in *E. coli*. Conversly, *E. coli* promoters that were tested were not recognized in *D. radiodurans,* suggesting that Deinococcal promoters might be different from the classical *E. coli* promoters [17, 88]. It is indeed very important to study the promoter structure and function to understand the regulatory mechanism. So far only a few promoters of *D. radiodurans* have been studied [11, 15, 16]. As a radiation resistant microbe *D. radiodurans* is an ideal organism to study the promoter structure to decipher the gene regulatory mechanism, especially the induction of gene expression in PIR.

Bacterial cells are constantly exposed to various stresses in the environment. To survive against these stresses, bacteria respond by changing its gene expression pattern. The highly radiation resistant bacterium *D. radiodurans* also responds to radiation stress by distinct radiation responsive gene expression. It was reported that 832 genes (28%) of its genome were induced and 451 genes (15%) of genome were repressed during post irradiation recovery of *D. radiodurans* from exposure to 15kGy dose of gamma radiation [6]. The radiation responsive up-regulation of *D. radiodurans* genes was further substantiated by real time PCR data [89] and proteomic data [8]. But the underlying gene regulatory mechanisms are not clearly known.

The upstream regions of many *D. radiodurans* R1 genes do not have typical *E. coli*-like promoter sequences i.e consensus hexameric sequences TTGACA at -35 and TATAAT at -10 position upstream of TSS. Almost 60% of *D. desert* genes are “leader less” [9]. Many of the *Deinococcus* genes have only an AT rich motif instead of -10 like consensus sequences and completely lack -35 sequence. Many deinococcal genes reportedly, do not function in *E. coli* and *vice versa*. But how gene repression is regulated in *Deinococcus* is yet to be revealed. The analysis of *D. radiodurans* genome sequence has revealed only three putative sigma factors, one classified with vegetative sigma^{70} (rpoD/sigA, DR0916) sequences, and two classified with extra-cytoplasmic

function (ECF) related alternative transcription factors (*sig1* DR0180 and *sig2* DR0804). *D. radiodurans* lacks orthologs for nitrogen-starvation, general starvation, and heat shock sigma factors of *E. coli*, such as rpoN, rpoS, and rpoH, respectively [90]. Mutation study showed that sig1 plays a positive role in regulation of heat shock response genes in *D. radiodurans* whereas *sig2* has a minor role in heat shock [90].

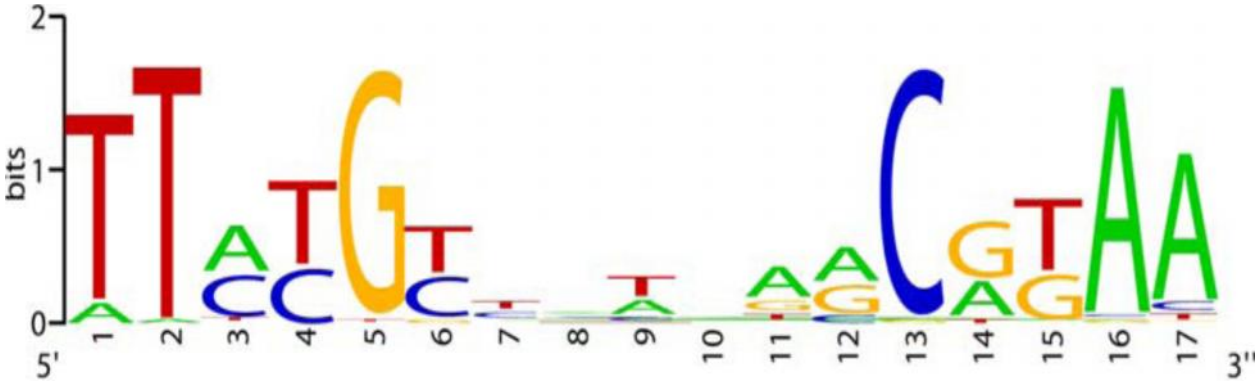

Fig. 1.7. The RDRM sequence. Radiation and Desiccation response motif in *D. radiodurans.* The height of the bases represent the extent of their conservation. (Source: Makarova *et. al.* 2007[10]).

The whole genome search of *D. radiodurans* and *D. geothermalis* found a 17 bp palindrome like sequence called radiation and desiccation response motif (RDRM) in several radiation inducible genes (Fig 1.7) [10]. This motif is present only in 24 genes of *D. radiodurans* and 29 genes of *D. geothermalis* shown in Table 1.1 [10]. Later RDRM motif was found in other *Deinococcus* species also [9]. Location of TSS is not known for many of Deinococcal genes, the position of RDRM with respect to translation start codon is very different in RDRM-based promoters ranging from -3 to -257bp.

Table 1.2. List of RDRM containing genes of *D. radiodurans* and their sequences.

(Source: Makarova *et al* 2007 [10])

S.no	Gene	Function	RDRM seq	Position
1	DR0070	ddrB protection DNA 3' end	TTATGTTATTtACgTAA	+4
2	DR0099	ssb	TTATGTcATTgACATAA	-28
3	DR0219	ddrF (hyp)	TTATGTTATatACgTAA	-41
4	DR0326	ddrD	TTcTGcTAAaAACAgAA	-26
5	DR0423	ddrA	TTATGTctTgAcCgTAA TTcTGTTcTaAACtaAA	-40-20
6	DR0171	irrI	aTcTGgccTgtACtgAA	-19
7	DR0596	ruvB	TTtcGcaAATAgCgTAA	-25
8	DR0659	FrnE	TTATtTTcTaAACtgAt	-29
9	DR0906	gryB	TTcTGTaAgagACgTAA	-257
10	DR1143	Similsr 1142	TTATGTTtTaAgCgTAA	+49
11	DR1039	mutS	TTtcGcTcAgAACgTAA	-42
12	DR1262	Rsr Rho like RNABinding protein	TTccGTctgTtgCgTcA	-122
13	DR1289	recQ	TTcTGcccAcAACgTAA	-34
14	DR1696	hexB/mutL	aTATGcTcAcAACAgAA	-17
15	DR1771	UvrA	TTAcGcgccTgcCgTAA	-63
16	DR1775	UvrD	TTAcGcTccTggCAgAA	-40
17	DR1913	gyrA	TTAcGTgATTAACATAA	-116
18	DR1921	sbcD	TTccGTcATgcgCgTAc	+61
19	DR2256	tkt	TTcTGTctTTAcCggAA	-180
20	DR2275	uvrB	TTAcGcTgTgggCgTAA	+74
21	DR2338	cinA	TTATGcTgcTAgCAgAA	-3
22	DR2574	ddrO	TTcTGTatTgAcCgTAg	-149
23	DRA0151	hutU	TTccGgatAgtgCggAA	-115
24	DRA0346	pprA	aTcTGTTcAgggCATAA	+22

Recent studies showed that *D. radiodurans* has a distinct repressor protein DdrO (DR2574) which binds to RDRM sequence in the promoter region of radiation inducible genes and represses their expression under normal growth conditions. During post irradiation recovery PprI protein, a metalloprotease which is a general switch for expression of several radiation inducible genes,

clears the DdrO repressor by cleaving it [12, 24], thereby derepressing transcription. To understand the radiation inducible gene expression it is important to study the structure of radiation-responsive promoters. The highly radiation resistant *Deinococcus* is an ideal choice to study promoter structure and function and to understand the regulatory mechanisms underlying radiation responsive regulation of gene expression.

1.10 Promoter analysis in *D. radiodurans*

Many systems of promoter detection using bioinformatic analyses have been developed. Computer-assisted searches for promoter sequences in ten bacterial species, using genomic distribution of hexanucleotide pairs within intergenic regions, has been described as a promising general tool for the prediction of promoters [91]. However, such analyses are based only on the presence of putative -10 and -35 hexamers and generate many false positives in their promoter predictions. There are some studies on deinococcal promoters by a few groups. The study of *D. radiodurans* promoters of *amyE*, *groESL*,and *lexA* genes was reported by Meima *et. al* [15], *pprA* by Obha H *et al* [16] and *ssb* promoter by Ujaoney *et al* [11]. But these did not reveal how promoter structure/function regulates expression of these genes. It is important to understand the *D. radiodurans* promoters, especially their role in radiation responsive gene expression.

There is a constant need for a wider range of genetic tools to facilitate deeper understanding of the mechanism, underlying regulation of gene expression. A common mechanism for the regulation of gene expression is by means of promoters. Promoter probe vectors are important tools for studying gene expression *in vivo* and have been extensively used to isolate and analyze regulatory sequences in many bacteria [92]. The study of promoter structure and activity of *in vivo* needs a suitable promoter-less plasmid shuttle vector, which can replicate both in the original host and the

cloning host. Reporter systems represent an important tool for isolating and characterizing promoter regions. Various promoter-less reporter genes, coding for easily detectable and quantifiable proteins and their activators have been used for the construction of a number of promoter-probe vectors to evaluate the activity of promoters and their regulation in bacteria such as *C. glutamicum*, *Methylococcus* etc [93, 94]. The insertion of promoter-containing DNA fragments upstream of the reporter genes create transcriptional fusions, which drive expression of the reporter genes. The application of such tools to *D. radiodurans* would facilitate the study of genetic mechanisms regulating gene expression in response to radiation stress. For *D. radiodurans* few shuttle vectors (Table 1.2) pI3, pRAD1, pROBe1, pRN1 etc. are currently available [17, 18]. The first *E. coli-D. radiodurans* shuttle plasmids were derived by fusing the *D. radiodurans* strain SARK natural plasmids pUE10 and pUEl1 with the *E. coli* vector pS27 or pS28 [95].These plasmids could be replicated both in *E. coli* and *D. radiodurans* strains SARK and Rl. The presence of *aphA* gene, with a promoter that works in both hosts, conferred selectable kanamycin resistance both in *D. radiodurans* and *E. col* [17]. However some of them were large in size (6.3- 17kb).

A few of the vectors had *cat*, *lacZ*, *phoN* [11, 15, 19], luciferase [96] as reporter genes These reporter proteins did not allow real time expression studies or microscopic visualization and it was rather time and labor intensive to measure them, although several improvements were reported. In other systems, green fluorescent protein encoding *gfp-mut2* gene [97] has been used successfully as reporter, that allows microscopic visualization and fluorimetric quantitative assessment of reporter gene expression. No such promoter probe vector is currently available for *D. radiodurans*. Therefore, using GFP as a reporter in *D. radiodurans* was considered desirable for understanding and deriving information on radiation inducible promoters.

Table 1.3 List of Shuttle vectors that work between *E. coli* and *D. radiodurans*

S. No.	Vector	Reporter	Reference
1	pS30	*cat* gene (Cm^R)	Smith *et.al* 1991 [95]
2	pI3, pI304	*cat* gene (Cm^R)	Master&Minton1992[17]
3	pRAD1	No reporter	Meima *et.al* 2000 [18]
4	pRADZ	*lacZ* (B-galactosidase)	Meima, *et.al* 2001 [15]
5	pROBe1	*xylE/lacZ*	Meima *et.al* 2001 [15]
6	pRN1	*phoN*	Appukuttam *et.al* 2006[19]
7	pRAD-gfp	*gfp-mut2*	This study [87]
8	pKG	*gfp-mut2*	This study [87]

Two kinds of reporter fusions with promoter DNA are possible (a) in transcriptional fusion the transcription of a reporter gene is placed directly under the control of a promoter and the reporter gene has its own ribosome binding site (RBS). The activity of the reporter gene product is proportional to the level of transcription initiation from the promoter. (b) The other type is translational fusion in which the reporter gene is placed under the control of a cloned promoter along with its RBS sequence. In this case the activity of the reporter gene product can be correlated with the activity of the native gene product that the promoter and RBS normally regulate [94].

1.11 Present work

The present work was initiated to understand the structure and function of selected radiation-responsive gene promoters of *D. radiodurans* by (a) construction and validation of a suitable promoter probe vector (b) use of such vector to analyze activity of several putative promoters under normal and radiation stress conditions, and (c) elucidation of the role of RDRM in radiation

responsive gene expression by constructing various point and deletion mutations in Deinococcal promoters. The work had the following defined objectives:

1. Design and construction of a new promoter probe shuttle vector, with GFP gene (*gfp*) as a reporter, and its validation using known *D. radiodurans* promoters.
2. Selection of radiation responsive genes based on bioinformatics, microarray, proteomic and mutational analysis data, for promoter analysis.
3. Cloning of selected promoters (up to 500bp upstream DNA sequences), containing/lacking RDRM motif, in promoter probe shuttle vector.
4. Assessment of promoter activity under ambient and radiation stress conditions by visualization and quantitation of reporter gene expression and activity.
5. Selective mutagenesis, insertion and reorientation of RDRM to elucidate its effect on corresponding gene expression.

The work carried out is presented in the thesis as following 6 chapters:

Chapter 1: General introduction.

Chapter 2: Materials and Methods.

Chapter 3: Construction of promoter probe shuttle vector and its validation.

Chapter 4: Cloning of selected putative *Deinococcal* promoters and assessment of their activity under ambient and radiation stress conditions.

Chapter 5: Mutagenesis, reorientation and swapping of RDRM and analysis of corresponding promoter activity under normal and radiation stress conditions in *D. radiodurans*.

Chapter 6: Summary and conclusions.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General laboratory chemicals and reagents

Bacterial growth media components Bacto-tryptone and Bacto-yeast extract in dehydrated form and Bacto-agar powder were procured from Difco Laboratories, USA. Fine biochemicals, and buffers were obtained from Sigma-Aldrich, USA. Organic solvents and acids of analytical (AR) or laboratory (LR) grade were purchased from Polypharm Pvt Ltd., India. Molecular Biology Grade reagents, agarose were procured from Sisco Research Laboratories Pvt. Ltd. (SRL), India. NBT/BCIP were obtained from Roche Biochemicals, Germany; Diethyl pyrocarbonate (DEPC), sodiumdodecylsulphate (SDS), 2-mercaptoethanol (2- ME), phenyl methane sulphonyl fluoride (PMSF), ethidium bromide, kanamycin, ampicillin, chloramphenicol, and spectinomycin from Sigma-Aldrich, USA; and lysozyme and protenase K were purchased from Hi Media Laboratories Pvt. Ltd. Nitrocellulose membrane were obtained from Millipore, India.

2.1.2 Enzymes and Kits

All restriction endonucleases, calf intestinal phosphatase, quick blunting kits and Bovine serum albumin (BSA) were purchased from New England Biolabs (NEB), UK. Quick blunting kit, T4 PNK kit, Quick ligation kit were obtained from Thermo Fisher scientific, USA. GFP monoclonal antibody was from Sigma Chemicals Co., USA. Taq DNA polymerase was obtained from Board of Radiation and Isotope Technology (BRIT), India. The genomic DNA isolation kit was procured from Hi Media Laboratories Pvt. Ltd, India. PCR purification kit, plasmid isolation kit, and gel extraction kit were obtained from BRIT, India. RNA isolation kits was purchased from Quiagen, Germany. RevertAid H Minus First Strand cDNA Synthesis Kit, DNA labeling kit and

sequencing kit were bought from Thermo Fisher Scientific, USA. RNase and DNase enzymes were obtained from Roche, Switzerland.

2.1.3 Oligonucleotides and Radionucleotides

Various custom made desalted oligonucleotides (primers), deoxyribo nucleotide-triphosphates (dNTP) and buffers for polymerase chain reaction (PCR) were obtained from BRIT, Mumbai, India; Sigma India; Eurofins, India; and Shrimpex, Chennai India. DNA electrophoresis markers (100 bp and 1 kb ladders) were obtained from New England Biolabs (NEB), UK. [^{32}P] γ-ATP was obtained from BRIT, India.

2.1.4 Antibiotics

The antibiotics used in this study included carbanicillin, kanamycin, spectinomycine and chloramphenicol. Their stock solutions were prepared in sterile distilled water, except for chloramphenicol where absolute ethanol was used as solvent.

2.1.5 Bacterial strains and plasmids

The various wild type and recombinant bacterial strains and recombinant plasmids used or constructed in this study are listed in Table 2.1

Table 2.1 List of plasmids and recombinant strains used in this study

Bacterial strain/ plasmid	Description	Source
Bacterial strains		
Deinococcus radiodurans R1 (DRA)	ATCC BAA-816, Wild type strain	M. Daly
Recombinant DRA strains	*D. radiodurans* harboring one of the recombinant plasmids listed below, DRA::pKG-$P_{(Gene\ no.)}$	This study
DRA Δ*pprI*	*pprI* knockout mutant of *D. radiodurans*	This study
E. coli (EC)		
DH5α (EC)	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Lab collection
E. coli BL21	F^- ompT gal dcmhsdS_B(r_B^- m_B^-) λ(DE3) pLysS, Amp^R, Cm^R	Lab collection
Recombinant EC strains	*E. coli* harboring one of the plasmids listed below, annotated as EC::pKG-$P_{(Gene\ no.)}$	This study
Plasmids		
pAM1956	EC-Anabaena shuttle vector with a promoter less *gfp-mut2*, Kan^R	Yoon and Golden, 1998
pRAD1	6280 bp, EC-DRA shuttle vector, Amp^R, Cm^R	Meima and Lidstrom, 2000
pRAD-gfp	pRAD1 with *gfp* gene, Amp^R, Cm^R	This study
pBluescriptII SK(+) (pBS)	2961 bp, Amp^R	Stratagene
pBS-ter	pBS with transcriptional terminator *term116,* Amp^R	This study
pBS-ter-gfp	pBS-ter with gfp gene, Amp^R	This study
pRAD-ter-gfp	pRAD1 with with transcriptional terminator *term116 and gfp* gene, Amp^R, Cm^R	This study
pUC4K	3914 bp, Kan^R	Pharmacia Biotech
pRAD-Kan	The *amp* and *cat* genes from pRAD1 was replaced with *aphII* (Kan^R) gene, Kan^R	This study
pKTG	pRAD-Kan with term116 and gfp gene**,** Kan^R	This study
pKGX	pKTG with new multiple cloning site (MCS), Kan^R	This study
pKG	4745 bp, pKGX after removal of 386 bp between term116 and EC Ori and 305bp downstream to *aphII* ORF, Kan^R	This study
pBS-Spc	pBS with spectinomycin cassette, Amp^R, Spc^R	This study
pBS-Δ*pprI*	pBS-Spc carrying 500bp up and down stream DNA regions of *pprI* gene of DRA, Amp^R, Spc^R	This study
pKG-P_{DR0053}	367 bp putative promoter sequence of DR0099 (Chr. I, 49915-49549) cloned in pKG (EcoRI/SpeI), Kan^R	This study

pKG-$P_{DR0070-1}$	433bp promoter sequence (Chr. I, 65604-66037) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR0070-2}$	Same as pKG-$P_{DR0070-1}$ but with G5A and C13T mutations in RDRM, Kan^R	This study
pKG-$P_{DR0070-3}$	Same as pKG-$P_{DR0070-1}$ but with 5bp deletion from 5' end of RDRM, Kan^R	This study
pKG-$P_{DR0070-4}$	Same as pKG-$P_{DR0070-1}$ but with complete RDRM deletion, Kan^R	This study
pKG-$P_{DR0070-5}$	Same as pKG-$P_{DR0070-1}$ but RDRM replaced with nonspecific sequence, Kan^R	This study
pKG-P_{DR0099}	367 bp putative promoter sequence of DR0099 (Chr. I,100091-100458) cloned in pKG (EcoRV), Kan^R	This study
pKG-P_{DR0219}	300bp promoter sequence (Chr. I, 219026-219326) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-P_{DR0423}	300 bp promoter sequence (Chr. I, 424005-423705) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-P_{DR0596}	506bp promoter sequence of DR0596 (Chr. I, 609955-609448) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR0606-1}$	247bp promoter sequence (Chr. I, 617624-617869) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR0606-2}$	Same as pKG- $P_{DR0606-1}$ but with addition of17bp RDRM sequence at -14 from ATG start codon of DR0606 ORF, Kan^R	This study
pKG-$P_{DR0606-3}$	Same as pKG- $P_{DR0606-1}$ but with addition of17bp RDRM sequence at -120 from ATG start codon of DR0606 ORF, Kan^R	This study
pKG-$P_{DR0606-4}$	Graft of 141bp from $P_{DR0906-1}$ (Chr. I, 911842-911982) on 5' side and125bp from $P_{DR0606-1}$ (Chr. I, 617746-617869) on 3'side of promoter cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR0606-5}$	Same as pKG- $P_{DR0606-4}$ but with deletion of 17bp RDRM sequence, Kan^R	This study
pKG-P_{DR0694}	348 bp putative promoter sequence of DR0694 (Chr. I, 709674-710021) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR0906-1}$	375 bp promoter sequence (Chr. I, 911842-912217) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR0906-2}$	Same as pKG- $P_{DR0906-1}$ but with 17bp RDRM sequence deleted, Kan^R	This study
pKG-$P_{DR0906-3}$	Same as pKG- $P_{DR0906-1}$but with reverse orientation of RDRM sequence, Kan^R	This study

pKG-$P_{DR0906-4}$	Same as pKG- $P_{DR0906-1}$but with RDRM sequence mutated at 2 most conserved positions (G5A and C13T), Kan^R	This study
pKG-$P_{DR0906-5}$	Same as pKG- $P_{DR0906-1}$but with 5 bases from 5' end of RDRM sequence deleted, Kan^R	This study
pKG-$P_{DR0906-6}$	Same as pKG- $P_{DR0906-1}$ but with 5 bases from 3' end of RDRM sequence deleted, Kan^R	This study
pKG-$P_{DR0906-7}$	Same as pKG- $P_{DR0906-1}$ but with deletion of 109bp from 5' end of the promoter sequence, Kan^R	This study
pKG-$P_{DR0906-8}$	Same as pKG- $P_{DR0906-1}$ but with deletion of 141bp from 5' end of the promoter sequence, Kan^R	This study
pKG-$P_{DR0906-9}$	Same as pKG- $P_{DR0906-1}$ but with deletion of 173bp from 5' end of the promoter sequence, Kan^R	This study
pKG-$P_{DR0906-10}$	Same as pKG- $P_{DR0906-1}$ but with additional RDRM downstream to -10 sequence (RDRM duplication), Kan^R	This study
pKG-$P_{DR0906-11}$	Same as pKG- $P_{DR0906-10}$ but with deletion of native RDRM sequence, Kan^R	This study
pKG-$P_{DR0906-12}$	Same as pKG- $P_{DR0906-2}$ but with introduction of RDRM in between -10 and -30 sequences, Kan^R	This study
pKG-$P_{DR0906-13}$	Same as pKG- $P_{DR0906-2}$ but with introduction of RDRM on extreme up in the $P_{DR0906-2}$ promoter, Kan^R	This study
pKG-$P_{DR0906-14}$	Same as pKG- $P_{DR0906-2}$ but with introduction of RDRM on extreme down in the $P_{DR0906-2}$ promoter, Kan^R	This study
pKG-$P_{DR1143-1}$	390bp promoter sequence (Chr. I, 1153428-1153038) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR1143-2}$	Same as pKG- $P_{DR1143-1}$ but with 17bp RDRM sequence deletion, Kan^R	This study
pKG-P_{DR1720}	515 bp promoter sequence (Chr. I, 1743794-1744309) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-P_{DR1913}	260bp promoter sequence (Chr. I,1932139-1932399) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-P_{DR2220}	300 bp putative promoter sequence of DR2220 (Chr. I, 2217380-2217680) cloned in pKG (EcoRI/EcoRV), Kan^R	This study
pKG-P_{DR2275}	177bp promoter sequence (Chr. I,2272809-2272986) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR2238-1}$	315bp promoter sequence (Chr. I, 2335583-2335896) cloned in pKG (EcoRI/SpeI), Kan^R	This study
pKG-$P_{DR2238-2}$	Same as pKG-$P_{DR2238-1}$, but with RDRM deletion, Kan^R	This study
pET21a (+)	Protein overexpression vector for *E. coli*	Lab collection

pET21a-ddrO	The *D. radiodurans* gene *ddrO* cloned in pET21a(+) vector, Amp^R, Cm^R	This study
pET21a-pprI	The *D. radiodurans* gene *pprI* cloned in pET21a(+) vector, Amp^R, Cm^R	This study
P13840	p11830 PSpac-term 116, Spc^R	Nguyen HH

2.1.6 Primers used for amplification and cloning of DNA.

The various primers used in this study were designed based on published genome sequence of *D. radiodurans* [40]. The primer synthesis was outsourced to different commercial suppliers and the primers used are listed in Table 2.2.

2.2 Microbiological Methods

2.2.1 Bacterial cultures and growth conditions

The cultures of *D. radiodurans* or *E. coli* were grown aerobically in TGY medium (1% bactotryptone, 0.1% glucose, and 0.5% yeast extract) at 32°C or in Luria Bertani (LB) medium at 37°C, respectively, with shaking at 150 rpm. If required, different antibiotics were supplemented to the growth media as follows, carbenicillin (100 µg ml^{-1} for *E. coli*), chloramphenicol (3 µgml^{-1} for *D. radiodurans*), kanamycin (5 µg ml^{-1} for *D. radiodurans* or 50 µg ml^{-1} for *E. coli*) or spectinomycin (100 µg ml^{-1} for *D. radiodurans* or 50 µg ml^{-1} for *E. coli*). Bacterial growth was monitored spectrophotometrically by measuring OD_{600nm} or by determining colony forming units (CFUs) by incubating *D. radiodurans* on TGY-agar plates (1.5% bactoagar) for 48h at 32°C or *E. coli* on LB-agar plates for 18h at 37°C.

2.2.2 Maintenance and disposal of recombinant bacterial cultures

For long term storage of bacterial cultures, different recombinant clones of *D. radiodurans* and *E. coli* were grown overnight in appropriate rich media with/without antibiotic. From these overnight grown cultures, glycerol stocks were made as follows: Cultures were harvested, suspended in fresh growth medium and 800µl of bacterial culture was mixed with 200µl of sterile glycerol (final concentration 20%) in a sterile microfuge tube. The suspension was vertexed, snap frozen in liquid nitrogen and stored at -70^0C in a deep freezer. The tubes were properly labeled and details of the clones were entered in the bacterial stock book in the laboratory. For revival of the culture from frozen stock, an aliquot was streaked on agar plates with a nichrome inoculation loop and grown at appropriate temperature with appropriate antibiotic for required time. A single colony from these plates was inoculated in appropriate liquid broth, grown overnight and used for different experiments as specified. These streaked cultures and colonies on agar plates were maintained for a short time (1-2 weeks) by storing at 4^0C. After completion of each experiment the bacterial cultures in broth or on agar plates were pooled in a closed container, autoclaved at 121^0C, 15 psi for 15 min and disposed off.

2.2.3 Radiation stress and post irradiation recovery

Recombinant *D. radiodurans* or *E. coli* cells were grown in rich media to early stationary phase (OD_{600nm} = 2.7 ± 0.2). The cells were pelleted down by centrifugation at 5000rpm for 5 min and the pellet was resuspended in fresh medium at an inoculum density of 3 $OD_{600/}$ ml. The cell suspension was subjected to 6 kGy (*D. radiodruans*) or 300 Gy (*E. coli*) of ^{60}Co gamma radiation (Gamma Cell 220, Bhabha Atomic Research Centre, Dose rate = 5 Gy/ min). Following irradiation, the cells were pelleted, resuspended in fresh TGY or LB medium, as appropriate, at an inoculum density (OD_{600nm}) of 0.5 and allowed to recover under optimal growth conditions. During post irradiation recovery (PIR) samples were withdrawn at different time points for analysis. Each

experiment was repeated at least three times with technical duplicates. Observed variation in viability between different experiments was less than 10%.

2.2.4 Competent cell preparation and bacterial transformation

2.2.4.1 Escherichia coli

Competent cells of various *E. coli* strains were prepared by calcium chloride ($CaCl_2$)-rubidium chloride ($RbCl_2$) method. The overnight grown *E. coli* pre-culture was reinoculated in fresh LB medium at 1:100 dilution (100 µl pre-culture in 10 ml of medium) and allowed to grow at 37°C with agitation at 150 rpm to OD_{600} of 0.3-0.5. The cells were pelleted down by centrifugation at 5000rpm for 5 min at 4^0C. The pellet was washed with cold solution-A [10 mM 3-(N-morpholino) propanesulfonic acid (MOPS)], 10 mM $RbCl_2$, pH 7.0). The washed cells were gently resuspended in solution-B (100mM MOPS, 50mM $CaCl_2$ and 10mM $RbCl_2$, pH 7.5) and incubated for 30 mins on ice. The cells were centrifuged and the pellet was again resuspended in minimum volume (0.5 – 1.0 ml) of fresh solution-B. For immediate use, 150 µl of the resuspended culture was aliquoted in 1.5 ml microfuge tubes. For storage, 15-20% glycerol was mixed, 150 µl aliquots were made, snap frozen in liquid nitrogen and stored at -70°C.

Transformation of *E. coli* cells was carried out by adding 100 ng of plasmid DNA or whole ligation mixture (~20µl) to the above aliquoted competent cells. After addition of DNA the cells were incubated on ice for 1h, then subjected heat shock by incubating at 42^0C for 90sec followed by 5 min incubation on ice. The cells were recovered by addition of 1 ml fresh LB media and further incubating it at 37°C for 1 h with shaking. For transformation with intact plasmid 100 µl of the recovered culture was directly spread on to LB agar plates containing appropriate antibiotic. For transformation with ligation mixture, the cells were gently pelleted by centrifugation at 5000 rpm

for 5 min, resuspended in 100µl of fresh LB and plated on LB agar plates. The plates were incubated at 37°C overnight and colonies obtained were scored and characterized.

2.2.4.2 Deinococcus radiodurans

The competent cell preparation and transformation of *D. radiodurans* was carried out as described earlier [19]. In brief the overnight grown *D. radiodurans* culture was inoculated in the fresh TGY medium (1:100 dilution) and incubated at 32^0C with shaking (150 rpm) for 3-4 h until the reach OD_{600} 0.3-0.5. The culture was then incubated on ice for 10 min and centrifuged at 5000rpm for 5min at 4^0C. The pellet obtained was resuspended in fresh TGY broth containing 30mM CaCl2 and 15% glycerol. The cell suspension was aliquoted into sterile microfuge tubes, 150 µl each. The vial were snap frozen in liquid nitrogen and stored at -70 ^{0}C untill further use. The transformation was performed by adding 1-3 µg of plasmid DNA to 150 µlof competent cells and incubating tubes on ice for 30 min. Cells were then subjected to heat shock at 32^0C for 45 min. The cells were recovered by adding 1ml of TGY medium and incubating the tubes at 32^0C for 4h with shaking at 150 rpm. The cells were pelleted down by centrifuging at 5000 rpm for 5 min, the pellet was resuspended in 100 µl of TGY and spread on to TGY agar plate containing appropriate antibiotic. The plates were incubated at 32^0C for 48-72 hr and transformants were scored and characterized.

2.2.5 Reporter gene assays, microscopy and fluorescence spectrophotometry

The promoter driven GFP expression and activity (fluorescence) in recombinant *E. coli* or *D. radiodurans* cells was monitored qualitatively by fluorescence microscopy and quantitatively by fluorescence spectrophotometry, as described earlier [87]. Bright-field and fluorescence microscopy was carried out on live cells using the Axioscop 40 microscope (Carl Zeiss, Germany).

At appropriate time points samples were withdrawn cells were washed with PBS, resuspended in PBS and 5– 10 µl of cell suspension was spread on a glass slide and covered with cover slip. Individual cells exhibiting GFP fluorescence were visualized under fluorescence microscope equipped with 100× oil immersion objective, GFP filter (cy2) set, and Axiocam MRc CCD camera controlled by AxioVision software. For each recombinant *E. coli* or *D. radiodurans* clone, at least 25 fields were examined from each biological replicate experiment.

For fluorescence quantification 1 ml aliquot was withdrawn from the recombinant *D. radiodurans* or *E. coli* cultures and spun down by centrifugation at 10000 rpm for 1 min. The pellet was resupended in phosphate buffer saline (10 mM Na_2HPO_4, KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). GFP fluorescence was quantified by fluorescence spectrophotometer (Jasco, FP-6000, Japan) at an excitation wave length (λex) of 489nm, and an emission wavelength (λem) of 509nm. When required, samples with high GFP activity were diluted in PBS buffer to prevent saturation of the detector. The fluorescence intensity was represented as arbitrary fluorescence units (a.u). Radiation inducible fold change in promoter activity was expressed in terms of fold induction. The fold induction (F_I) was calculated as described earlier in [87] brief $F_I = (Fl_I \times OD_C)/(Fl_C \times OD_I)$, where Fl_I was the fluorescence intensity of irradiated sample, Fl_C was the fluorescence intensity of unirradiated (control) sample, OD_I was OD_{600nm} of irradiated sample and OD_C was OD_{600nm} of control sample. For each recombinant *E. coli* or *D. radiodurans* sample, the fluorescence pectrophotometry experiments were performed with 3 independent biological replicates and at least 2 technical replicates for each biological replicate.

2.3 Molecular biology methods

2.3.1 Chromosomal DNA isolation

The chromosomal DNA from *D. radiodurans* was isolated as described earlier [98] with some modifications. Cells grown in rich media overnight were pelleted down by centrifugation of 5ml culture at 5000 rpm for 5 mins at 4°C and washed with PBS. To remove the pigment the cell were incubated with absolute ethanol for 5 min with intermittent vertexing. The step was repeated twice. The depigmented cells were washed with PBS again to remove traces of alcohol. The cells were resuspended in 200 µl TE buffer (10mM Tris-HCl and 1mM EDTA pH 8.0) containing lysozyme (10 mg/ml) and incubated at 37^0C for 30mins followed by addition of freshely prepared 20 µl proteinase K solution (2% sodium dodecyl sulphate, 0.1M EDTA, pH 8.0, 4 mg of proteinase K) and further incubated at 50^0C for 15 mins to remove proteins. Phenol chloroform solution at a ratio of 1:1 was then added to the mixture and aqueous phase was extracted into a fresh tube. The genomic DNA was precipitated by adding 0.1 ml of 3M sodium acetate (pH 7) and 2 ml of ice-cold absolute ethanol. The DNA was spooled out with a curved glass rod. The spooled DNA was washed twice with 70% ethanol, air dried and resuspended in 100 µl of TE buffer and stored at -20^0C. The purity and quantity of DNA was measured spectrophotometer by taking the ratio of OD260/280nm. The ratio obtained was 1.8, indicative of the purity of sample. The purity of DNA was also checked by electrophoresis on agarose gel as described in the following Section 2.3.2. Alternatively, the genomic DNA was also prepared using Genomic DNA preparation kit (Hi Media lab Pvt Ltd, India) as per manufacturer's protocol.

2.3.2 Agarose gel electrophoresis

Agarose gels of different porosities were used to resolve different kinds of DNA (Genomic DNA, plasmid DNA, PCR amplified DNA etc) or RNA. The agarose gels were prepared by dissolving appropriate amount of molecular biology grade agarose in TBE (0.09 M Tris, 0.088 M boric acid and 0.002 M EDTA, pH 8.0) buffer by boiling. The solution was cooled to 45-50^0C, ethidium

bromide (0.5ug/ml final concentration) was added, solution poured in a gel caster and allowed to solidify at room temperature. The gel along with the caster was kept in the running tank and submerged with TBE running buffer. The DNA or RNA samples were mixed with loading dye (composed of 0.2% Bromophenol Blue, 0.2 M EDTA, pH 8.0 and 50% Glycerol in sterile distilled water) and were loaded into the wells. A 100bp or 1kb DNA ladder was used as molecular size markers. The gel electrophoresis was carried out by applying constant voltage (8V/cm). The electrophoresis was continued till the dye front reached bottom edge of the gel. The DNA or RNA bands were visualized by UV transilluminator (UVP Model, UK). The gels were photographed using a gel documentation system (SYNGENE, Diversity model, UK).

2.3.3 PCR amplification

The DNA sequence of the *D. radiodurans* was obtained from online data base KEGG website (http://www.genome.jp/dbget-bin/www_bget?dra) [99]. The primers for polymerase chain reaction (PCR) were designed based on the DNA sequence of *D. radiodurans* [40]. The primer secondary structure, GC percentage, Tm values and dimer formation among primers were analysed using DNA MAN software (Lynnon Biosoft, Germany). Wherever possible the Tm values of primers were kept around $55^0 \pm 5^0$C. Desired restriction endonuclease sites were incorporated at 5' end of each primer for directional cloning. Various primers used in this study are listed in Table 2.2. PCR amplification of desired DNA was carried out using genomic DNA of *D. radiodurans* as template. For 50µl of PCR reaction, 100-200ng of genomic DNA, 2U of Taq DNA polymerase, 0.2 µM of primers and 200 µM dNTPs were used. For GC-rich templates, GC-rich resolution buffer or Q-buffer was added to PCR reaction mix. The PCR was carried out for 30 cycles involving initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 94^0C annealing at $55^0 C \pm 5^0$C depending on individual primer and extension at 72°C, followed by 10 min

for final elongation at 72°C. The products obtained were analysed by agarose gel electrophoresis (0.8-2.0% depending on the size of the PCR product) and DNA bands were visualized on a UV illuminator. If required the amplicon was excised from the gel and purified using affinity columns (Gel extraction kit, BRIT, India) by following the protocol as described by the manufacturer.

Table 2.2 List of primers used in this study

Sr. No.	Name	Sequence	Restriction sites used for cloning
1	Gfp-F	5′-GCTCGAGGGAATTCCATATGAGTAAAGGAGAAGAACTTTTCACTG-3′	XhoI/EcoRI
2	Gfp-R	5′-CGGGATCCTTATTTGTATAGTTCATCCATGCCAT-3′	BamHI
3	P5-F	5′-GGAGCGGATAACAATTTCACACA-3′	-
4	Term116-F	5′-AAAAAATCCCCCCGGTGGCAATCCGGGGGGTTTTTT-3′	
5	Term116-R	5′-TTTTTTAGGGGGGCCACCGTTAGGCCCCCCAAAAAA-3′	-
6	Kan-F	5′-TATTCGTTACAACCAATTAACCAATTCTG-3′	-
7	Ori-R	5′-GGAAGTAGACCCGTAAAAAGGCCG-3′	-
8	pRAD-F	5′-TCGCGAGGCCTCGAGGTCAAAA-3′	-
9	pRAD-R	5′-GGTCAGGCCTGCTTCTGTGCTTCCCT-3′	-
10	MCS-F	5′-ACTAGTAGCGGCCGCTCTAGAGTCGACGATATC-3′	-
11	MCS-R	5′-GATATCGTCGACTCTAGAAGCGGCCGCACTAGT-3′	-
12	pKG-1	5′-CTGTCATGTTTCAGATAAGT-3′	-
13	pKG-2	5′-TTCCAAATGAGTTTCCGCC-3′	-
14	pKG-3	5′-TCGTAGAAGGCGGACTTCT-3′	-
15	pKG-4	5′-TTGTCACGCAAAGGCCCG-3′	-
16	pKG-5	5′-AGGCGGTGCTACAGAGTTCT-3′	-
17	pKG-6	5′-CTTGCTCGAGGCCGCGA-3′	-
18	pKG-7	5′-GGCTGGCCTGTTGAACAAG-3′	-
19	pKG-8	5′-GACGGTATCGATAAGCTTGA-3′	-
20	pKG-9	5′- ACAACATTAAAGATGGAAGCG-3′	-
21	P0053F	5′-GGACTAGTAGTTCGGCAGGTTGG-3′	SpeI
22	P0053R	5′-GGAATTCTTCCTTTTCCTCCTGA-3′	EcoRI
23	P0070F	5′-GGACTAGTTCGCAGCGTAAAGGCA-3′	SpeI
24	P0070R3	5′-GGAATTCCTGCCTCCTCCTTACGTA-3′	EcoRI
25	P0070R4	5′-GGAATTCCTGCCTCCTCCTTACATAAATAATATAACACATCGGGGA AGCCGGTGC-3′	EcoRI
26	P0070R5	5′-GGAATTCCTGCCTCCTCCTTACGTAAATAACACATCGGGGAAGCCGGTGC-3′	EcoRI
27	P0070R6	5′-GGAATTCCTGCCTCCTCCCACATCGGGGAAGCCGGTGC-3′	EcoRI
28	P0070R8	5′-GGAATTCCTGCCTCCTCCTCATGCGCGCACGAGAGCCACATCGGGGAAGCCG3′	EcoRI
29	P0070Rseq	5′-GCCGATCAGCGACCAGT-3′	
30	Pssb-F	5′-CCGAGAAGGATTACAATCTAGAACG -3′	XbaI
31	Pssb-R	5′-CATGCCTCGGGCCATATGAAATTCT -3′	NdeI
32	P0219-F	5′-GGACTAGTACATGGCCCAACTGAGG-3′	SpeI
33	P0219-R	5′-GGAATTCGCTTAGGGATTATATCT-3′	EcoRI

34	P0326-F	5′-GGACTAGTGGCCTTTTTCACCCG-3′	SpeI
35	P0326-R2	5′-GGAATTCCGTTCCAGCTTTTTTCAG-3′	EcoRI
36	P0423-F	5′-GGACTAGTCGTGCCCGGTGGCGG-3′	SpeI
37	P0423-R	5′-GGAATTCGCATTTAGTTTAGAA-3′	EcoRI
38	P0596-F	5′-GGACTAGTAACTCGTGACGGTGGA-3′	SpeI
39	P0596-R2	5′-GGAATTCGCCCAGCTTGTCCTTG-3′	EcoRI
40	Pgro-F	5′-GTGGCCGCCAGATCTGTTCAGG-3′	BglII
41	Pgro-R	5′-GTTTCAGCATCTAGAGTCCTCCTG-3′	XbaI
42	P0606-F	5′-GGACTAGTGTATTGTCGCCCTA-3′	SpeI
43	P0606-F3	5′-GTGGCCGCCACTAGTGTTCAGGGAT-3′	SpeI
44	P0606-R	5′-GGAATTCGTGGGGTCCTCCTGT-3′	EcoRI
45	P0606-R2	5′-GGAATTCGTGGGGTCCTCCTTTACGTAAATAACATAAGGAGAGTCGTGCCGG3′	EcoRI
46	P0606-F4	5′-AG**A**TTGTCAGCTTCGTTCTGTAAGAGACGTAAGTCAGTTGACATTTT-3′	
47	P0606-R3	5′-AAAATGTCAACTGACTTACGTCTCTTACAGAACGAAGCTGACAATCT-3′	
48	P0606-F5	5′-CTGTAAGAGACGTAAGTCAGTTGACATTTT-3′	
49	P0606-F6	5′-AACCGTCCGAGTTCAGTCAGTTGACATTTT -3′	
50	P0694-F	5′-GGACTAGTAGCATCTGACAACAGAC-3′	SpeI
51	P0694-R	5′-GGAATTCCTCCCTCCTCCTTTC-3′	EcoRI
52	P0906F1	5′-GGACTAGTTGACCTTTCCCGGCA-3′	SpeI
53	P0906R1	5′-GGAATTCCTGCTCATTGGGTTC -3′	EcoRI
54	P0906F2	5′-GGACTAGTTTGATATTTTTCGTGTC-3′	SpeI
55	P0906F3	5′-GGACTAGTTGCACCGCCGGAGCCTTG-3′	SpeI
56	P0906F8	5′-AACCGTCCGAGTTCATAAGATTGATATTTT-3′	
57	P0906R3	5′-AAAATATCAATCTTATGAACTCGGACGGTT-3′	
58	P0906F9	5′-AACCGTCCGAGTTCATTACGTCTCTTACAGAATAAGATTGATATTTT-3′	
59	P0906R4	5′-AAAATATCAATCTTATTCTGTAAGAGACGTAATGAACTCGGACGGTT-3′	
60	P0906F10	5′-ACCGTCCGAGTTCATTCTATAAGAGATGTAATAAGATTGATATTT-3′	
61	P0906R4a	5′-AAATATCAATCTTATTACATCTCTTATAGAATGAACTCGGACGGT-3′	
62	P0906F11	5′-AACCGTCCGAGTTCATAAGAGACGTAATAA-3′	
63	P0906R5	5′-TTATTACGTCTCTTATGAACTCGGACGGTT-3′	
64	P0906F12	5′-TCATTCTGTAAGAGATAAGATTGATATTTT-3′	
65	P0906R6	5′-AAAATATCAATCTTATCTCTTACAGAATGA-3′	
66	P0906F13	5′-CGGAGCCTTGACAGTTTCTGTAAGAGACGTAATAAGCTCTCTCCGCC-3′	
67	P0906R7	5′-GGCGGAGAGAGCTTATTACGTCTCTTACAGAAACTGTCAAGGCTCCG-3′	
68	P0906F-16	5′-GGACTAGTTGACCTTCTGTAAGAGACGTAAGGCCCCCAGGCTTTATGC-3′	SpeI
69	P0906R-10	5′-GGAATTCCTGCTCATTACGTCTCTTACAGAAGCTTCCGATCGGTCTGGT-3′	EcoRI
70	P0906R11	5′-AAAATGTCAACTGACTGAACTCGGACGGTT-3′	
71			
72	P0906R seq3	5′-ATGCCTTCGAGGACACTGATCTGGT-3′	
73	P1143F	5′-GGACTAGTAGTGATGCTTATCCGC-3′	SpeI
74	P1143R2	5′-GGAATTCAGCTTCTCCTTTAAAACCC-3′	EcoRI
75	DR1143R-4	5′-GGAATTCGCTTCTCCTTTAAAACCCGCTTTGTTTCACGGCGGGAGGACTT-3′	EcoRI
76	P1262-F	5′-GGACTAGTCGCCTGATTTCCTCA-3′	SpeI
77	P1262-R	5′-GGAATTCGGTCGGCCCTCCTTG-3′	EcoRI
78	P1314-F	5′GGACTAGTGGCAATCGCCACCTGGT-3′	SpeI
79	P1314-R2	5′-GGAATTCGGTATTTCCTCCTCCGGGAA-3′	EcoRI
80	P1358-F	5′-GCCGGATATCCGATGGGCGCGAGCA-3′	EcoRV
81	P1358-R	5′-TCTTGAATTCGGTCGGCTCCTTGAG-3′	EcoRI

82	P1720-F	5′-GGACTAGTAAGTGGGCAGCGGC-3′	SpeI
83	P1720-R	5′-GGAATTCGGGTAAATCCTCCTTG-3′	EcoRI
84	P1913-F	5′-GGACTAGTCTCAATGGGGGTACA-3′	SpeI
85	P1913-R2	5′-CGGATATCGGCACTCCAATCGGG-3′	EcoRV
86	P2220-F	5′-ACCCGATATCGCGAGGTGCAGCAGG-3′	EcoRV
87	P2220-R	5′-AAAAGAATTCTGCGAGTCCTCCCGA-3′	EcoRI
88	P2275-F	5′-GGACTAGTTTCGACATGCGCGAC-3′	SpeI
89	P2275-R	5′-GGAATTCCCCGTGCCCACCGGC-3′	EcoRI
90	P2238-F	5′-GGACTAGTCCTCGTGCACGAAAC	SpeI
91	P2238-R	5′-GGAATTCTGATGATTTCTGCTA-3′	EcoRI
92	P2338-R2	5′-GGAATTCCAACCTCACCGGAGT-3′	EcoRI
93	P2574-F	5′-GGACTAGTCGTCCTTATCTGCGGGAGCC-3′	SpeI
94	P2574-R	5′-GGAATTCTCACCTCCTGGGCTGCGGCG-3	EcoRI
95	DdrO-F	5′-GCCATATGACATTGAAACTGCACGA-3′	NdeI
96	DdrO-R2	5′-CGAAGCTTTCACCTCCTGGGCTGCGGCG-3′	HindIII
97	pprI-up-F	5′-CCCAAGCTTCCTCAAGCTCTACGCCCTTTAC-3′	HindIII
98	pprI-up-R	5′-AACTGCAGATGGCAGTGATTTCGCCTGTTTTGC-3′	PstI
99	pprI-dn-F	5′-CGGGATCCACTGGACGGGCCGTATCCACGAGC-3′	BamHI
100	pprI-dn-R	5′-GCTCTAGACTGGTGCTGCATGGTGCGCGGCTC-3′	XbaI
101	pprI-F	5′-CGCATATGCCCAGTGCCAACGTCAGCCCCCCTT-3′	NdeI
102	pprI-R	5′-CGAAGCTTCTGTGCAGCGTCCTGCGGCTCGTC-3′	HindIII

The restriction site sequence contained in the primer, and used for cloning subsequently, is underlined.

2.3.4 Restriction digestion and Ligation

Restriction endonuclease digestion of PCR amplified products and plasmids was carried out as per the manufacturer's protocol (New England Biolabs Ltd., UK). The DNA was incubated with restriction enzyme in appropriate buffer for 3-4h at 37^0C. To see whether DNA was digested properly or not, it was resolved on 1% agarose gels containing ethidium bromide and visualized on UV transilluminator. To avoid UV exposure, which causes mutations in nucleic acids the digested DNA mixed with green view^{TM} dye (Chromas Biotech, India) and resolved on 1% agarose gel without ethidium bromide. The DNA bands were visualized on blue light illuminator (Chromas biotech, India) and appropriate DNA bands were excised from the gel and eluted using gel elution kit (BRIT, India). For cloning of PCR amplified product in appropriate plasmid the ligation reaction was carried out by T4 DNA ligase, as per the manufacturer's protocol. For blunt end

ligation, if the insert was a PCR amplified product, it was first blunted by quick blunting kit or if insert was two primer annealed product the 5'phosphorylation was carried out by T4 polynucleotide kinase, as per manufacturer protocol. To prevent self-ligation of blunt end digested vector was dephosphorylated by calf intestinal phosphatase (CIP) as for manufacturer protocol. The insert to vector ratio was maintained at 3:1 for sticky end ligation or at 1:1 for blunt end ligation. Total DNA concentration was kept at 150 ng/20 µl of ligation reaction mix. The ligation mix was incubated for 1-2h at RT and used for transformation.

2.3.5 Plasmid Isolation

2.3.5.1 E. coli

Plasmids were isolated using plasmid isolation kit (BRIT, India) as per manufacturer's protocol. Briefly, recombinant *E. coli* cells were grown overnight and 3ml culture was pelleted down by centrifuging at 10000 rpm for 1 min. The pellet was resuspended in 200 µl of PA buffer (supplied by manufacturer) and cells were lysed by adding equal volume of PB buffer and gentle mixing by inverting the tube 3-4 times. Proteins and chromosomal DNA were precipitated by adding 300µl of PC buffer and gently mixing the solution by inverting the tube 3-4 times. The precipitate was separated by centrifugation at 13000 rpm for 10min. The supernatant which contained plasmids was passed through affinity column to bind plasmid DNA. The column was washed twice with wash buffer containing ethanol, followed by elution of the pure plasmid with TE buffer or nuclease free water (supplied with kit). The purity and quantity of plasmid was analyzed by 0.8% agarose gel electrophoresis and spectrophotometry (measuring ratio of $A_{260nm/280nm}$). The purified plasmid preparation was stored at -20^0C for further use.

2.3.5.2 D. radiodurans

D. radiodurans cells are more rigid and difficult to break open and therefore require pretreatment steps. Overnight grown culture was pelleted down and washed twice with 1 ml absolute alcohol to remove pigments. Pellet was resuspended in 180µl lysozyme (from 10mg/ml stock solution) and incubated at 37^0C for 30min. Proteinase K (from 20mg/ml stock solution) was then added (20 µl) and suspension further incubated at 37^0C for 15min. The remaining steps were same as described for *E. coli* plasmid isolation. The yield of the plasmid from *D. radiodurans* was less compared to *E. coli* wherein the plasmid copy number is very high.

2.3.6 Cloning and overexpression of Deinococcal *pprI* and *ddrO* genes in *E. coli*

The *pprI* (DR0167) and *ddrO* genes (DR2574) were individually PCR amplified from *D. radiodurans* genomic DNA using pprI-F/ppri-R or DdrO-F/DdrO-R primer pairs (Table2.2), repectively. The amplified DNA fragments were individually cloned in the overexpression vector pET21a at NdeI/HindIII restriction endonuclease sites. The recombinant plasmids generated were individually transformed into *E. coli* overexpression strain BL21 (DE3) pLysS (here after called as BL21). The cells were grown till OD_{600}nm reached 0.5, when overexpression of the PprI or DdrO proteins was induced by adding 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cultures. At different time intervals cell aliquots were pelleted down by centrifugation, washed with PBS and stored at -70^0C until further use.

Protein overexpressing cells were resupended in 20mM Tris-HCl pH 8.0 and lysed by sonication (Branson, UK), with a continuous pulse of 1sec ON/2 sec OFF for a total time of 2 min. The cell-free extract was prepared by centrifugation at 25000g for 30min at 4^0C. The supernatant was transferred to fresh tube. Proteins were estimated by Folin's Lowery method with BSA used as standard in the lower range of 2-25µg. About 30 µg of protein from the above cell free extract was resolved by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in the following Section 2.3.6.1. Intense bands of PprI and DdrO proteins were visualized after staining and destaining the gel.

2.3.6.1 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method used to separate proteins from a mixure of proteins based on their size. The gel solution was made by appropriately diluting 30% acylamide-bis acrylamide solution (29.2% Acrylamide, 0.8% Bis-acrylamide), 1.5M Tris-HCl buffer pH 8.8, 10% SDS, 1.5% APS and absolute TEMED (Table 2.3) to get desired percentage of resolving gels (10-14%) (Table 2.4). The final concentration of gel components are 375mM Tris-HCl pH 8.8; 0.1% SDS; 10µl of TEMED/20ml; 0.075% ammonium per sulphate (APS).

Table 2.3 List of reagents used for SDS-PAGE gels

Reagent	Composition
30% Acrylamide stock solution	29.2% Acrylamide, 0.8% Bis-acrylamide in 100ml distilled water, filter sterilized
1.5M Tris, pH 8.8	18.3g Tris in 100ml distilled water, pH 8.8 with HCl
0.5M Tris, pH 6.8	6.1g Tris in 100ml distilled water, pH 6.8 with HCl
10% SDS	10g SDS in 100ml distilled water
1.5% APS	0.15g APS in 10ml distilled water
TEMED	Absolute
Running Buffer	0.3% Trizma Base, 1.44% Glycine, 0.1% SDS
Coomassie Brilliant Blue G250 (CBB)	0.2% Coomassie Brilliant Blue G 250 in 40% Methanol, 20% Acetic Acid
Destaining Solution (DS)	DS-I: 50% Methanol, 10% Acetic Acid DS-II: 10% Methanol, 2 % Glycerol, 10% Acetic Acid

The gels were prepared by pouring the above prepared gel solution between two glass plates in the cassette with 1mm thick spacers and allowed to polymerize for 1h at room temperature. The gel was overlaid with water saturated butanol to remove air bubbles and smoothening of gel surface. After polymerization of resolving gel, butanol was removed by washing the gel surface with distilled water. A comb was fitted on to the gel cassette to form wells for sample loading and 4% stacking gel mix, prepared as shown in the Table 2.4 was poured on to the resolving gel in cassette and allowed to polymerize for at least 45 min at RT.

Table 2.4 Solutions required for preparation of resolving and stacking gels.

Reagent	Amount of reagent for 10% gel	Amount of reagent for 12% gel	Amount of reagent for 14% gel	Stacking gel 4%
Acrylamide-bis acrlylamide	6.675 ml	8.0 ml	9.3 ml	1.25 ml
1.5M tris (pH 8.8)	5.0 ml	5.0 ml	5.0 ml	2.5 ml*
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.1 ml
Dist. Water	7.125 ml	5.8 ml	4.5 ml	5.65 ml
1.5% APS	1.0 ml	1.0 ml	1.0 ml	0.5 ml
TEMED	10 µl	10 µl	10 µl	7.5 µl
Total	**20 ml**	**20 ml**	**20 ml**	**10 ml**

* The concentration and pH of Tris buffer for stacking gel was 0.5 M Tri-HCl, pH 6.8

Protein sample for loading on to SDS-PAGE gel was prepared by mixing equal volumes of cell-free extract (30 µg) solution and 2X Laemmli buffer (Solution contains 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8.) [100] mixed by vortexing. The mixture was boiled for 5 min on water both and after a short spin loaded onto the gel (about 10µl). The gel was run at 50V till dye front crossed the stocking gel and voltage was then increased to 100V. When the dye front reached the bottom edge of the gel, electrophoresis was stopped, gel was removed from glass cassette, rinsed with water and stained for 30min in CBB and de-stained in DS-I for 5 min fallowed by DS-II till the gel background become clear. The gel image was recorded with Gel documentation system (SYNGINE, Diversity model, UK).

2.3.6.2 Native-PAGE and western blotting.

To detect the GFP protein, Native PAGE and western blotting techniques were used. Native-PAGE was prepared exactly same as SDS-PAGE, except that SDS was replaced with Tween 20 in the polyacrylamide mix. Irradiated and unirradiated *D. radiodurans* cells carrying pKG with various promoters were withdrawn at different time points during PIR and cells were pelleted. Whole cell protein was extracted and estimated as described in section 2.3.6. The protein was mixed with protein loading dye (Laemmli buffer without SDS and β-mercaptoethanol), loaded onto the gel, and electrophoresis was carried out till the dye front reached the edge of the gel, at 4^0C. The gel was removed from gel cassette and the green fluorescent bands (GFP protein) were detected using cy2 filter in Gel documentation system (SYNGINE, Diversity model, UK).

After the image was documented the proteins from Native-PAGE gel was electro-blotted on to nitrocellulose membrane. The membrane was blocked with 1.5% gelatin solution. The membrane was incubated with anti-GFP antibody (1:5000) overnight at 4^0C. After washing, the membrane was incubated with the secondary antibody (anti-IgG antibody raised in rabbit, conjugated with alkaline phosphatase enzyme) for 1.5h at 4^0C. GFP protein bands were developed using NBT/BCIP substrate for alkaline phosphatase enzyme. The image was grabbed by a camera.

2.3.7 Construction of *pprI* knockout mutant of *D. radiodurans*

The *pprI* knockout mutant of *D. radiodurans* was constructed as per the strategy reported earlier [87, 101], but with some modifications. In brief, the 1104 bp spectinomycin antibiotic cassette (Spc^R) was excised from the p13840 plasmid [102] by digesting it with EcoRI/HindIII restriction enzymes followed by blunt end ligation to SmaI restriction digested pBluescript plasmid to generate the pBS-*spc* construct. The 500bp immediate upstream and downstream sequences of

pprI (DR0167) gene of *D. radiodurans* were PCR amplified using the pprI-up-F/pprI-up-R and pprI-dn-F/pprI-dn-R primers (Table 2.2), respectively. The *pprI*-up and *pprI*-down DNA fragments were cloned in HindII/PstI and BamHI/XbaI restriction sites in the pBS-*spc* construct, upstream and downstream to spectinomycin cassette respectively, to generate the pBS-Δ*pprI* plasmid. Cloning of correct insert, its orientation and sequence was confirmed by PCR and DNA sequencing (The schematic illustration of construction is given as Fig. 5.6 in Chapter 5). The pBS-Δ*pprI* construct, which is a suicide vector for *D. radiodurans*, was transformed into *D. radiodurans* competent cells and selected on the TGY agar plate containing spectinomycin (100 µg/ml). Positive transformant colonies were passaged once in every 48h on fresh TGY agar plates containing appropriate antibiotic for 15 generations to obtain homozygous *pprI* deletion mutant (Δ*pprI*). The mutation was ascertained by PCR amplification by using (a) either the pprI-up-F/pprI-dn-R primers which give ~2.1kb DNA fragment which contains 500bp each of the up and down fragments of *pprI* gene in wild type, or 1.9kb fragment which contains 500bp up and down frgamens and spc^R cassette in *pprI* mutant, or both the fragments in case of heterozygous mutant, or (b) by using pprI-F/pprI-R primers to ascertain absence of *pprI* gene from *pprI* knockout mutant.

2.3.8 Co-transformation of pKG with gene promoters in *E. coli* strains overexpressing either DdrO or PprI proteins from *D. radiodurans*.

To evaluate the effect of *D. radiodurans* regulatory protein DdrO and PprI on promoter activity, each promoter clone was transformed into *E. coli* BL21 cells, regulatory proteins were individually overexpressed and promoter activity was assessed by monitoring change in GFP fluorescence. The pKG vector carrying various *D. radiodurans* promoters were transformed into *E. coli* BL21 cells, carrying either pET21 plasmid or pET21-*pprI* or pET21-*ddrO* plasmids (Fig.2.1).

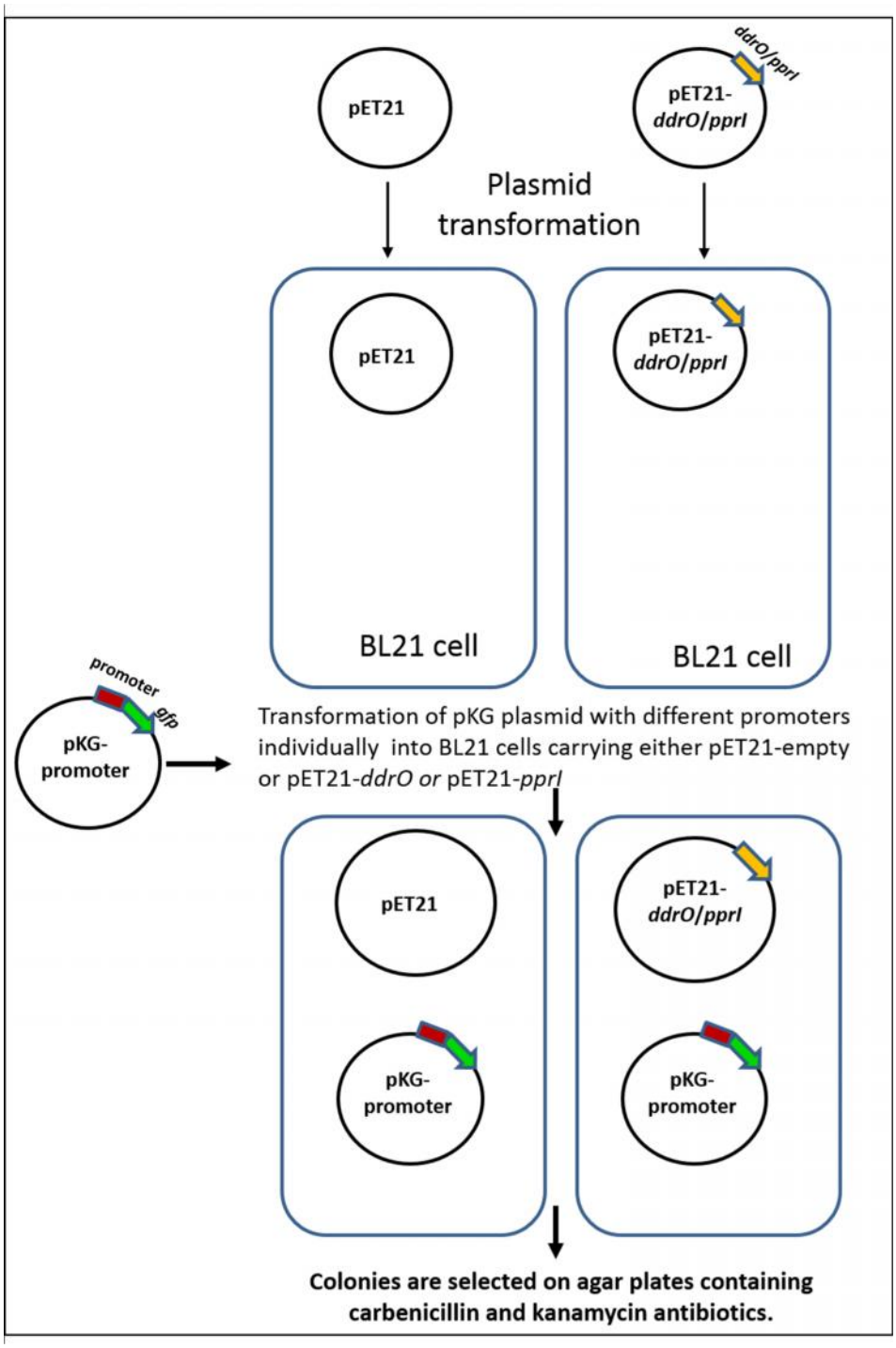

Fig. 2.1. Schematic representation of co-transformation. Plasmids pET21 or pET21carrying *ddrO* or *pprI* genes (pET21-*ddrO*/*pprI*) respectively, were individually transformed into competent BL21 cells. Plasmid pKG carrying different Deinococcal promoters were transformed into *E. coli* BL21strain cells carrying either pET21 or pET21-*ddrO* or pET21-*pprI* plasmids. The transformants were selected on agar plate containing carbenicillin and kanamycine antibiotics for both pET21 and pKG plasmids.

The transformants were selected on LB agar plates containing carbenicillin and kanamycin (double antibiotics for both pET21 and pKG plasmids respectively). The recombinant *E. coli* cells were inoculated in LB broth with appropriate antibiotics and grown overnight. An aliquot of overnight culture was reinoculated again and allowed grow at 37°C till OD_{600} reached 0.5 and then 1mM of IPTG was added and incubation continued for 3 more hours. From these cultures a 0.5 ml culture aliquot in 1.5 ml microfuge tube was pelleted down, the pellet was resuspended in 1 ml PBS and GFP fluorescence was quantified as detailed earlier in section 2.2.5. The expression of PprI and DdrO proteins in *E. coli* BL21 cells was ascertained by extracting whole cell proteins from the cultures and resolving on the by SDS PAGE followed by staining with Coommassie Brilliant Blue G-250 (CBB).

2.3.9 RNA isolation and transcription start site (TSS) mapping

Total RNA was isolated from unirradiated and irradiated *D. radiodurans* cells, using RNeasy mini kit and its quality/quantity measured exactly as described earlier [103]. In brief, the cultures of irradiated and unirradiated *D. radiodurans* were first recovered for 30 min at 32^0C. About 5ml of each culture was pelleted down, washed with PBS and resuspended in 0.5 ml of RNA protect solution (supplied with kit) and incubated for 10 min at room temerature. The cells were centrifuged at 5000rpm for 10 min, supernatant was discarded and tubes dabbed on tissue to remove traces of RNA protect solution. The pellet was resuspended in 180µl of lysozyme solution (10mg/ml) and incubated at 37^0C for 30 min. Then 20 µl of proteinase-K (20mg/ml) was added and further incubated for 15 min. Further steps were followed as per manufacturer's protocol. The quantity and quality was checked by measuring the absorbance at 260nm and 280nm. The value of ~2.0 obtained from ratio of 260/280nm ascertained the purity of RNA.

Transcription start site (TSS) for two genes was mapped by primer extension technique as described earlier [104]. The gene specific reverse primer that is complementary to an mRNA sequence ~100 bases downstream of the anticipated 5' end was designed. The primer (10 pM) was radio labeled at it 5' end with 120 µCi of [γ-^{32}P] ATP (BRIT, India) using T4 polynucleotide kinase. The unincorporated [γ-^{32}P] ATP was removed by passing the reaction mix through Microspin G-25 column (Roche, India). The sequence cycle reaction was carried out using Thermo Sequenase cycle sequencing kit, as per manufacturer instructions. In brief, the sequence reaction was set up in four tubes in which chain elongation inhibitors ddATP, ddGTP, ddCTP and ddTTP were added separately. The gene along with 500bp upstream sequence wherein TSS was to be found was amplified by PCR and used as template to generate single base ladder. PCR reaction was done with radiolabeled primer (1 pM) containing 400000cpm radio activity, reaction was allowed to continue for 40 cycles. The c-DNA was synthesized by RevertAid H minus M-MulV reverse transcriptase as per manufacturer protocol, from 3µg of total RNA using same ^{32}P-labelled primer which was used for generation of ladder. The c-DNA and PCR sequencing reaction products were heat denatured at 94^{0}C for 3min, immediately cooled on ice for 3min and resolved by denaturing polyacrylamide gel (6% acrylamide-8M urea) electrophoresis using 50w constant power. The electrophoresis was continued for 4h till the dye front reached the bottom edge. The gel was removed from the cassette and vacuum-dried onto a filter paper (Whatman No1) and exposed to phosphor screen for 24h and image was captured by Typhoon Trio Variable mode imager (GE-Healthcare).

2.4 Bioinformatic analyses

D. radiodurans genome sequence and map was accessed through KEGG website (http://www.genome.jp/dbget-bin/www_bget?dra) [99]. Prediction of presence or absence of *E.*

coli like gene promoters and transcription start site in *D. radiodurans* in 500bp upstream DNA sequence of selected ORFs was carried out by using fruitfly software (http://www.fruitfly.org/seq_tools/promoter.html) [105] or BPROM software (http://linux1.softberry.com/berry.phtml). Sequence alignments were performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) or BioEdit softwares (Ibis biosciences, CA). The plasmid restriction map and primer design was done using DNAMAN software (Lynnon Biosoft, Germany). Cropping and resizing of the capture images was done using the online tool Resizeimage.net (http://resizeimage.net/). All statistical analyses and graphs were plotted using Origin-8 software (http://www.originlab.com/).

Chapter 3

Construction of a promoter probe shuttle vector and its validation

The study of promoter structure and activity in bacteria *in vivo* needs a suitable promoter-less reporter gene in a plasmid shuttle vector which can replicate both in the original host and the cloning host. Reporter systems represent an important tool for identifying and characterizing promoter regions in DNA sequences. Various promoter-less reporter genes, coding for easily detectable and quantifiable proteins, have been used for construction of a number of promoter-probe vectors to test the activity of promoters and their regulation in bacteria [20] and plants [106]. The transcriptional and translational fusion of promoter-containing DNA fragments immediately upstream of the reporter gene drives expression of the reporter genes as governed by the regulatory sequences present in the promoter fragment and the regulatory proteins present in the host. Application of such tools to *D. radiodurans* would facilitate the study of genetic mechanisms regulating gene expression during normal and stress affect growth including post irradiation recovery.

A number of different reporter genes like β-galactosidase (*lacZ)*, Luciferase (*lux*), acid phosphatase (*phoN*) and others are available for visual and quantitative assessment of gene expression in prokaryotes. The quantification of gene expression or promoter activity *in vivo*, in real time, and at the level of single cells is problematic with the *lacZ* and *phoN* reporter systems, while the *lux* reporter requires very expensive equipment. Recently, the *gfp* reporter gene encoding the light-emitting green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* [107, 108] has been used which allows non-invasive *in vivo* analysis and automated quantification [109]. Promoter-probe vectors based on the streptococcal pMV158 plasmid and carrying the gene encoding the highly fluorescent GFP have been used and validated in *E. faecalis* [110] and *L. lactis* [111]. The advantage of this system is that it works without additional expensive substrates and, unlike other reporter proteins, and does not suffer from background problems [109, 112]. The

study showed that the widely used GFP system could be applied in pigmented bacteria also regardless of the accessory pigments like chlorophyll that might absorb some of the emitted light. The *gfp-mut2* (with S65A, V68L, S72A mutations) gene has been successfully used as reporter in the intensely blue-green coloured cyanobacteria [22, 113].

3.1 Vector construction

To investigate radiation induced gene expression in *D. radiodurans*, a green fluorescent protein (GFP) based promoter probe shuttle vector was constructed, using the existing *Deinococcus/E. coli* shuttle vector pRAD1 [114] as a template. The promoter-less *gfp-mut2* gene, from plasmid pAM1956 [22], was PCR amplified using Gfp-F and Gfp-R primers (Table 2.2), restriction digested with XhoI/BamHI restriction endonucleases and cloned in pRAD1 at identical sites. The resultant plasmid was termed pRAD-gfp (Fig 3.1A). This recombinant plasmid vector was transformed into competent *E. coli* (DH5α) and *D. radiodurans* cells. The cloning of *gfp-mut2* gene was confirmed by colony PCR using primers P5-F and Gfp-R primers (Table 2.2), which yielded a single band of ~0.7 kb size confirming the cloning (Fig3.1B). The correctness of the sequence was confirmed by DNA sequencing. This plasmid vector showed strong GFP green fluorescence in *E. coli* but a very weak fluorescence in *D. radiodurans*, even in the absence of any cloned promoter (Fig 3.1C). Such leaky GFP expression suggested a possible read-through by RNA polymerase possibly from the *amp* (ampicillin) gene promoter, which is active in *E. coli* but not in *D. radiodurans*. Prevention of leaky background fluorescence and further improvisation of vector involved sequential cloning of desired DNA fragments encoding *term116* transcription terminator, *gfp*-mut2 gene, a new multiple cloning site (MCS) and the kanamycin resistance (*aphII*) gene at suitable restriction sites. These efforts ultimately yielded a zero background promoter probe shuttle vector for *D. radiodurans*.

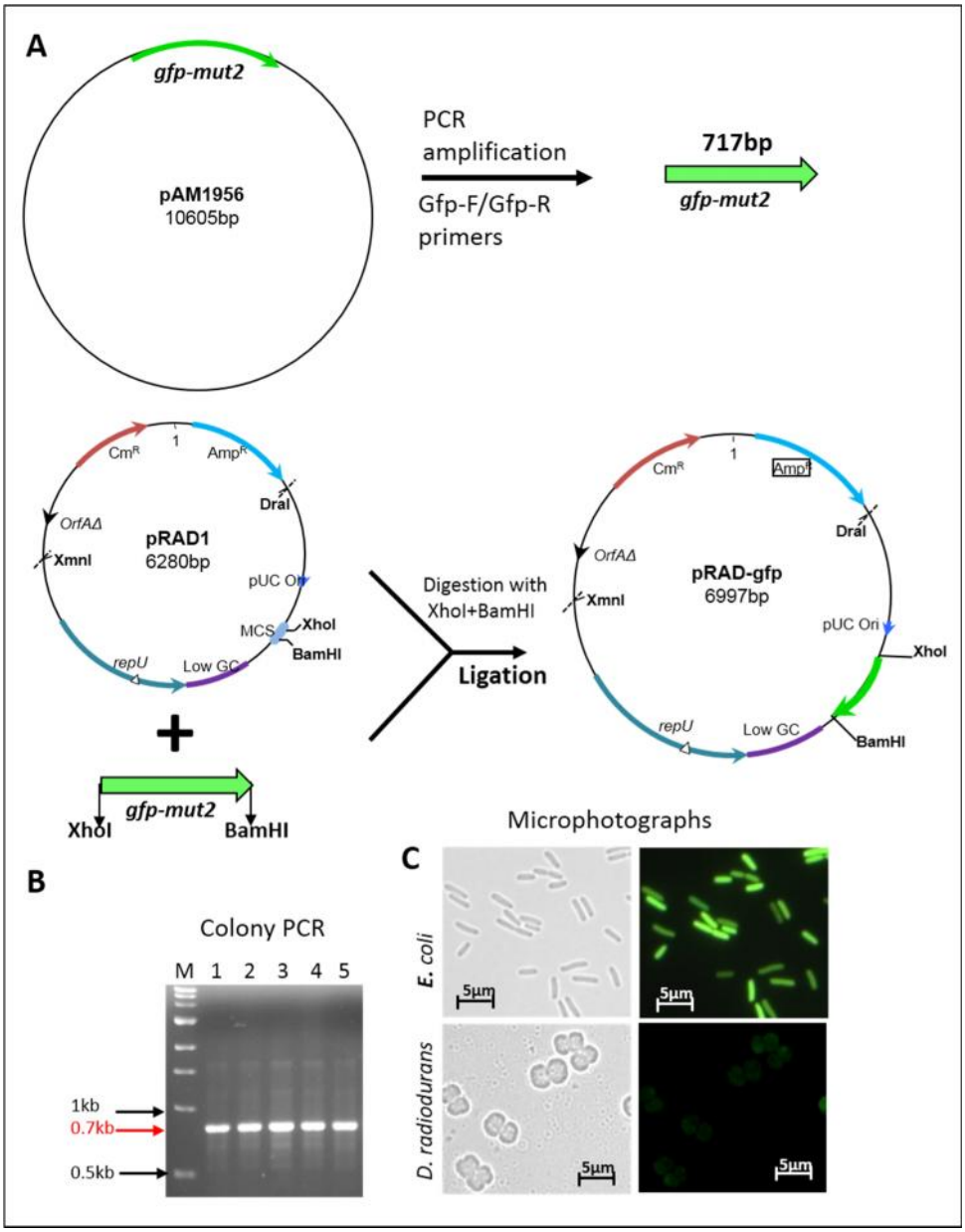

Fig. 3.1 Cloning of *gfp-mut2* gene in pRAD1 plasmid. (A) Schematic representation showing the PCR amplification of 717bp *gfp-mut2* gene from pAM1956 plasmid using Gfp-F/Gfp-R primer set and it's cloning in the pRAD1 plasmid at XhoI/BamHI sites to obtain pRAD-gfp plasmid. (B) The colony PCR of the pRAD-gfp plasmid transformants, performed using P5/Gfp-R primers. The amplicon was resolved on 1% agarose gel. The various lanes contained 1kb marker (M) and colony PCR products of 5 different random colonies (lanes1-5). (C) Bright field and fluorescence microphotographs of *E. coli* and *D. radiodurans* harboring pRAD-gfp plasmid (magnification: 100X, oil immersion).

3.1.1. Sequential cloning of term116 transcriptional terminator and green fluorescent protein (*gfp-mut2*) gene as a reporter

It was necessary to clone a suitable transcriptional terminator upstream of promoter sequence to ensure unambiguous detection and quantification of promoter activity in both *E. coli* as well as *D. radiodurans*. To stop the leaky expression, a 36bp *rho* independent transcription terminator *term116* [21] from DR_0116 gene of *D. radiodurans* was chosen. Direct cloning of term116 in pRAD1 was not possible due to unavailability of suitable restriction site upstream of MCS. Therefore, terminator and *gfp* gene were first cloned in pBlueScript (pBS) and then moved to pRAD1 at suitable restriction sites. The 36bp terminator was synthesized as two complementary oligonucleotides using primers term116-F and term116-R (Table 2.2**)** and annealing them (boiling for 5 min, cooling at room temperature) to get double stranded *term116* terminator. This was cloned by blunt end ligation in the pBS plasmid, which was previously digested with HincII restriction endonuclease. The resultant plasmid was termed pBS-ter (Fig 3.2A). The insertion of *term116* in pBS-ter was confirmed by colony PCR with M13 forward (M13-F) and M13 reverse (M13-R) primers, which correspond to pBS plasmid (Fig 3.2B) and its correctness was ascertained by DNA sequencing.

The PCR amplified *gfp-mut2* gene (hereafter called *gfp*) was digested with EcoRI/BamHI and cloned in pBS-ter plasmid at identical restriction sites, downstream to the *term116* transcription terminator (Fig 3.2A). After confirmation of cloning by colony PCR using Gfp-F, Gfp-R primer set (Fig 3.2C) and DNA sequencing, the *ter116-gfp* fragment was excised from pBS-ter-gfp using XhoI/BamHI restriction enzymes and cloned in the same restriction sites in pRAD1 vector. The resultant construct pRAD-ter-gfp (Fig 3.3A) was confirmed by PCR amplification using pRAD1 forward (P5-F) and *gfp* reverse (Gfp-R) primer set (Fig 3.3B).

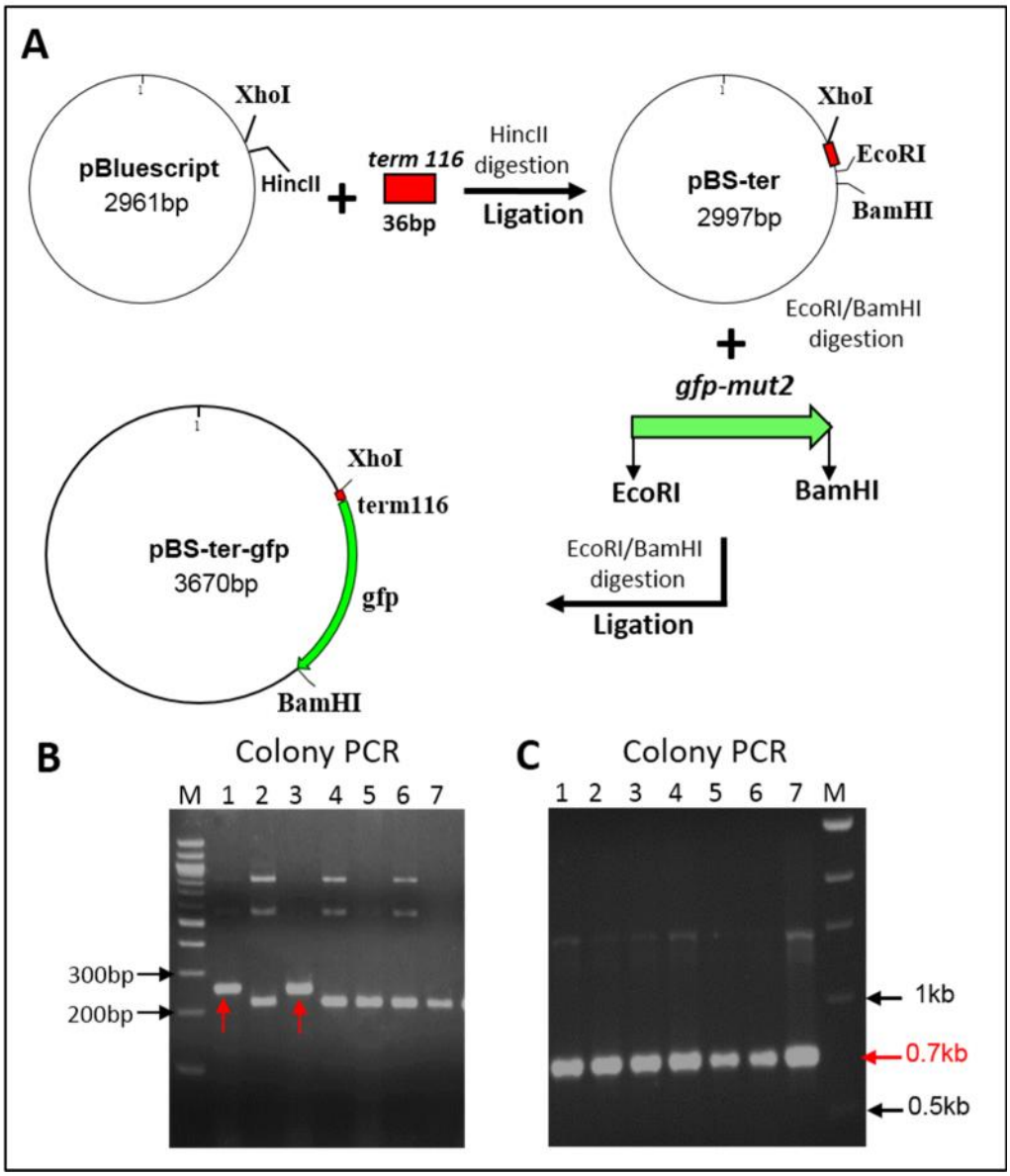

Fig. 3.2 Cloning of *term116* and *gfp-mut2* in the pBS plasmid. (A) Schematic representation of cloning of 36 bp transcription terminator (term116) obtained by annealing of primers term116-F and term116-R. Term116 was cloned in pBlueScript (pBS) plasmid at HincII site by blunt end ligation to obtain plasmid pBS-ter. The *gfp-mut2* gene was amplified from pAM1956 plasmid, using Gfp-F/Gfp-R primers digested with EcoRI/BamHI and cloned in pBS-ter plasmid at identical sites to obtain pBS-ter-gfp plasmid. (B) The colony PCR of pBS-ter transformants using M13-F and M13-R primers. Lane M contained 100bp DNA marker and lanes 1-7 carried PCR product from 7 randomly picked up colonies. The appearance of expected size (~250 bp) bands in positive clones is indicated with red arrows. (C) The colony PCR of pBS-ter-gfp transformants by Gfp-F and Gfp-R primers. Lanes 1-7 contained amplicons from 7 different transformants. Single band of expected size (~0.7 kb) is marked. Lane M: 1 kb DNA marker

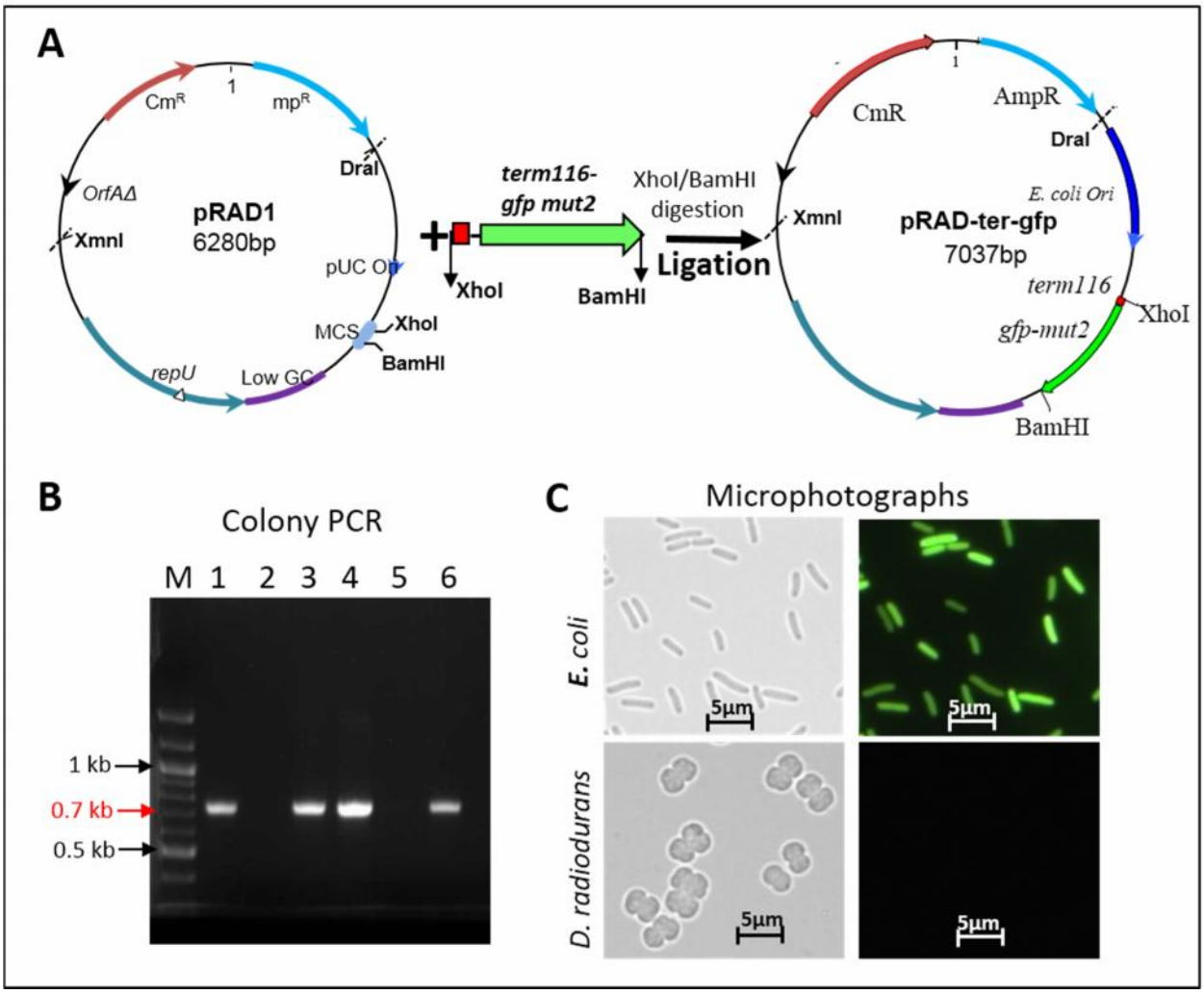

Fig. 3.3 Cloning of *term116-gfp* in pRAD1. (A) Schematic diagram of cloning of *term116-gfp* excised from pBS-ter-gfp plasmid with XhoI/BamHI in pRAD1 plasmid at identical sites to obtain pRAD-ter-gfp vector. (B) Confirmation of pRAD-ter-gfp plasmid by colony PCR with P5-F and Gfp-R primers. The various lanes contained 100 bp DNA marker (lane M), and PCR products of 6 randomly picked colonies (lanes 1-6). Appearance of a single band of ~0.7 kb size in lanes 1, 3, 4 and 6 is marked. (C) Bright field and fluorescence microphotographs of *E. coli* and *D. radiodurans* harboring pRAD-ter-gfp plasmid (magnification: 100X, oil immersion).

However, this plasmid construct continued to give leaky GFP fluorescence in *E. coli* (DH5α) cells (Fig 3.3C), though not in *D. radiodurans.* It appeared that *term116* may be specific for *D. radiodurans* but might not be active in *E. coli*. For accurate and consistent results one needs a

shuttle vector with clean background in both the hosts. Plasmid pRAD-ter-gfp also lost all the restriction sites in MCS during its construction. Therefore it was necessary to further modify the vector to (a) achieve zero background reporter expression, and (b) provide it with a new MCS for further cloning. The plasmid pRAD-ter-gfp was, therefore, not used any further and a new approach was undertaken, as described in Sections 3.1.2 to 3.1.4 below.

3.1.2 Replacement of ampicillin and chloramphenicol resistance genes with *aphII* gene, as a single selection marker for the plasmid in both the hosts

The *amp* gene promoter was suspected to be responsible for leaky expression of GFP reporter in *E. coli*. Therefore *amp* gene, which works only in *E. coli*, and chloramphenicol acetyltransferase (*cat*) gene, which works only in *D. radiodurans*, were excised from pRAD1 to be replaced with single antibiotic marker whose promoter works in both the organisms and thereby gives resistance in both the hosts. The aminoglycoside phosphotransferase-II gene (*aphII*) conferring kanamycin (Km) resistance was excised from pUC4K plasmid using HincII site at both the ends to get the 1.25kb *aphII* gene (Fig. 3.4) which has its own promoter. A 305bp extra sequence downstream of stop codon, which has no function in antibiotic resistance, was also excised along with *aphII* gene from pUK4K while digesting with HincII enzyme.

To excise *amp* and *cat* genes (total ~2.9 kb) the pRAD1 plasmid was digested with XmnI/DraI (Fig3.4). These genes could have been excised directly from pRAD-ter-gfp plasmid to save one extra step of cloning of *term116-gfp*, but the XmnI/DraI sites are present in *term116-gfp* also. Therefore first *amp* and *cat* genes were excised out from pRAD1 and replaced by *aphII*, and then term116-gfp was re-cloned in it. After deleting the *amp* and *cat* genes from pRAD1, the

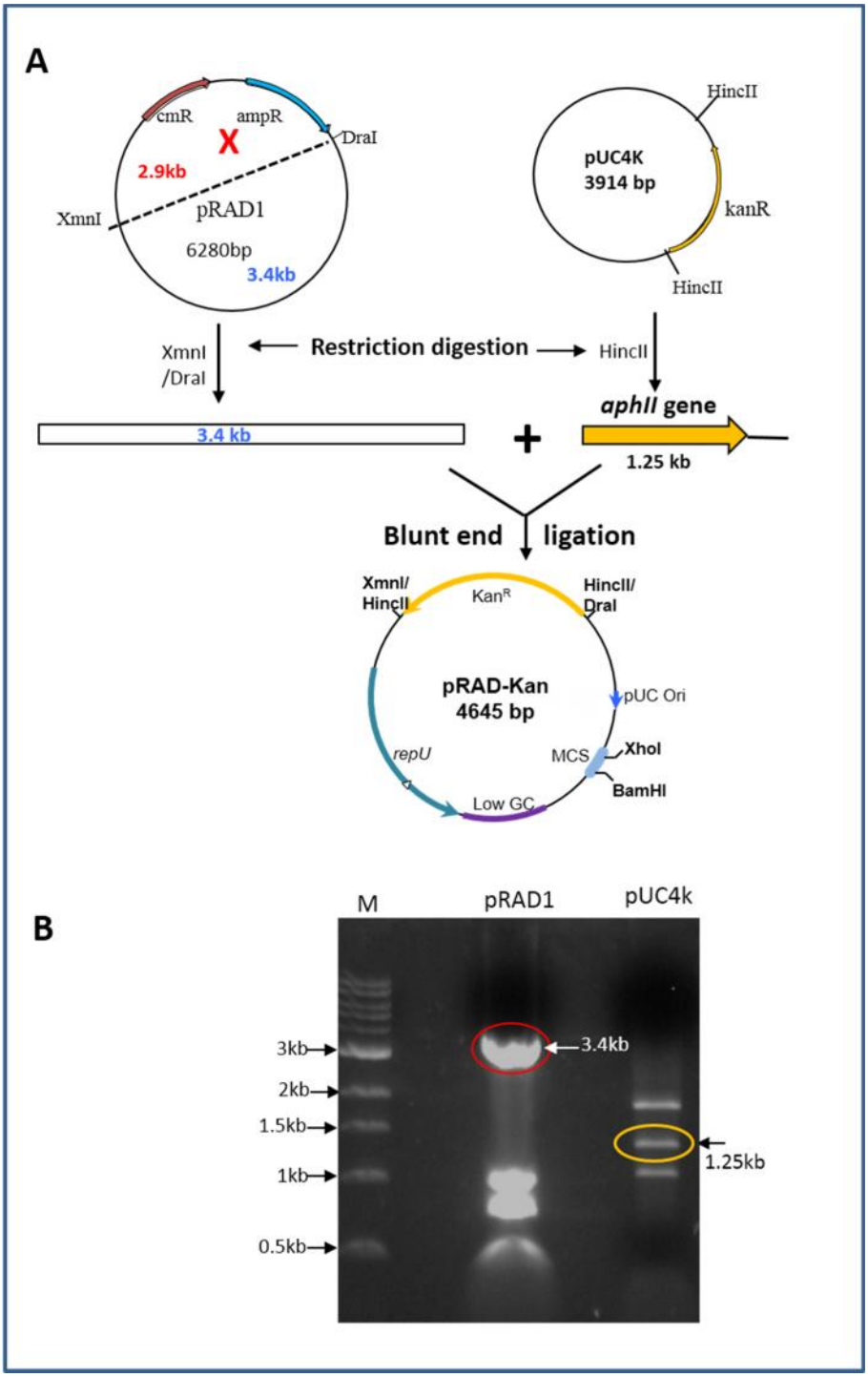

Fig. 3.4. Replacing the *amp* and *cat* genes with *aphII*. (A) Schematic diagram showing construction of pRAD-Kan. Plasmids pRAD1 and pUC4K were digested with XmnI/DraI and HincII restriction endonucleases, respectively. The 3.4 kb DNA fragment from pRAD1 and *aphII* gene from pUC4K were blunt end ligated to obtain pRAD-Kan. (B) The digested products from pRAD1 and pUC4K [mentioned in (A)] were electrophoretically resolved on agarose gel. The 3.4 kb and 1.25 kb bands are circled.

remaining 3.4 kb DNA fragment (Fig 3.4B) was blunt end ligated to the 1.25 kb *aphII* gene (Fig 3.4B) to generate the plasmid pRAD-Kan (Fig3.4). This vector provided kanamycin resistance in both *E. coli* and *D. radiodurans*.

3.1.3 Re-cloning of *term116-gfp* and new MCS in pRAD-Kan plasmid

To reintroduce the reporter gene along with transcription terminator, *term116-gfp* (hereafter called as *ter-gfp*) DNA fragment was excised from pBS-ter-gfp using XhoI and BamHI as shown in the Fig. 3.3 and cloned in pRAD-Kan plasmid, which yielded the plasmid pKTG (Fig 3.5A). This step of cloning of *ter-gfp* fragment in XhoI/BamHI sites of pRAD1 deleted almost all the unique restriction sites from the multiple cloning site (MCS) from pKTG. To reintroduce the MCS, two complementary oligonucleotides MCS-F and MCS-R (Table 2.2) (44b each) containing eight unique restriction endonuclease sites were synthesized. The double stranded MCS was obtained by annealing of the two primers (1:1 ratio) by boiling at 100^0C for 5 minutes followed by cooling at room temperature. This double stranded MCS was cloned by blunt end ligation in the EcoRV site between the *term116* and *gfp* gene in the pKTG plasmid, to obtain the plasmid pKGX (Fig 3.5A). The construct was transformed to competent *E. coli* cells. The kan^r positive clones were confirmed by colony PCR with Gfp-F and Gfp -R primers (Table 2.2) (Fig 3.5B). When observed under fluorescence microscope, these recombinant *E. coli* cells were found to still fluoresce brightly even in the absence of a promoter sequence (Fig 3.5C). Thus while *term116* is effective in *D. radiodurans* the sequence responsible for leaky expression of GFP in *E. coli* seemed to be still present in the pKGX plasmid.

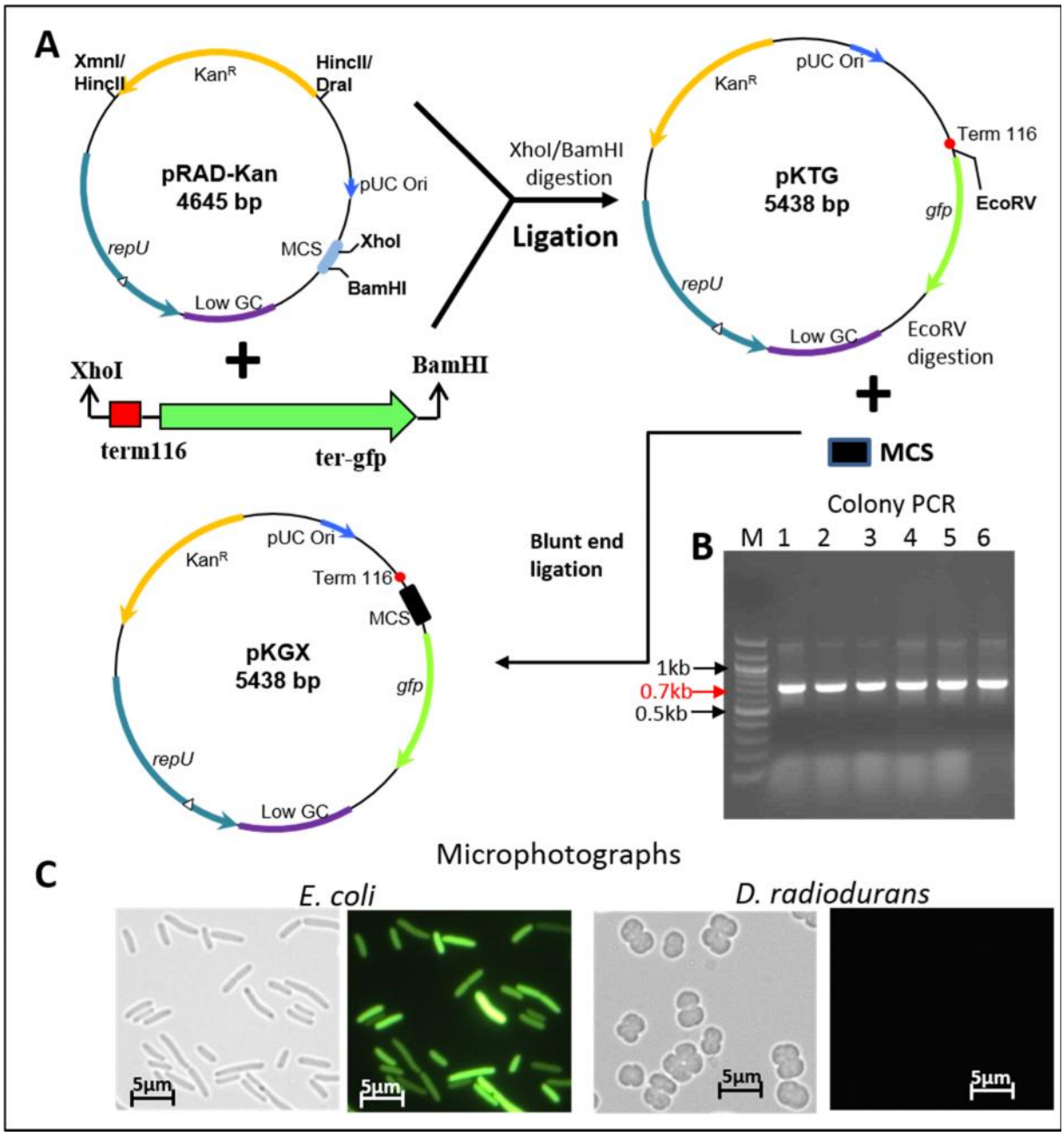

Fig. 3.5 Cloning of *term116-gfp* and MCS in pKTG plasmid. (A) The *gfp* gene along with *term116* was excised from pBS-ter-gfp with XhoI/BamHI restriction enzymes and cloned in pKTG. The 44bp MCS was synthesized by two primer (MCS-F and MCS-R) annealing and cloned in between term116 and *gfp* gene by blunt end ligation, to yield pKGX. (B) Colony PCR with Gfp-F/Gfp-R primers confirming presence *term116-MCS-gfp* fragment in the pKGX plasmid as a single intense band (~700bp). (C) Light and fluorescence microphotographs showing the leaky GFP expression (fluorescence) in *E. coli* cells and its absence in *D. radiodurans*.

3.1.4 Deletion of a 386bp sequence from the plasmid pKG eliminates leaky expression

A 386 bp uncharacterized DNA sequence was located between *E. coli* origin of replication and *term116* in plasmid pKGX. Bioinformatic analysis of this sequence revealed presence of a potential *E. coli* promoter-like sequence (-10 and -35 hexamers) present upstream of *ter116* in pKGX, which could be the possible cause of leaky expression of GFP in *E. coli* cells (Fig 3.6).

E. coli repication origin ----- 5'- TGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGT
TATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCG
CAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATAC
GCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCC
CGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGC
ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGGAGCGGATAAC
AATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGC –3'-------*term116-MCS-gfp*

Fig. 3.6 The 386 DNA sequence in pKGX. The sequence upstream of *term116* in pKGX plasmid. The *E. coli* -10 and -35 promoter-like sequences which were suspected to cause leaky expression of GFP are highlighted and underlined.

There were no suitable restriction sites available in the sequence to remove this 386 bp DNA sequence from pKGX. The 386 bp DNA sequence between *term116* and *E. coli* replication origin (*ori*) and an additional 305bp DNA sequence (Fig. 3.7A) downstream to *aphII* gene (derived from pUC4K along with *aphII* gene) which has no role in kanamycin resistance were removed by (a) first PCR amplifying entire pKGX as two fragments, one consisting of *ter-mcs-gfp*-pRAD1 DNA region (~3kb) and the other consisting of Kan^r-Ori (~1.7kb), using pRAD-F/pRAD-R and Kan-F/Ori-R primer pairs, respectively, and (b) blunt end ligating the two fragments to generate the pKG vector minus the 386 bp and 305 bp sequences (Fig. 3.7). The pKG vector (with no cloned

promoter) was transformed into *E. coli* and *D. radiodurans*. The positive clones were confirmed by colony PCR with Gfp-F/Gfp-R primers (Table 2.2). The accuracy of pKG vector sequence was verified by nucleotide sequencing. The promoter probe shuttle vector pKG was completely sequenced using primers pKG-1 to pKG-9 (Table 2.2) and the sequence (Appendices) was deposited in the public database (GenBank™ Accession number: KF975402**)**.

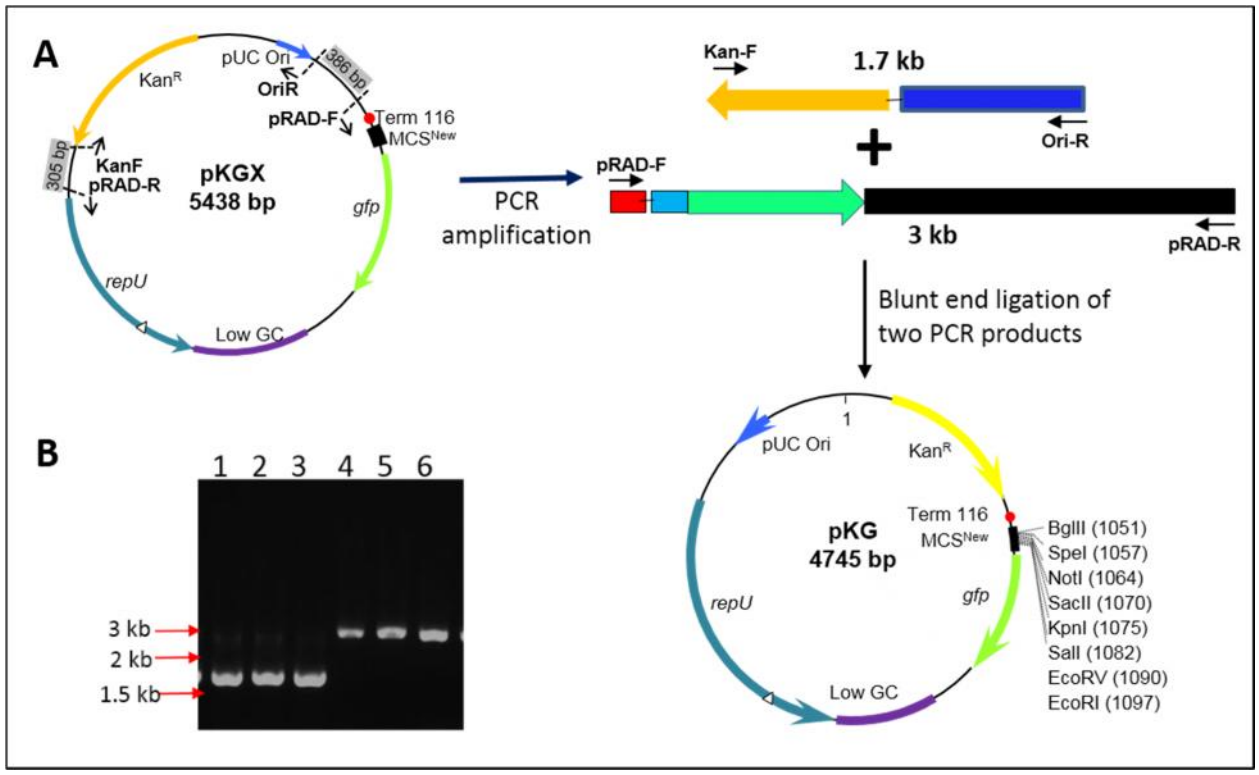

Fig. 3.7 Removal of unwanted extra sequences from pKGX plasmid. (A) Schematic showing deletion of the 386 bp and 305 bp undesirable extra regions in the pKGX plasmid. The 1.7 kb and 3kb DNA fragments, obtained by PCR with Kan-R/Ori-R and pRAD-F/pRAD-R primer sets respectively, were ligated to obtain the final vector pKG. (B) The PCR amplified products shown in (A) were electrophoretically resolved. Lanes 1-3 show amplified product of 1.7 kb and lanes 4-6 show the 3 kb DNA band.

3.2 Validation of the promoter probe shuttle vector pKG

The key features of promoter probe shuttle vector constructed were as follows (i) plasmid maintenance in both the hosts (*E. coli* and *D. radiodurans*), (ii) no reporter expression/activity in the absence of promoter, (iii) availability of suitable restriction endonuclease sites in MCS, for directional cloning of desired DNA fragments to be tested for promoter activity (iv) ease of detection and quantification of reporter expression and activity, and (v) provision for monitoring of reporter expression and in turn promoter activity, in real time. These features needed to be validated before using the vector for promoter screening. The replication of the plasmid in both *E. coli* and *D. radiodurans* was tested by transforming the pKG vector in both organisms. In both cases, colonies were observed on agar plate with appropriate antibiotic. Presence of plasmid in these recombinants was confirmed by colony PCR for *gfp* gene with pKG-F8/Gfp-R primers (Table 2.2) (Fig. 3.8A), and plasmid isolation from both organisms (Fig. 3.8B). The vector has eight unique sites for common restriction endonuclease in MCS for easy cloning of any DNA fragment for promoter screening. Digestion of pKG at each of these sites was individually ascertained (Fig. 3.8C). Fluorescence microscopic observation of both *E. coli* and *D. radiodurans* cells harboring pKG plasmid showed clear background (Fig. 3.8D), thereby confirming zero background fluorescence of GFP reporter from pKG in both the hosts. The GFP does not require any substrate for its fluorescence and it also offers *in situ* real time monitoring, by visual fluorescence microscopic detection. It is also easy to quantify GFP expression and in turn promoter activity of cloned DNA fragment by fluorescence spectrophotometry.

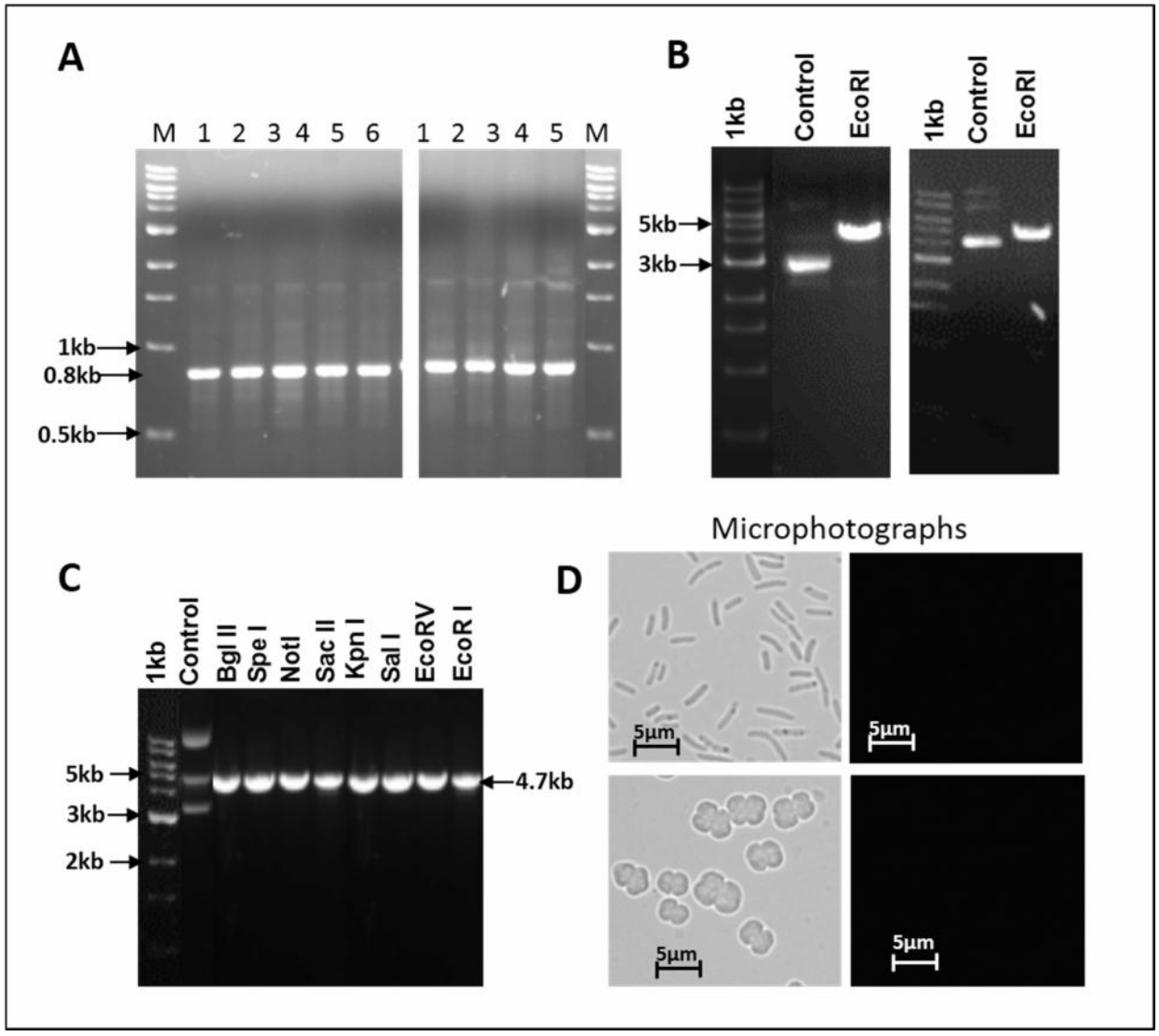

Fig. 3.8 Validation of pKG plasmid vector. (A) Confirmation of plasmid in both *E. coli* and *D. radiodurans* transformants by colony PCR using pKG-F8 and Gfp-R primer set. Lane M contained 1 kb DNA marker and lanes 1-6 in first gel and lanes 1-5 in second gel contained PCR product from random colonies picked up from *E. coli* and *D. radiodurans*, respectively. (B) The uncut pKG plasmid (control) and its EcoRI digested product isolated from *E. coli* or *D. radiodurans* cells was resolved on agarose gels. The 1kb DNA marker was used. (C) The pKG vector was digested with eight restriction enzymes for which unique restrictions sites were present in MCS. The digested products were resolved on agarose gel along with undigested (Control) DNA and 1 kb DNA marker. (D) Bright field and fluorescence microphotographs of *E. coli* and *D. radiodurans* clones harboring pKG plasmid.

3.2.1 Assessment of promoter activity using pKG vector

Two known *D. radiodurans* promoter P*groESL* and P*ssb* were used to validate the pKG vector in both *E. coli* and *D. radiodurans*. P*groESL* is a strong deinococcal promoter whose expression does not change significantly in radiation stress. The activity of this promoter was reported earlier by Appukuttam *et al* 2006 [15, 19]. P*ssb*, the promoter of single stranded DNA binding protein gene (*ssb*), is a known radiation inducible promoter reportedby Ujaoney *et al* 2011. Six fold induction of the *ssb* promoter has been shown during post irradiation recovery (PIR) following 7 kGy gamma radiation [11].

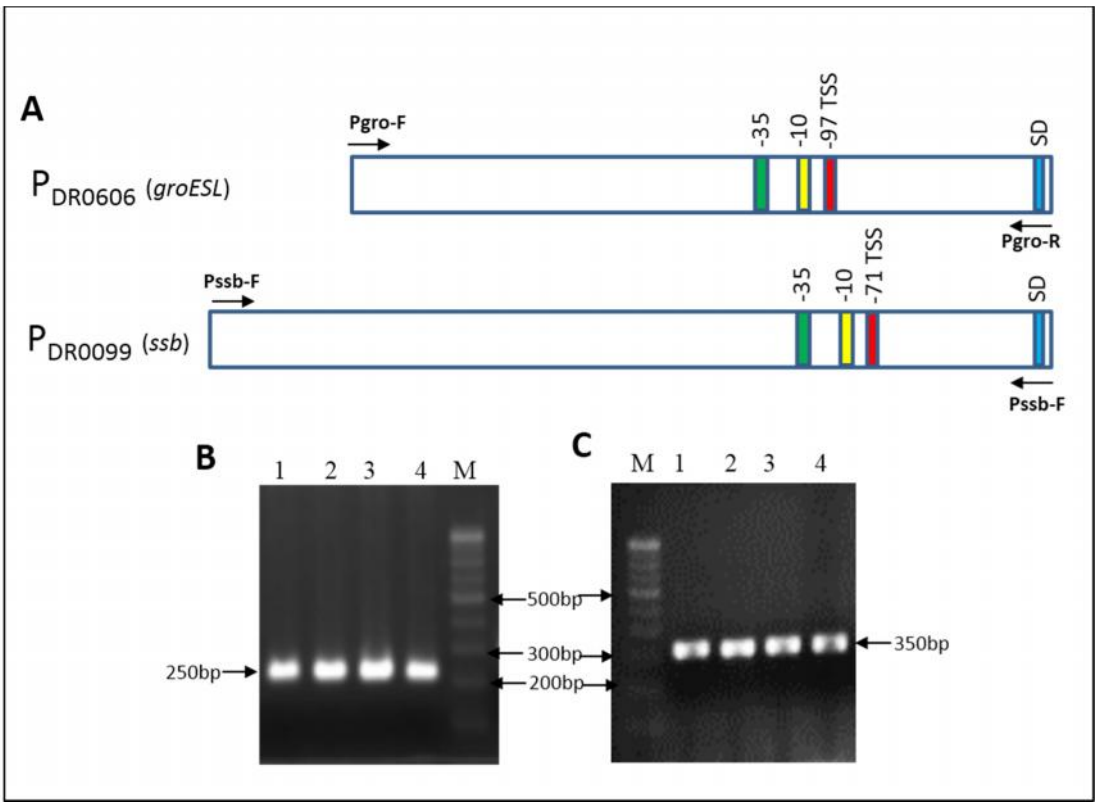

Fig. 3.9 The structure of P_{DR0606} and P_{DR0099} promoters. (A) Schematic representation of the P_{DR0606} and P_{DR0099} promoters. Shine-Dalgarno sequence (SD), transcription start site (TSS), *E. coli* like promoter sequences -10 and -35 are indicated in different color strips. Position of TSS is shown from the first ATG codon. (B and C) The P_{DR0606} (B) and P_{DR0099} (C) promoters were amplified in four tubes with Pgro-F/Pgro-R and Pssb-F/Pssb-R primer sets, respectively and electrophoretically resolved on agarose gels along with 100 bp DNA marker.

3.2.1.1. Assessment of *groESL* gene promoter activity in pKG vector

About 250bp (Fig. 3.9A) DNA sequence (see Appendix), immediately upstream of *groESL* operon was amplified from *D. radiodurans* chromosomal DNA by PCR using Pgro-F/Pgro-R primers (Table 2.2). The pKG plasmid was digested with EcoRV restriction endonuclease. PCR amplified P*groESL* DNA fragment was cloned in EcoRV restriction site of pKG vector by blunt end ligation. The resultant construct was termed pKG-P*gro* (Fig 3.10A). The ligation mixture was transformed into *E. coli* cells and the cloning of P*groESL* in the correct orientation was confirmed by colony PCR using pKG-F8 primer (pKG vector background) and Pgro-R primers (Fig. 3.10B).

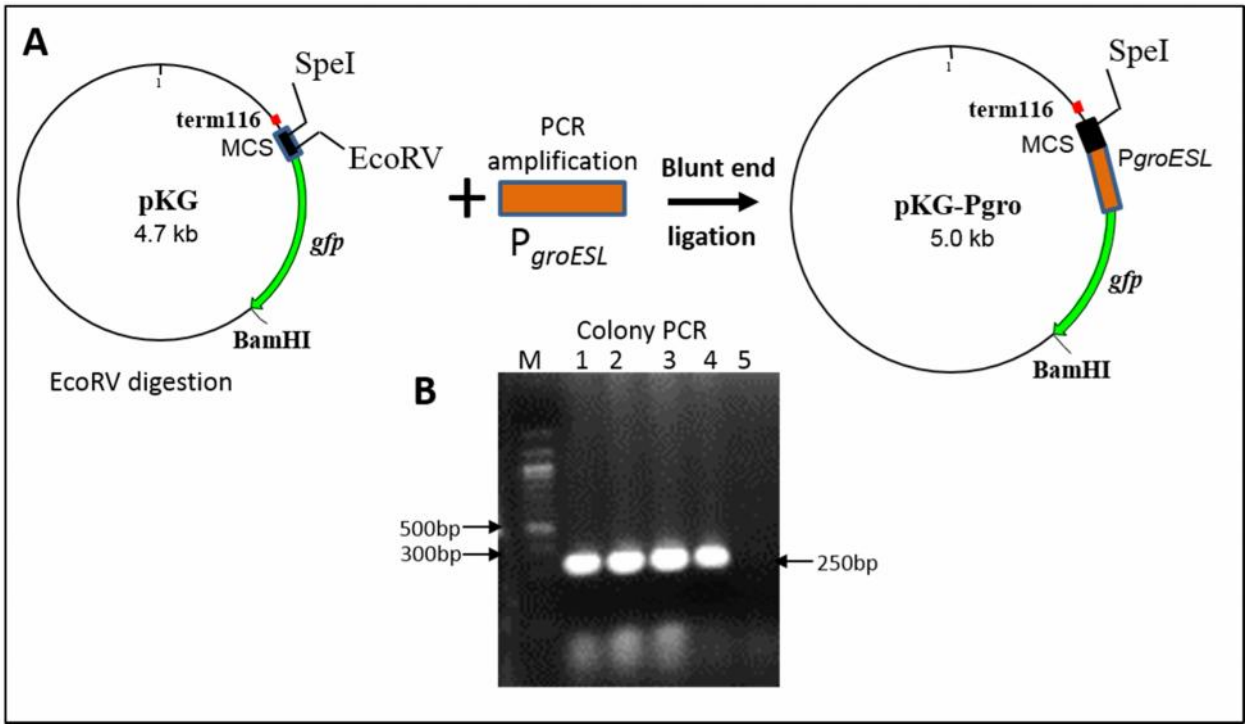

Fig. 3.10 Cloning of PgroESL promoter in pKG vector. (A) Schematic diagram showing PCR amplification of ~250 bp P_{groESL} promoter with Pgro-F/Pgro-R primers and its cloning in pKG vector at EcoRV site by blunt end ligation to generate pKG-P*gro* vector. (B) The colony PCR of pKG-P*gro* transformants by pKG-F8 and Pgro-R primers. Lane M: 100 bp DNA marker, Lanes 1-5: colony PCR amplicons from 5 different colonies. A single band of ~ 250bp is marked in lanes 1-4.

The pKG-P*gro* plasmid was transformed into competent *E. coli* and *D. radiodurans* cells. Recombinant cells of both the bacteria fluoresced brightly when observed under fluorescence microscope (Fig 3.11 A&B) indicating that Deinococcal P*groESL* is also functional in *E. coli*. During post irradiation recovery following 6kGy gamma radiation of *D. radiodurans*, no change in GFP expression was observed (Fig 3.11 C&D). This result is in agreement with the earlier reported data [6, 19].

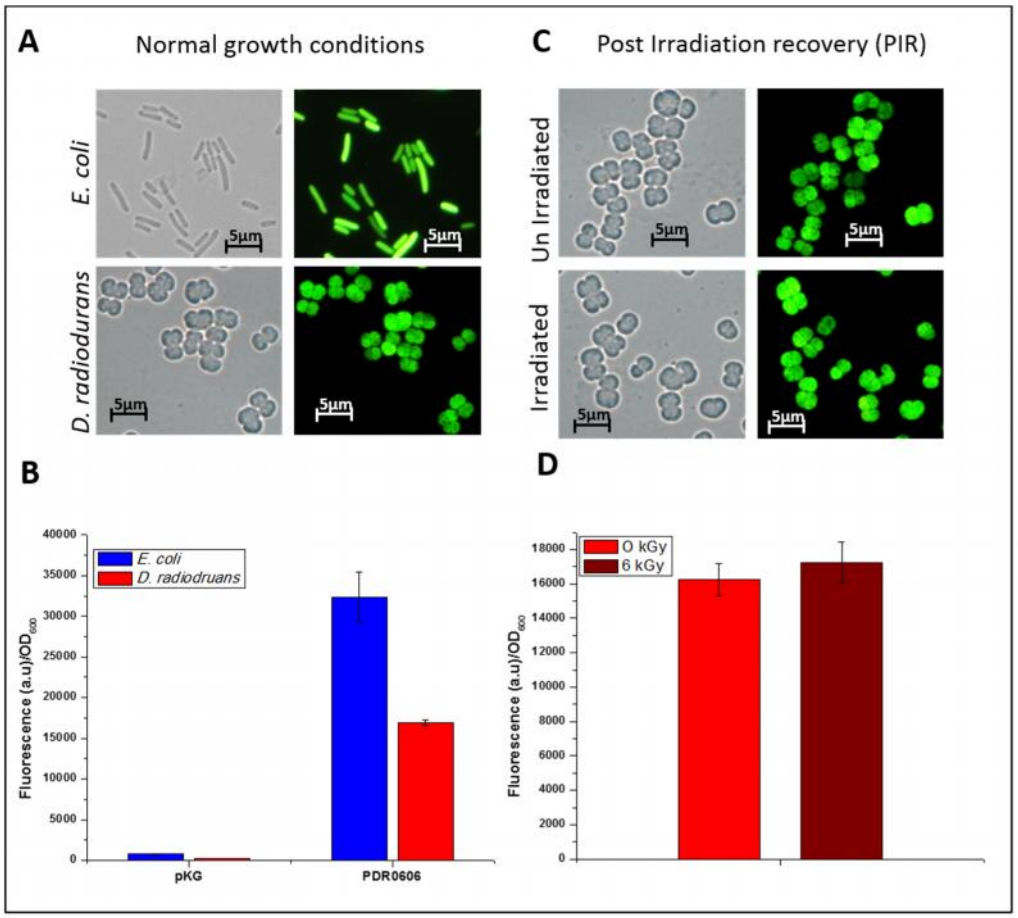

Fig 3.11 Assessment of P_{groESL} promoter activity. (A) Light and fluorescence microphotographs of *E. coli* and *D. radiodurans* harboring pKG-Pgro vector in normal growth conditions. (B) Basal promoter activity (GFP fluorescence) in *E. coli* and *D. radiodurans* in normal growth conditions. (C) Light and fluorescence microphotographs of *D. radiodurans* at 4h of PIR. (D) PgroESL promoter activity in *D. radiodurans* at 4h of PIR. (Radiation was given in GC-220 cell, radiation source Co^{60} and dose rate 5 Gy/min).

3.2.1.2. Assessment of radiation stress inducibility of *ssb* gene promoter in pKG vector

The *ssb* gene promoter of *D. radiodurans* is known to be inducible during PIR [6, 8, 11]. The 350bp upstream DNA sequence of *ssb* gene (see Appendix) was amplified using Pssb-F/Pssb-R primers (Table 2.2) and cloned in EcoRV restriction site of pKG vector (Fig 3.12 A). The insert in the resultant vector pKG-P*ssb* was confirmed by colony PCR using pKG-F8/Pssb-R primers (Table 2.2) (Fig 3.12B) and DNA sequencing for correct orientation.

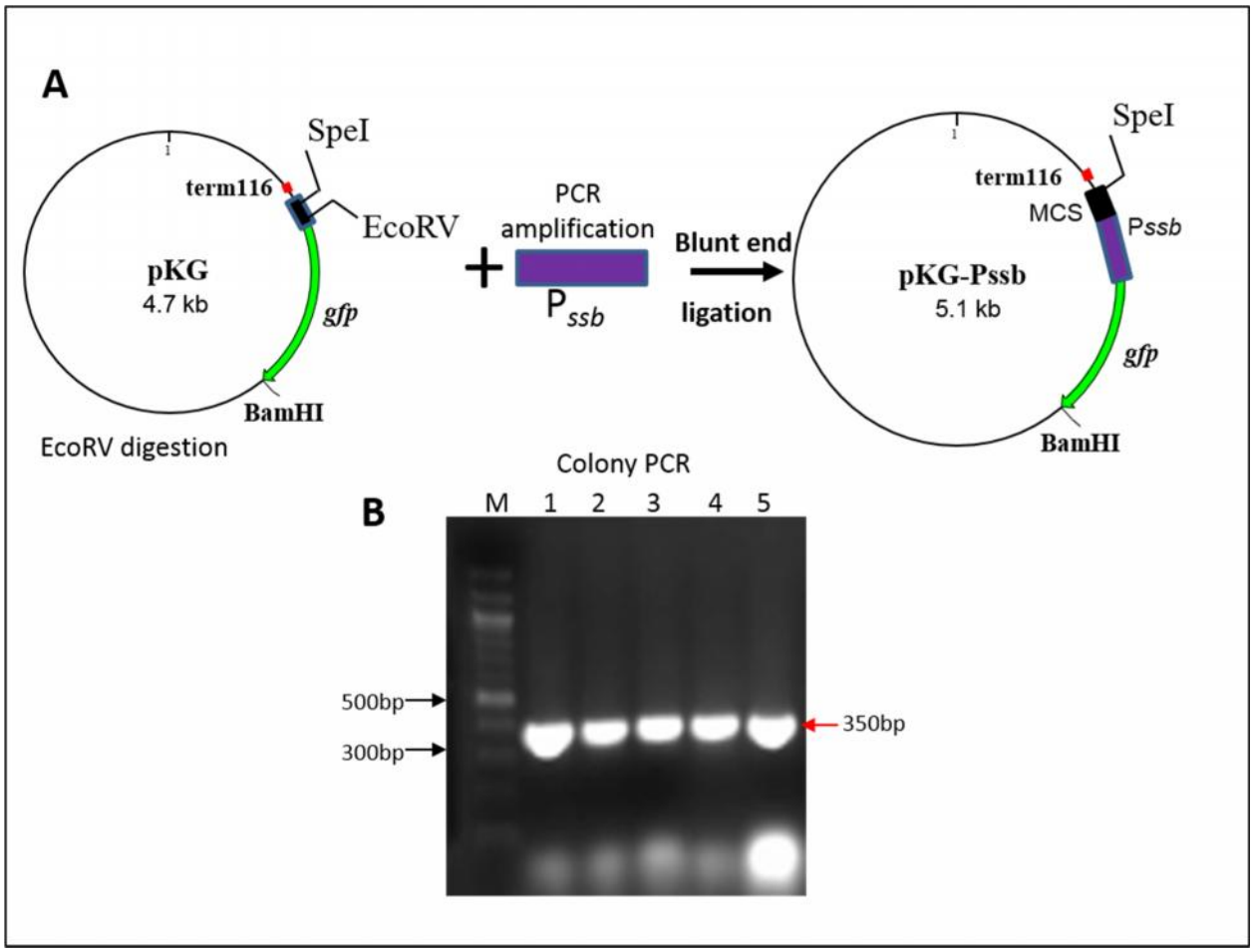

Fig. 3.12 Cloning of Pssb promoter in pKG vector. (A) Schematic diagram represents the PCR amplification of ~350 bp Pssb promoter with Pssb-F/Pssb-R primers and its cloning in pKG vector at the EcoRV site to obtain pKG-Pssb vector. (B) Electrophoretic resolution of PCR products of 5 randomly picked up colonies of pKG-Pssb transformants with pKG-F8 and Pssb-R primers. Lane M: 100 bp DNA marker; Lanes 1-5: 5 different colonies.

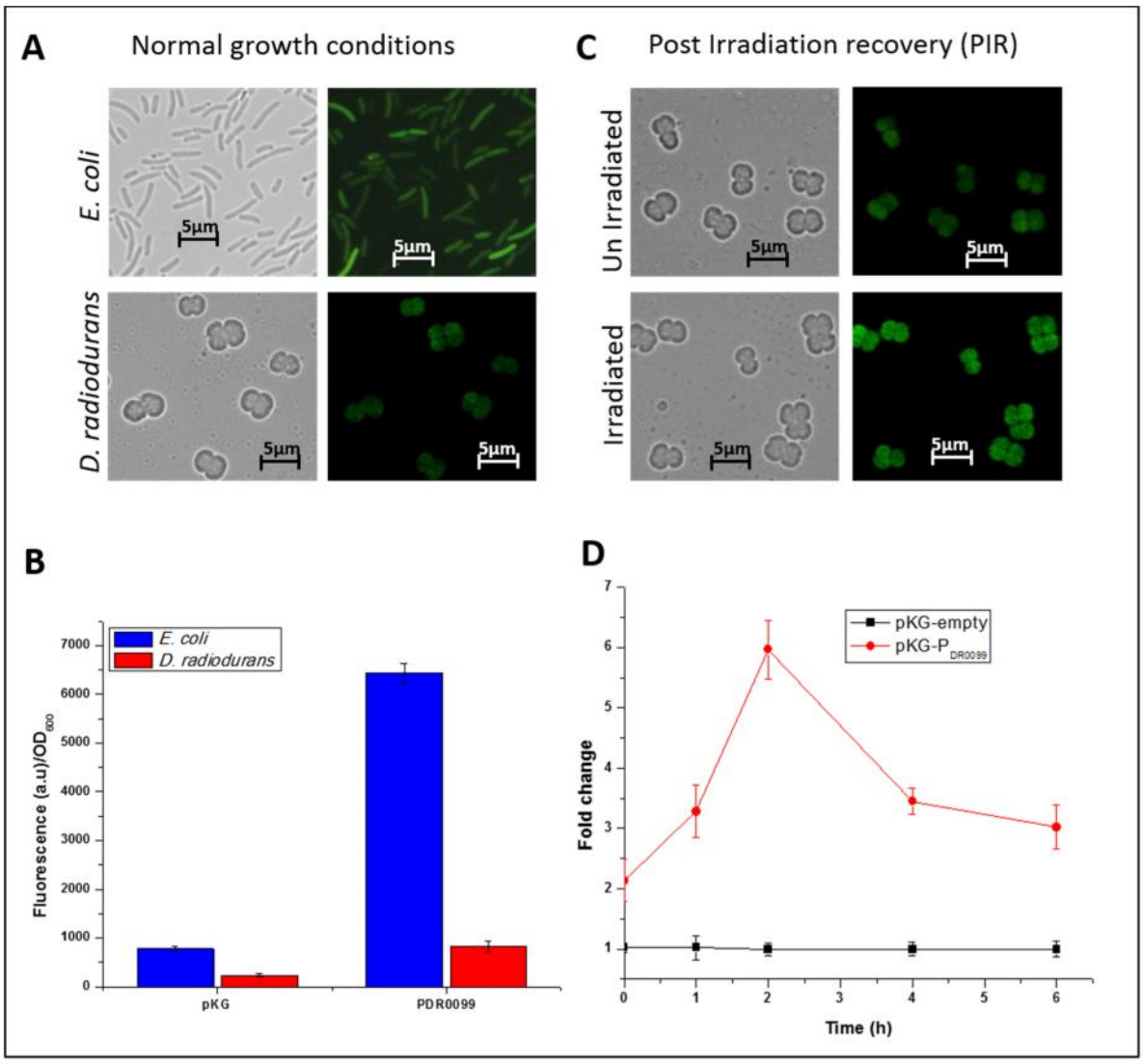

Fig. 3.13 Evaluation of P*ssb* promoter activity. (A) Fluorescence and light microphotographs of *E. coli* and *D. radiodurans* under normal growth conditions. (B) The basal promoter activity of *ssb* gene in *E. coli* and *D. radiodurans*. (C) Fluorescence and white light microphotographs of *D. radiodurans* during 4h PIR of 6 kGy gamma irradiated *D. radiodurans*, as compared to unirradiated controls. (D) The kinetics of P*ssb* promoter activity during post irradiation recovery (PIR) following 6 kGy gamma radiation stress in *D radiodurans.*

The plasmid pKG-P*ssb* was transformed into competent *E. coli* and *D. radiodurans* cells. Recombinant *E. coli* and *D. radiodurans* cells showed moderate fluorescence when observed under fluorescence microscope (Fig. 3.13 A&B). The deinococcal transformants were subjected

to 6 kGy of gamma irradiation. The radiation inducibility of P*ssb* was assessed by measuring GFP expression during PIR. The GFP fluorescence was ~5 fold induced in irradiated cells at 2h of PIR when compared with corresponding control cells (Fig. 3.13 C&D). These results substantiate the data published earlier [8, 11]. The results demonstrate that the vector pKG is suitable for promoter screening in *D. radiodurans*. Vector pKG was found to be useful for non-invasive, real time and *in situ* expression studies as well as for an easy quantification of gene expression.

3.3 Discussion

The complete genome sequence of the highly radiation resistant bacterium *D. radiodurans* was published in the year 1999 [40]. More than 15 years have passed since, but only 2 or 3 promoters of this microbe have been studied, partly because of lack of suitable bio-molecular tools. Although there are several bioinformatic tools available, they have failed to derive any consensus promoter sequence from whole genome sequence of *D. radiodurans*. Many of the promoter prediction bioinformatic tools have been designed based on *E. coli* promoters, whereas many of the *D. radiodurans* promoters lack such -10 and -35 like hexameric sequences. Even if the tools correctly predict any consensus promoter sequence or motif, it is very important to validate it by wet biology experimentation. It is, therefore, necessary to have a suitable promoter probe shuttle vector.

A few promoter probe shuttle vectors, with different reporters like *cat*, *lacZ*, *phoN* etc. [11, 15, 17, 19] exist for *D. radiodurans*. But the quantification of their reporter activity is rather laborious and requires expensive substrates and equipments. There is need to design a new promoter probe vector for *D. radiodurans*, which can facilitate a non-invasive or non-destructive study of several promoters at a time easily, without need for any expensive chemicals. In this study a third generation promoter probe shuttle vector pKG was constructed, using the green fluorescent protein

(GFP) as a reporter, for *in situ* evaluation of Deinococcal promoter activity in *E. coli* and in *D. radiodurans*, in real time. The promoter-less pKG plasmid conferred zero background fluorescence in both the organisms.

The pKG vector has a single antibiotic selection marker which expresses in both bacteria. A Rho independent transcription terminator *ter116* from *D. radiodurans* was cloned upstream of reporter gene to stop any readthrough by RNA polymerase from genes upstream of the reporter and the promoter to be cloned in the vector. A new multiple cloning site (MCS) with eight common restriction endonuclease sites has been introduced just immediately upstream of reporter gene for easy cloning of candidate promoter DNA fragments. All restriction sites of MCS in the pKG plasmid belong to common restriction enzymes and are usable.

Mere zero background of reporter expression is not sufficient for a promoter probe vector. The reporter has to express in the desirable host when a suitable promoter is tagged to it. The GFP was tested for its expression in both bacteria using two known *D. radiodurans* promoters P*groESL,* a strong promoter and P*ssb* a radiation inducible promoter. Ability to visualize and quantitate the promoter activity by GFP fluorescence provided an easy, convenient and accurate handle (Fig. 3.14). The vector's functionality or utility was validated with both the promoters, both in *E. coli* and in *D. radiodurans*. With P*groESL* promoter the GFP was expressed strongly in both *E. coli* and *D. radiodurans* colonies (Fig. 3.14A) but no radiation induction of this promoter was observed in *D. radiodurans* (Fig. 3.14B). The P*ssb* promoter also showed basal GFP expression in both organisms but also displayed radiation induction during PIR (Fig. 3.14A and B), as was reported earlier [11], thereby confirming the utility of pKG vector for screening of radiation-responsive promoters in *Deinococcus radiodurans*.

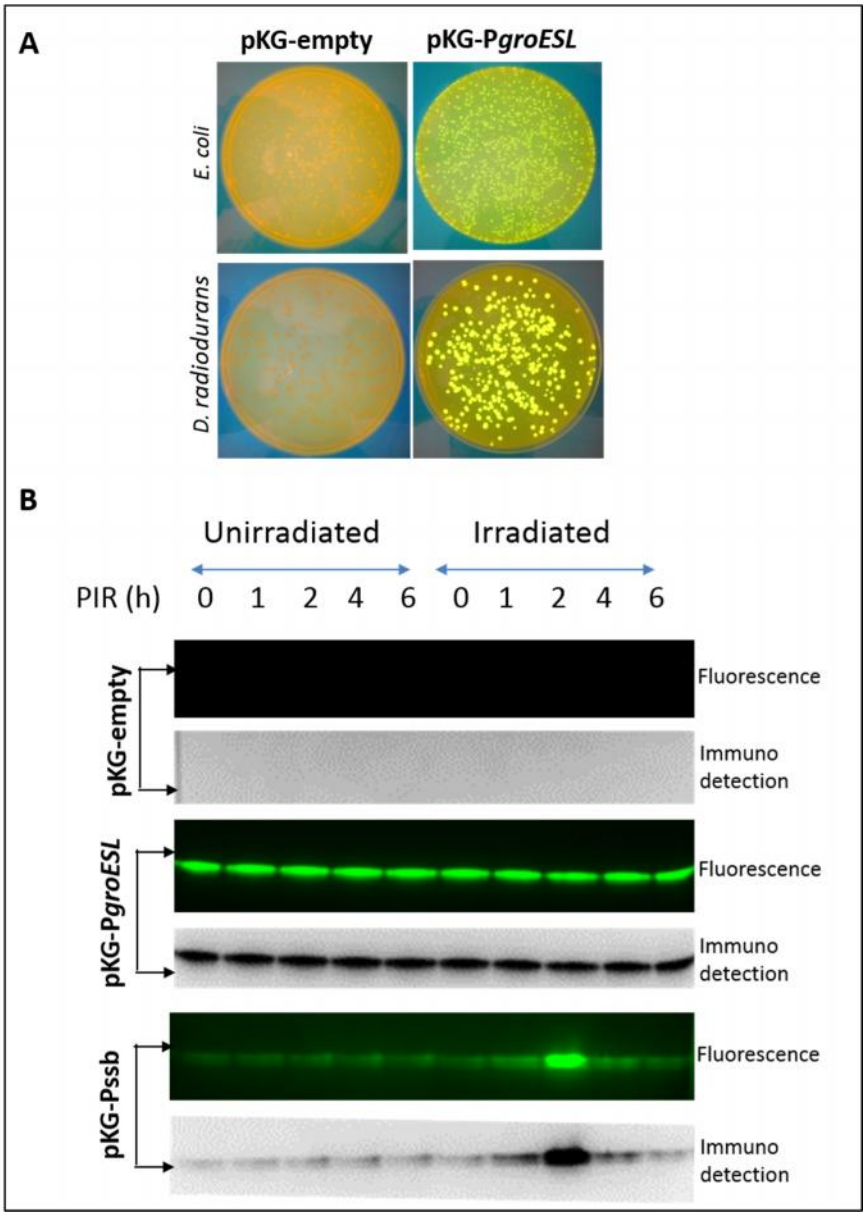

Fig. 3.14. Analysis of GFP expression and activity in *D. radiodurans.* (A) Agar plates showing recombinant *E. coli* and *D. radiodurans* carrying pKG-empty vector (control) or pKG-P*gro* plasmids. GFP fluorescence from colonies was visualized by blue light illuminator (B) *In gel* analysis of GFP expression and activity by immuno-detection and fluorescence. Protein extracts (30 µg) from unirradiated or 6kGy γ-rays irradiated recombinant *D. radiodurans* strains carrying pKG-empty, pKG-P*gro* or pKG-P*ssb* plasmids were electrophoretically resolved on 12% native gels at different time points during PIR. *In gel* fluorescence was visualized in Geldock equipped with cy2 filter. Proteins from the gels were elctroblotted onto nitrocellulose membrane and GFP protein was immuno-detected using primary anti-GFP antibody and secondary anti-rabbit-IgG-coupled to alkaline phosphatase, followed by color development using NBT/BCIP substrate.

The next chapter describes utilization of pKG for cloning and analysis of several radiation-induced Deinococcal promoters in *D. radiodurans* as well as in *E. coli*.

Chapter 4

Promoter Selection, cloning and evaluation of their activity

Bacteria respond to various environmental stresses such as heat, cold, salt, oxidation and radiation stress, by differential expression of their genome [115, 116]. The stress responsive genes/proteins are not needed in normal growth conditions or needed only at basal level. Elevated expression of such genes under normal conditions is often detrimental for cell survival and cells do not waste their energy in synthesis of unnecessary proteins [115]. Expression of stress responsive genes is therefore under tight control under normal growth conditions. Constitutively expressed and stress responsive genes have different type of *cis* and *trans* regulatory elements which control their expression under a given growth condition. The common *cis* elements are promoter sequences like -10 and -35 consensus hexamers present upstream of the gene in *E. coli* and many Gram negative bacteria. These consensus sequences are recognized by different transcription factors (TFs) or sigma factors (σ^{70}, σ^{54}) and RNA polymerase which initiates the transcription. Regulation of gene expression in prokaryotes primarily occurs at transcription level and is generally regulated by gene promoters and transcription factors which bind to the promoters. The study of promoter structure is very important to understand bacterial gene regulation.

D. radiodurans is a highly radiation resistant organism [25]. In response to radiation stress several genes have been shown to be induced in *D. radiodurans* [6, 8, 89], but the underlying regulatory mechanisms are not yet clearly understood. Unlike in *E. coli*, the promoters are poorly studied in *D. radiodurans*. Several *D. radiodurans* genes lack -10, -35 like consensus sequences upstream of their genes [87]. Earlier studies showed that *Deinococcus* promoter regions are poorly recognized in *E. coli*, and similarly *E. coli* promoters that were tested were not recognized in *D. radiodurans*, suggesting that Deinococcal promoters might be different from the classical *E. coli* promoter [17, 88].

A number of computational methods have been developed for promoter prediction from genomic sequences of bacteria [82]. A major problem of such bioinformatic tools is that they predict a fairly large number of false positives [82]. Majority of these tools are developed based on *E. coli* data and may not be useful for prediction of *D. radiodurans* promoters. Promoter-reporter systems are therefore important to characterize the promoters of organisms which do not have *E. coli*-like promoter sequences. In such promoter-reporter probing systems, a putative promoter is fused to a reporter gene on a plasmid. If the putative promoter has promoter activity, the reporter protein is expressed. The strength of the promoter can be estimated by quantifying the reporter expression. In the present study, the selected putative promoters carrying their own Shine-Dalgarno (SD) sequence were amplified by PCR and fused to a promoterless *gfp* gene in the pKG vector. The promoter activity was visualized by fluorescence microscopy in individual cells and quantified by measuring the GFP fluorescence under normal and radiation stress conditions.

4.1 Prediction of promoter sequences likely to be present upstream of *D. radiodurans* genes

Computer assisted search for promoter sequences in several bacterial species for finding hexanucleotide pairs within intergenic regions has been considered as a promising tool for the prediction of promoters [117]. The present study focused on radiation-responsive gene promoters in *D. radiodurans*. The whole genome search of two *Deinococcus* species, *D. radiodurans* and *D. geothermalis* found a 17 bp palindrome-like sequence, called radiation and desiccation response motif (RDRM) in 24 radiation inducible genes of *D. radiodurans* and 29 genes of *D. geothermalis* [10]. Later this RDRM motif was also found in other *Deinococcus* species, such as *D. deserti* [118]. Bioinformatic tools were used in the present study to predict radiation responsive gene promoters in *D. radiodurans.* Since the transcription start site (TSS) is not known for many Deinococcal gene promoters, about 500bp upstream DNA sequences from translation start codon

of several radiation inducible genes of *D. radiodurans* were downloaded from online database (KEGG website) [99]. The sequences of these genes were analyzed by online promoter prediction bioinformatic tools like Softberry, Fruitfly online bioinformatics tools [87, 105], for detecting presence of *E. coli*-like -10, -35 consensus sequences and other regulatory elements.

Fig. 4.1. Multiple sequence alignment. About 500bp sequence upstream of 25 different radiation inducible genes of *D. radiodurans* were aligned using BioEdit software. The *E. coli* promoter-like -10 and -35 hexamer sequences are highlighted. Several Deinococcal promoters only have an AT rich motif around -10 but do not have the -35 sequence. The numbers given on right side of each sequence represent distance between 5' end of -10 sequence and first base of ATG start codon. The red rectangular boxes indicate GC-rich sequences.

Among them several genes DR0070, DR0099, DR0219, DR0326, DR0423, DR0596, DR0606, DR0694, DR0906,DR1143, DR1913, DR2338, DR2574, DRA0346 and DR1771 were found to harbor *E. coli*-like -10, -35 consensus sequences upstream of the gene, though the distance between two hexamers varied from the reported 16-18bp. Several other genes DR0053, DR1262, DR1314,

DR1358, DR1720, DR2220 and DR2275 did not have the *E. coli* like -10, -35 consensus promoter sequences in their upstream 500bp DNA sequence. Recent reports also show that about 60% of the Deinococcal transcripts are either leaderless or have a very short 5'-UTR [9]. Most of them harbour AT-rich (*E. coli* -10 like) motifs, even though they lack the typical -35 sequence [9]. A manual search of non-*E. coli* promoter sequences found an AT rich motif in the non-*E. coli*-like Deinococcal promoters (Fig. 4.1). Apart from -10, -35 sequences or AT rich motifs, some putative promoters also showed repeats of G or C nucleotides (Fig. 4.1).

4.2 Selection of genes for promoter characterization

Based on existing microarray, transcriptome and proteomics data of *D. radiodurans* 25 genes were selected for promoter analysis. All the selected genes, except for DR1314 and DR0606, are reported to be radiation-induced either at transcriptional level [6, 89] or at protein level [8] (Table 4.1). DR1314 which is repressible in radiation stress and DR0606 whose level remains unchanged in radiation stress were included as negative controls. *In silico* analysis of these 25 putative promoter sequences revealed that 15 of them harbored *E. coli*-like -10, -35 consensus sequences, while the other 10 possessed only AT rich motif, but no -35 sequence (Fig. 4.1). Form these 25 genes, 20 genes were selected for promoter study based on their radiation induction level and mutational studies (Table 4.1). The other five genes were omitted because $P_{DRA0346}$ has already been reported [16] and the role of remaining 4 genes (DR0003,DR0207, DR1289 and DR1771) during radiation stress has not been studied. Among these, two known gene promoters P*groESL* (DR0606) and P*ssb* (DR0099) were initially used to evaluate and validate the pKG vector (see Section 3.2, Chapter 3). Among the selected 20 genes, 13 possessed RDRM sequence while 7 lacked RDRM sequences in their upstream regions. The DNA damage response (*ddr*) family genes

Table 4.1. List of genes selected for further study, their known phenotype.

S.No.	Gene ID	Gene name	Sensitivity of deletion mutant to ionizing radiation [ref]	Fold induction during PIR	
				Transcription level [ref]	Proteomic level [ref]
RDRM containing genes					
1	DR0070	*ddrB*	Sensitive [89]	3.3-13 [6, 89]	128.7 [8]
2	DR0099	*ssb*	NA	3.0 [6]	6.0-8.9 [8, 11]
3	DR0219	*ddrF*	NA	1.9-6 [6, 89]	NA
4	DR0326	*ddrD*	Normal growth[89]	2.5-13 [6, 89]	NA
5	DR0423	*ddrA*	Moderate [89]	4.5-18 [6, 89]	47.8 [8]
6	DR0596	*ruvB*	Moderate [119]	3.2-11 [6, 89]	NA
7	DR0906	*gyrB*	Sensitive [120]	4.4-8 [6, 89]	5.6 [8]
8	DR1143	*hypothetical*	NA	5-8.8 [6, 89]	NA
9	DR1262	*rsr*	NA	1.3-4 [6, 89]	NA
10	DR1913	*gyrA*	NA	3.2-13 [6, 89]	5.6 [8]
11	DR2275	*uvrB*	Sensitive [120]	4.9-7 [6, 89]	NA
12	DR2338	*cinA*	NA	10-14 [6, 89]	NA
13	DR2574	*ddrO*	Lethal [12]	5.2-8 [6, 89]	NA
Non-RDRM genes					
14	DR0053	*dinB*	Sensitive [121]	5-70 [6, 121]	Enhanced[121]
15	DR0606	*groES*	NA	No change	NA
16	DR0694	*hypothetical*	NA	5.5 [6]	Enhanced [122]
17	DR1314	*hypothetical*	Normal growth*	-4 [6]	Decreased [122]
18	DR1358	*ABC transporter*	NA	22.7 [6]	NA
19	DR1720	*acn*	NA	2.1 [6]	Enhanced [122]
20	DR2220	*terB*	NA	3.1-4[6, 89]	3.0 [8, 123]

NA: data not available, * temperature sensitive (>48^{0}C)

(*ddrA, ddrB, ddrC, ddrD, ddrF*) are known to be highly induced during PIR [6, 8, 89]. The DNA repair related genes *ssb*, *ruvB, gyrA, gyrB, uvrB* and *recA* (operon), some of the hypothetical genes (DR0694, DR1143) and tellurium resistance gene *terB* (DR2220) are also known to be induced in PIR. The details of 20 genes selected for promoter analysis are shown in Fig. 4.2 and are as follows:

1. **DR0053 *(dinB)*:** DinB is a DNA damage-inducible protein, which belongs to the DinB/YfiT protein family. More than 10 homologues of this protein were found in *D. radiodurans* [121]. Several fold induction was observed in gamma radiation and mitomycin-C stress [6, 89]. This gene neither has RDRM nor *E. coli* like -10 and -35 consensus sequences in its upstream region but manual search showed an AT rich motif near the translation start site as shown in Fig. 4.1. Two transcription start sites (TSS) were earlier mapped at -10 and -20bp upstream of the translation start site [121]. The deletion mutant of this gene showed no change in its growth under normal conditions, but growth was adversely affected under radiation and MMC stresses [121].

2. **DR0070 (*ddrB*):** This DNA damage response gene B is one of the 5 highly radiation inducible genes of the *D. radiodurans* [89]. Recent studies showed that DdrB protein acts like single stranded DNA binding protein (Ssb) and protects the single stranded DNA ends during radiation stress, thereby helping DNA repair [124, 125]. The gene has less conserved -10, -35 sequences [12] and a RDRM sequence in the upstream region. In normal growth conditions the expression of this protein is undetectable, but in PIR its level increases more than 100 fold [8]. This gene is unique to *Deinococcus*. A deletion mutant of this gene exhibits normal growth rate in rich media, but is sensitive to radiation stress [89].

3. **DR0099 (*ssb*):** Single stranded DNA binding protein encoding *ssb* gene is a versatile gene present in all living organisms. It is necessary for DNA replication and repair and has been shown

to be induced in *D. radiodurans* in radiation stress [8, 11]. It harbors *E. coli* like -10 and -35 consensus sequences and two RDRM sequences in its promoter. During PIR ~5 fold induction of Ssb protein and ~6 fold up-regulation of its promoter activity has been reported earlier [11].

4. **DR0219 (*ddrF*):** This uncharacterized gene is reported to be induced several fold at transcriptional level during PIR, following 15 kGy exposure to gamma radiation stress [6]. It possesses a RDRM and -10, -35 consensus sequences in its upstream region. No further information is available about this gene or its promoter.

5. **DR0326 (*ddrD*):** This gene is also one of the five highly radiation inducible genes of *D. radiodurans* [89]. Its deletion mutant shows no effect on growth under normal conditions or radiation sensitivity compared to its wild type parent organism [89]. It has -10, -35 sequences, and a RDRM in its promoter sequence and is reported to be radiation inducible [89].

6. **DR0423 (*ddrA*):** This is another of the five highly radiation induced genes of *D. radiodurans*. It is transcriptionally induced several fold during PIR [6, 89] and is also induced *de novo* at protein level [8]. It is distantly related to Rad52 protein of eukaryotes and Erf protein of cryptic phage. A deletion mutant of the gene shows no effect on normal growth of *D. radiodurans* but exhibits significant radiation sensitivity [89]. It is implicated in the single strand annealing (SSA) reaction during the RecA-independent phase of Deinococcal DNA repair [126]. The gene has two RDRM units and harbors both the *E. coli*-like -10 and -35 consensus sequences.

7. **DR0596 (*ruvB*):** The RuvB protein participates in recombination and DNA repair in bacteria [127]. Deletion mutant (Δ*ruvB*) of *D. radiodurans* shows moderate sensitivity to gamma radiation [119]. The gene is up-regulated during PIR [6, 89]. It possesses the RDRM sequence and -10, -35 consensus sequences like *E. coli*. Bioinformatic analysis has revealed another RDRM like motif upstream of the annotated *ruvB* gene.

8. **DR0606 (*groES*):** This is a chaperon protein encoded by the first gene of *groESL* operon of *D. radiodurans*. Corresponding heat shock protein shows strong expression at protein level. No change in expression level is observed in radiation stress. It has no RDRM sequence but has *E. coli*-like -10 and -35 consensus sequences.

9. **DR0694 (hypothetical):** This is one of the uncharacterized genes of *D. radiodurans* which exhibits radiation induction at transcriptional level [6]. It doesn't have any RDRM sequence, but possesses poorly conserved -10, -35 consensus sequences.

10. **DR0906 (*gyrB*):** DNA gyrase subunit B protein is necessary for DNA replication, recombination and repair in bacteria [128]. The gene is reported to be induced at transcriptional level during PIR [6, 89]. It has both -10, -35 and RDRM sequences upstream of the start codon.

11. **DR1143 (hypothetical):** This uncharacterized gene of *D. radiodurans* has been reported to be induced several fold at transcriptional level during PIR [6, 89]. It has both -10, -35 and RDRM sequences upstream of the start codon.

12. **DR1262 (*rsr*):** Ribonucleotide Ro/SS-A-related (Rsr) protein is normally bound to small RNAs known as Y RNAs. It was earlier found only in higher eukaryotes. Rsr protein of *D. radioruans* contributes resistance to UV radiation [129]. The gene has RDRM but no *E. coli*-like -10, -35 consensus sequences. Sequence analysis showed an AT rich motif upstream of the start codon. Minor radiation induction of the gene is reported during PIR [6].

13. **DR1314 (hypothetical):** This uncharacterized protein is reported to be induced during heat shock. Its deletion mutant grows normally under optimal growth conditions, but shows 10-fold reduction in growth at higher temperature (48^0C) [130]. This protein is also induced in heavy metal stress [131]. The gene has neither RDRM nor -10, -35 consensus sequences, but an AT rich motif was manually identified upstream of the start codon.

14. **DR1358:** This protein is a homolog of an outer membrane protein (ABC transporter) which is reported to be highly radiation induced during PIR [6]. The gene has neither RDRM nor the -10, or -35 consensus sequences. An AT rich motif was identified upstream of the start codon.

15. **DR1720 (*acn*):** Aconitase is a TCA cycle contributing metabolic enzyme and is moderately induced during PIR [6]. Its promoter has neither the RDRM nor the -10, -35 consensus sequences but an AT rich motif has been found upstream of the start codon.

16. **DR1913 (*gyrA*):** Gyrase subunit-A helps in replication, recombination and repair of DNA. The gene is induced at transcriptional level during PIR [6, 89], and has both RDRM and -10, -35 consensus sequences in its promoter region.

17. **DR2220 (*terB*):** Tellurium resistance genes are present in many microorganisms as operon of 2-6 genes [132]. In *D. radiodurans* there are 10 genes related to tellurium resistance and reduction. The TerB protein was shown to be induced at protein level in radiation stress [123] and tellurite stress [131]. Bioinformatic analysis showed that there is neither an *E. coli*-like promoter sequence nor a RDRM upstream of the *terB* gene.

18. **DR2275 (*uvrB*):** This subunit B of exinuclease is part of the nucleotide excision repair system. Several fold induction of *uvrB* gene has been reported at transcriptional level during PIR [6, 89]. The gene has a RDRM sequence but lacks the -10, -35 *E. coli*-like consensus sequences. Sequence analysis found an AT rich motif upstream the start codon.

19. **DR2338 (*cinA*):** Competence-inducible protein (CinA) is encoded by the first gene of *recA* operon which is reported to be several fold induced by gamma radiation, at transcript level [6, 89]. The RDRM sequence of this gene enters into the *cinA* ORF. It has a typical *E. coli*-like promoter.

20. **DR2574 (*ddrO*)**: DdrO is a repressor protein, which binds to RDRM sequence and represses gene expression under normal growth conditions. Upon radiation stress, the PprI (also called IrrE) protease cleaves the DdrO repressor and induces gene expression in *D. radiodurans* [12, 24]. The gene is also reported to be induced during PIR [6, 89]. The gene has RDRM as well as *E. coli*-like -10, -35 sequences in its promoter.

4.3 Categorization of randomly selected structurally distinct Deinococcal promoters

Many of the gene promoters of *D. radiodurans* are distinct from those of *E. coli* and several of them do not harbor -10 (TATAAT) and -35 (TTGACA) like consensus sequences that are present in typical σ^{70} dependent promoters in *E. coli*. Experimentally it has been demonstrated that some Deinococcal promoters are not functional in *E. coli*, and similarly some *E. coli* promoters do not function in *D. radiodurans* [17]. Additionally, *D. radiodurans* genome harbors RDRM sequences that are predicted to regulate gene expression following radiation and desiccation stress. Thus, Deinococcal promoters can be categorized in two ways; (i) depending on the presence/absence of RDRM, Deinococcal promoters were categorized in to two groups, (A) Promoters having RDRM and (B) Promoters lacking RDRM. (ii) Based on presence or absence of *E. coli* like -10, -35 sequences, *D. radiodurans* promoters again fall into two groups, (A) promoters having *E. coli*-like promoter sequences and (B) promoters lacking the *E. coli*-like promoter sequences. Some genes have both RDRM and -10, -35 sequences and some genes lack both. Structural features of promoters belonging to different groups are shown in Fig. 4.2. These were categorized into four groups termed as A, B, C and D (Fig. 4.2). Some details of the genes included in the four categories are as follows:

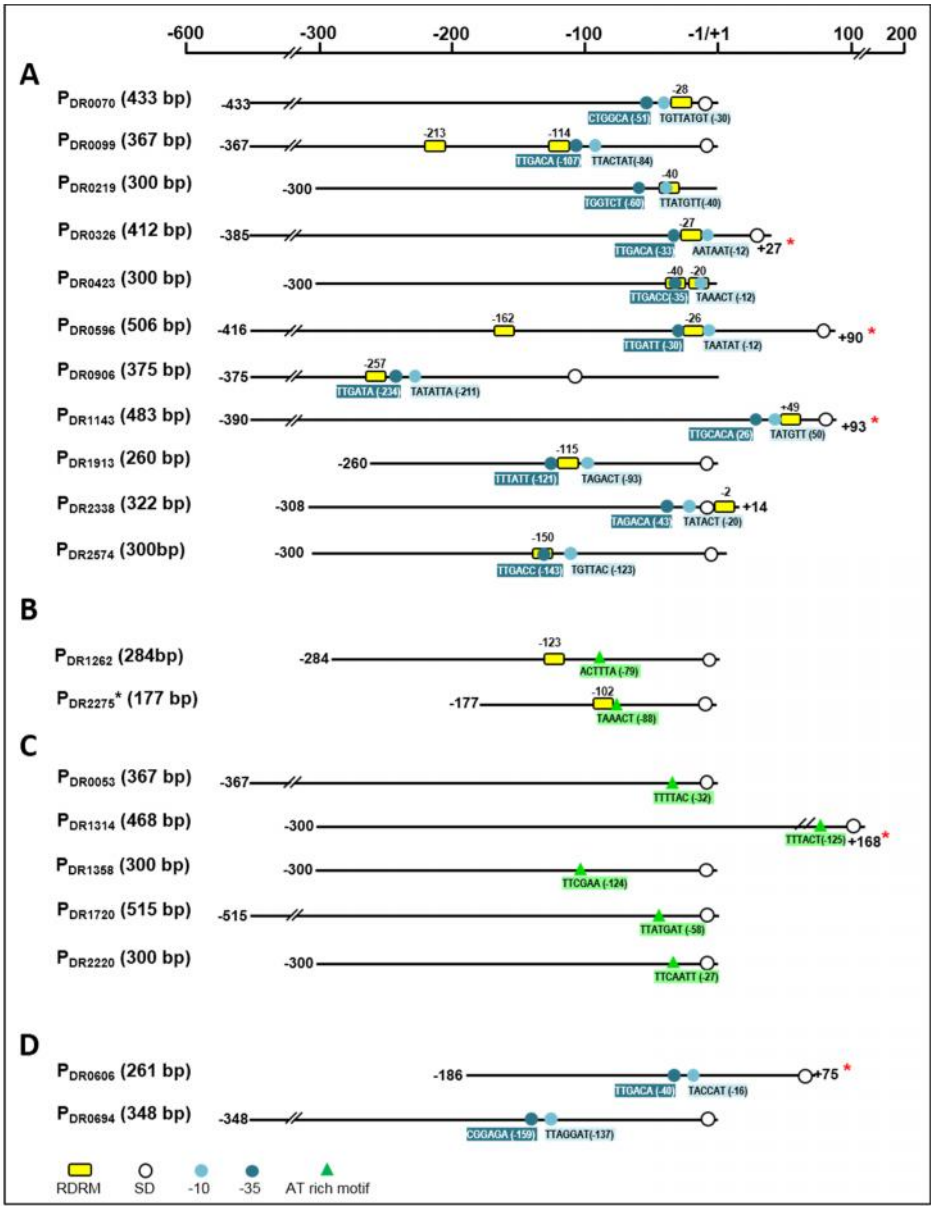

Fig. 4.2. Schematic representation of 20 structurally distinct gene promoters of *D. radiodurans*. (A) The promoters carrying both *E. coli*-like -10, -35 sequences and RDRM sequence(s), (B) Promoters harboring only RDRM sequence but not the -10, -35 sequences, (C) Promoters possessing neither RDRM nor -10, -35 sequences, and (D) promoter carrying only -10, -35 sequences but no RDRM sequence. The structural features of *D. radiodurans* promoters investigated shown include: RDRM: radiation desiccation response motif; SD: Shine Dalgarno sequence. Number above the RDRM indicates its position relative to the first base of the annotated start codon. The -10 and -35 sequences identified by BPROM software and AT-rich motifs detected manually are shown below the corresponding symbols and their position relative to start codon is given in parenthesis. Asterisks (*)indicate promoters for which a start codon downstream to the annotated start codon was found to be functional; however, the positions of -10, -35 and SD sequences are indicated respective to the annotated start codon for these promoters.

(A) Of the 20 putative promoters, 11 promoters P_{DR0070}, P_{DR0099}, P_{DR0219}, P_{DR0326}, P_{DR0423}, P_{DR0596}, P_{DR0906}, P_{DR1143}, P_{DR1913}, P_{DR2338} and P_{DR2574} harbor both RDRM and -10 and -35 consensus sequences (*E. coli*-like promoter sequences). Among these the promoters of *ssb* (P_{DR0099}), ddrA (P_{DR0423}) and *ruvB* (P_{DR0596}), show presence of two RDRM sequences each (Fig.4.2). (B) Two promoters P_{DR1262} and P_{DR2275} do not possess *E. coli*-like promoter sequences but do have RDRM and an AT rich motif. (C) The promoters P_{DR0053}, P_{DR1314}, P_{DR1358}, P_{DR1720} and P_{DR2220} possess neither *E. coli*-like promoter structure nor the RDRM sequence, but an AT rich motif is found in them. (D) Two remaining promoters P_{DR0606} and P_{DR0694} harbor -10 and -35 consensus sequences (*E. coli*-like promoter sequences) but do not possess RDRM. Some of the promoters in classes A, C and D (P_{DR0326}, P_{DR0596}, P_{DR0606}, P_{DR1143}, P_{DR1314}), have another translation start codon downstream to annotated one (Fig. 4.1).

4.4 Cloning of putative promoter DNA fragments in the pKG vector

The cloning and assessment of P_{DR0099} (P*ssb*) and P_{DR0606} (P*groESL*) were described earlier to validate pKG vector (Chapter 3, Section 3.2). The strategy followed for cloning the remaining 18 putative promoters in pKG promoter probe shuttle vector is described in Fig. 4.3. The primers were designed and synthesized based on the *D. radiodurans* genome sequence available online on the KEGG website (http://www.genome.jp/dbget-bin/www_bget?dra) [99]. In the forward primer, for all promoters except P_{DR1913}, SpeI site and in the reverse primer EcoRI site was, introduced. An EcoRI site was present in P_{DR1913} sequence, therefore an EcoRV site was introduced in the reverse primer of P_{DR1913}, instead of EcoRI. The upstream (upto ~ 500bp, from first base of the initiation codon of ORF), DNA sequences of the selected 18 genes were PCR amplified using promoter-specific forward and reverse primers (Table 2.2) from chromosomal DNA of *D. radiodurans*. The PCR amplified DNA fragments were purified and digested with SpeI/EcoRI

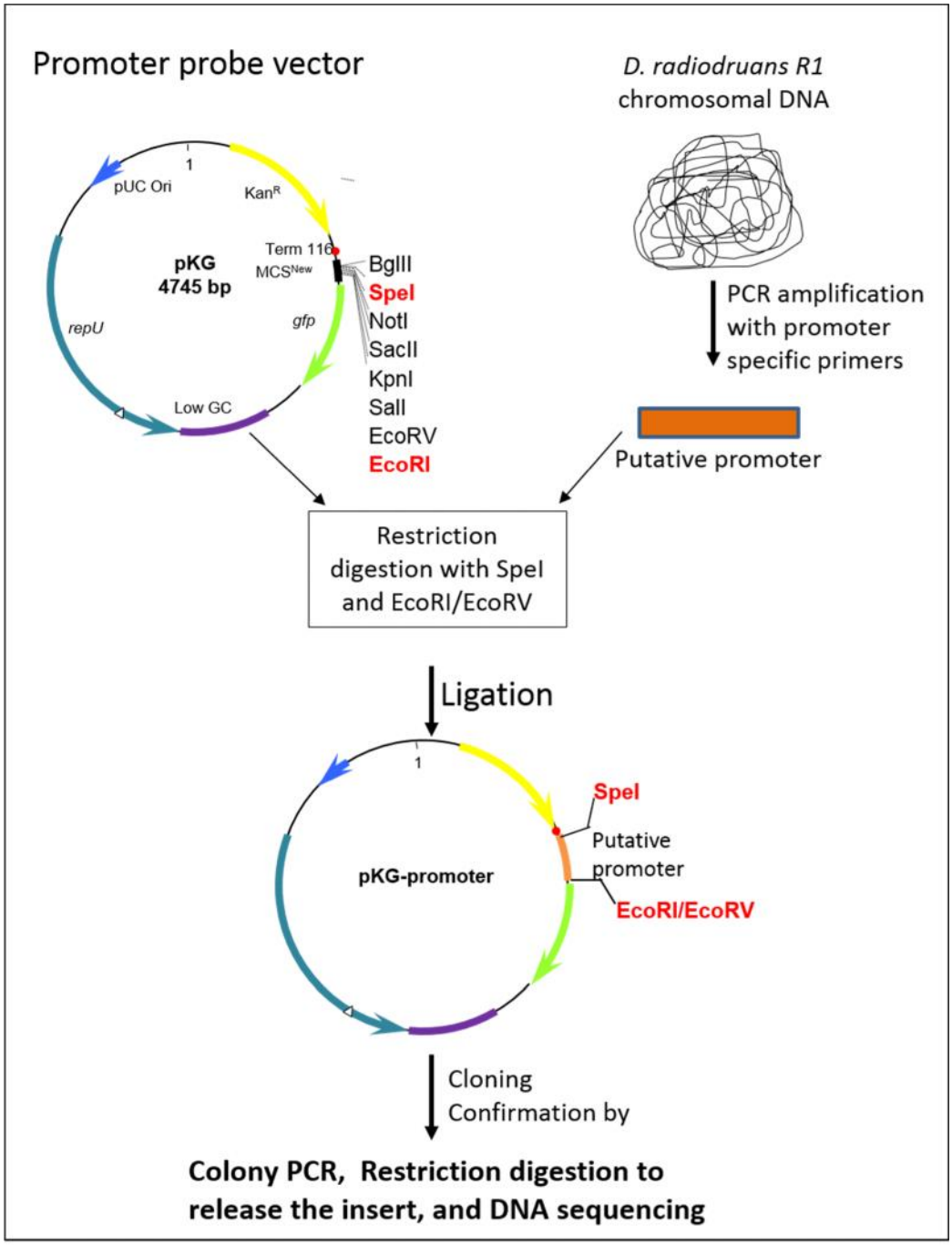

Fig. 4.3 General strategy for the cloning of promoters in the pKG vector. The schematic representation of cloning of different *D. radiodurans* promoters in promoter probe shuttle vector pKG. The putative promoters were amplified from *D. radiodurans* genomic DNA using relevant forward and reverse primers specific for each putative promoter. PCR amplified promoter fragments and the pKG vector were individually digested with SpEI and EcoRI/EcoRV restriction endonucleases and then ligated to obtain pKG-promoter vector. These pKG plasmid carrying different cloned promoters were transformed into *E. coli* and accuracy of cloning was confirmed by colony PCR, restriction digestion to release insert and DNA sequencing.

restriction endonucleases (SpeI/EcoRV for PDR1913). The restriction digested promoter fragments were cloned in the same restriction sites in the MCS of pKG promoter probe shuttle vector just upstream of the promoterless *gfp* reporter gene. The resultant 18 plasmids carried promoter-reporter translational fusions in which Shine-Dalgarno sequence (SD) was derived from individual promoter fragments. These were named as pKG-Pgene, where "gene" represents each individual gene, viz. pKG-P_{DR0606} (Table 2.1). The plasmids carrying various putative promoters were first transformed into *E. coli* and selected on agar plates containing kanamycin antibiotic. Cloning was confirmed by colony PCR with pKG-F8 vector forward primer and promoter specific reverse primers, followed by digestion with same restriction enzymes in which promoter was cloned to elute correct size promoter fragment and finally DNA sequencing (data not included). The confirmed plasmids were transformed into *D. radiodurans* cells and the positive clones were selected on TGY agar plate containing 5µg/ml of kanamycin and by detection of green fluorescent colonies when exposed to blue light. The presence of vector in *D. radiodurans* was confirmed by colony PCR with Gfp-F and Gfp-R primers. *E. coli* (DH5α) and *D. radiodurans* strains harboring pKG vector with different promoters were assessed for GFP fluorescence-based promoter activity. The GFP fluorescence intensity was a reflection of promoter activity and translation of GFP protein from the promoter-corresponding SD sequence.

4.5 Presence of consensus −10 and −35 sequences is essential for promoter activity in *E. coli*

Deinococcal promoters that have -10 and -35 *E. coli*-like sequences showed GFP fluorescence with varying intensity in *E. coli* (Figs. 4.4, 4.5). Promoter activity in *E. coli*, which lacks other Deinococcal factors, would solely depend upon similarity of the Deinococcal promoter to typical

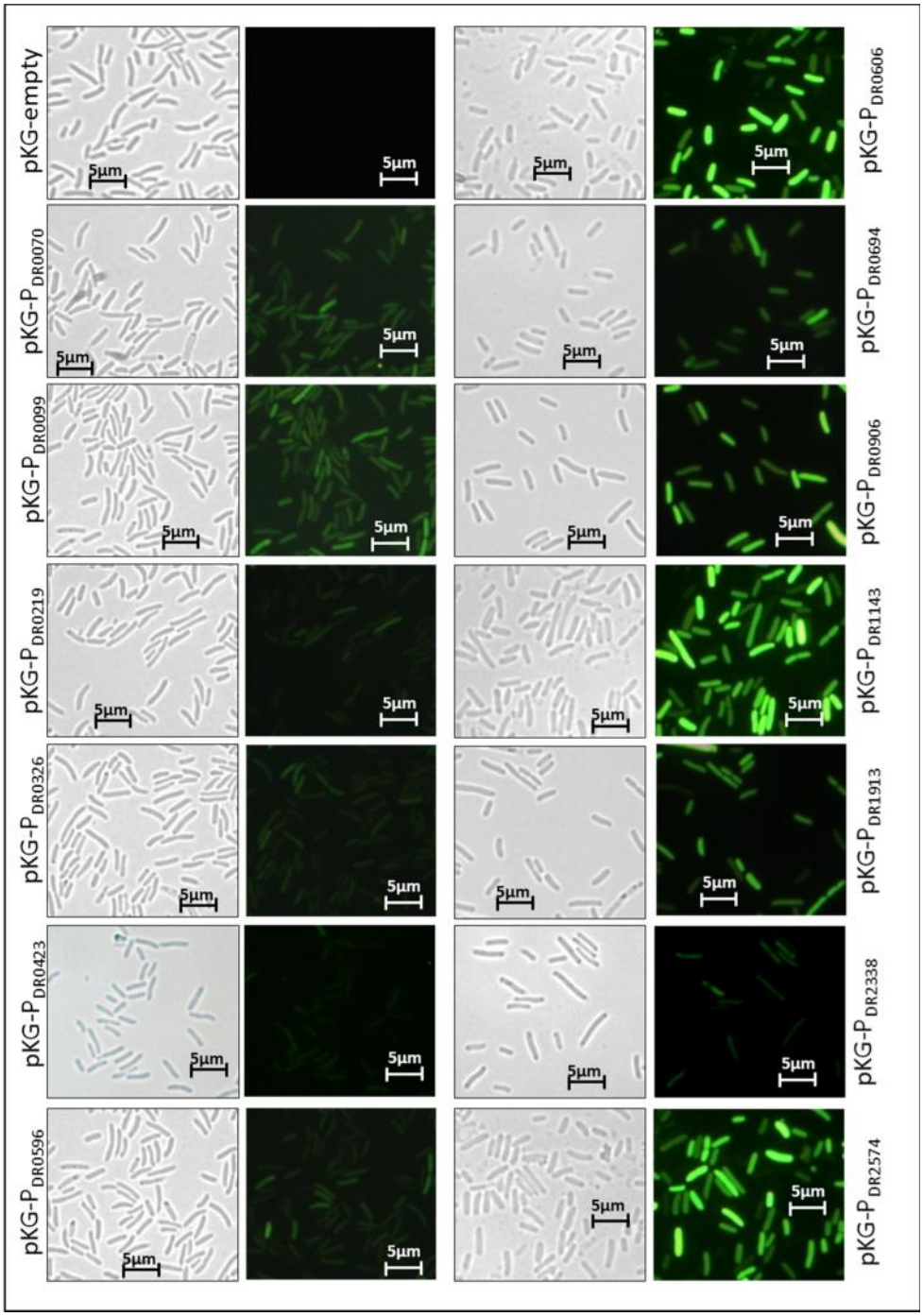

Fig. 4.4. Light and fluorescence microphotographs of recombinant *E. coli* cells carrying *E. coli*-like *D. radiodurans* promoters. *E. coli* cells carrying pKG with different *E. coli*-like putative Deinococcal promoters were grown overnight in rich media. The cells were pelleted down from 1ml culture, washed with PBS buffer and suspended in 50 µl of PBS. Aliquot of 5µl of cell suspension was spread on glass slide, covered with coverslip and observed under fluorescence microscope with 100x oil immersion objective. The cy2 filter was used for visualizing the GFP fluorescence in cells. Light and fluorescence photographs were captured using CCD camera attached to the microscope. At least 25 fields were examined from each biological replicate.

σ^{70} promoter of *E. coli*. Among the promoters that have −10 and −35 like consensus sequences, P_{DR1143} displayed best GFP expression (fluorescence 16843 ± 797 AU) in *E. coli*, followed by P_{DR0606} (fluorescence 5396 ± 506 AU) and P_{DR2574} (fluorescence 3793 ± 185 AU). In all other promoters containing −10 and −35 like sequences, GFP expression was moderate (fluorescence >350 AU to < 1500 AU) (Figs. 4.4, 4.5), while promoters P_{DR0053}, P_{DR1262}, P_{DR1314}, P_{DR1358}, P_{DR1720}, P_{DR2220} and P_{DR2275} that harbored only −10 like AT-rich motif, showed rather low to negligible GFP expression (fluorescence <350 AU) in *E. coli* (Fig. 4.5 and 4.6). The results clearly showed that the -10, -35 like hexamer sequences are essential for Deinococcal promoter expression in *E. coli*.

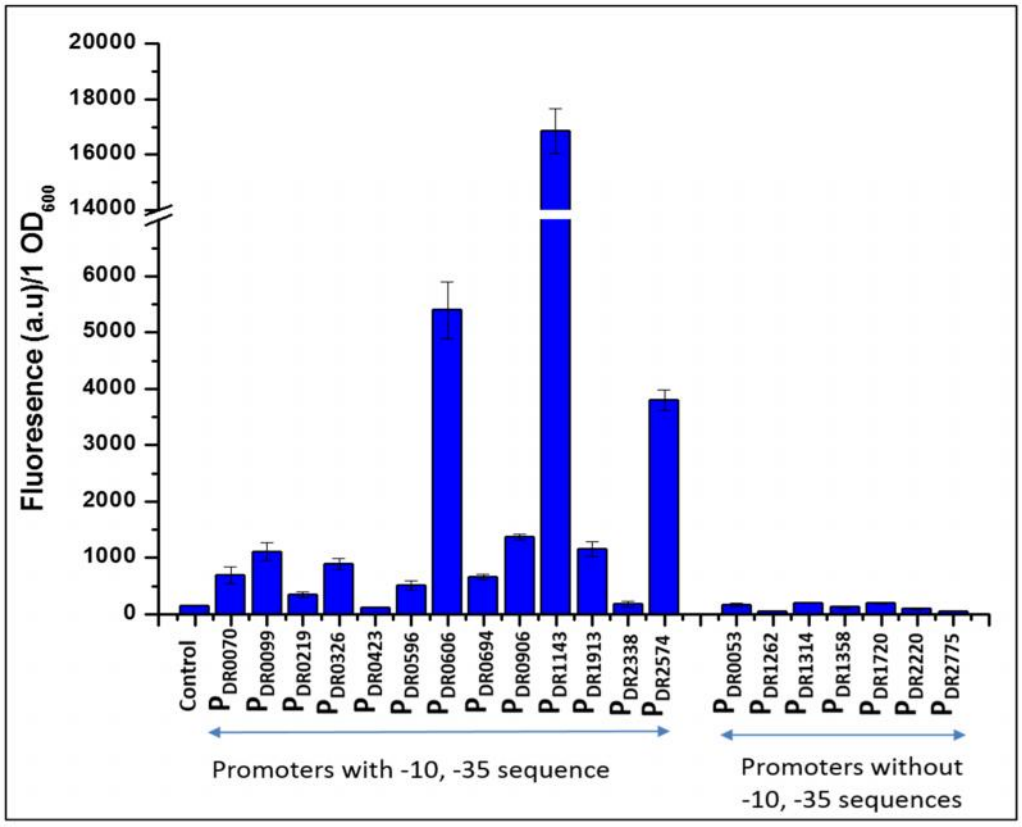

Fig. 4.5. Assesment of basal activity of Deinococcal promoters in *E. coli*. The recombinant *E. coli* cells carrying pKG with different deinococcal promoters were grown overnight. The GFP fluorescence, which reflects promoter activity, was quantified by fluorescence spectrophotometer (λex: 489nm and λem: 509nm). Fluorescence values (arbitrary units) were expressed per unit cell density (OD_{600nm}).

4.6 The -10 and -35 *E. coli*-like promoters are dispensable in *D. radiodurans*

The Deinococcal promoters which have *E. coli*-like promoters sequences showed various levels of GFP expression from zero (P_{DR1143}) to highest 3386 ±70AU (P_{DR0606}) promoter activity in *D. radiodurans* under normal growth conditions (Fig. 4.7). The highest promoter activity in *E. coli* was shown by P_{DR1143} (Fig. 4.5) which surprisingly did not show any activity in *D. radiodurans* (Fig. 4.7).

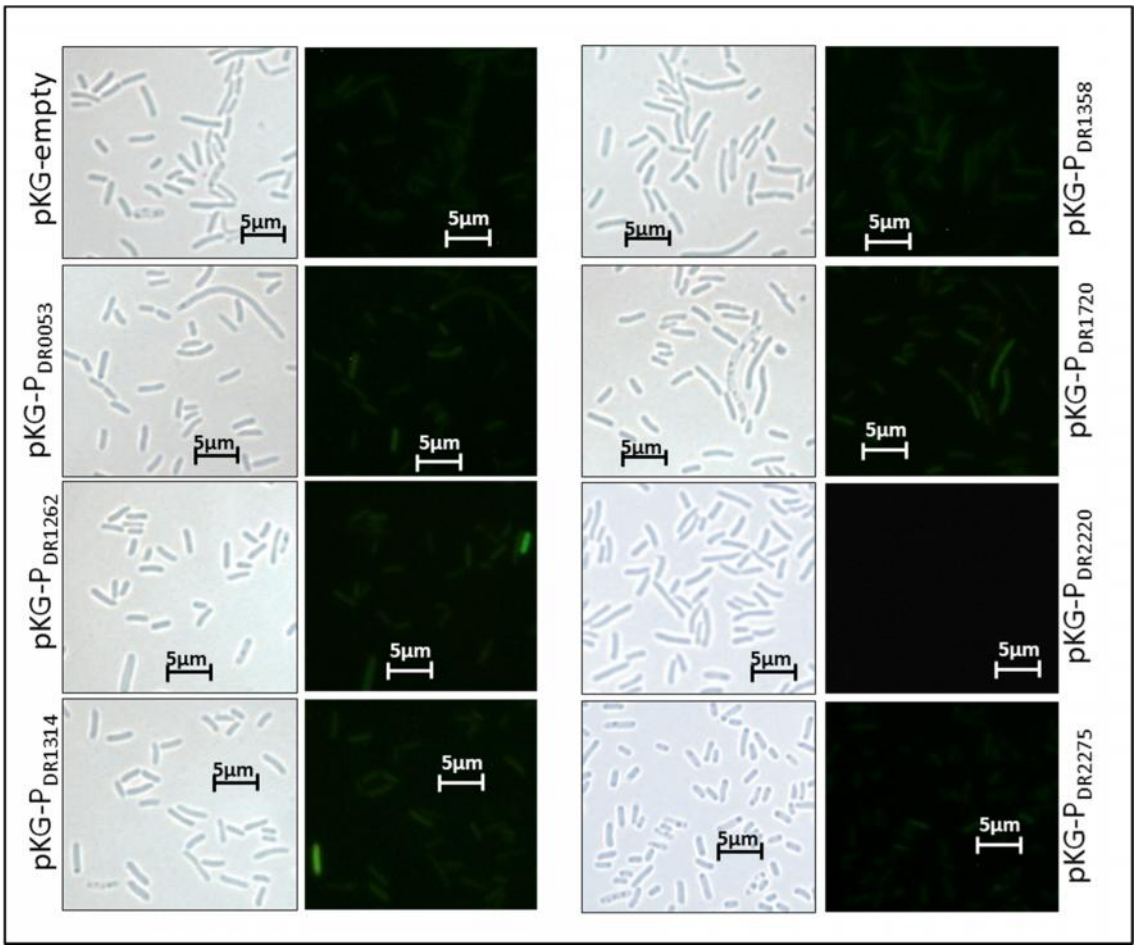

Fig. 4.6. Light and fluorescence microphotographs of recombinant *E. coli* cells harboring non-*E. coli*-like Deinococcal promoters. The representative microphotographs of *E. coli* cells carrying pKG with non-*E coli*-like putative *D. radiodurans* promoters. Other details were same as in the legend to Fig. 4.4.

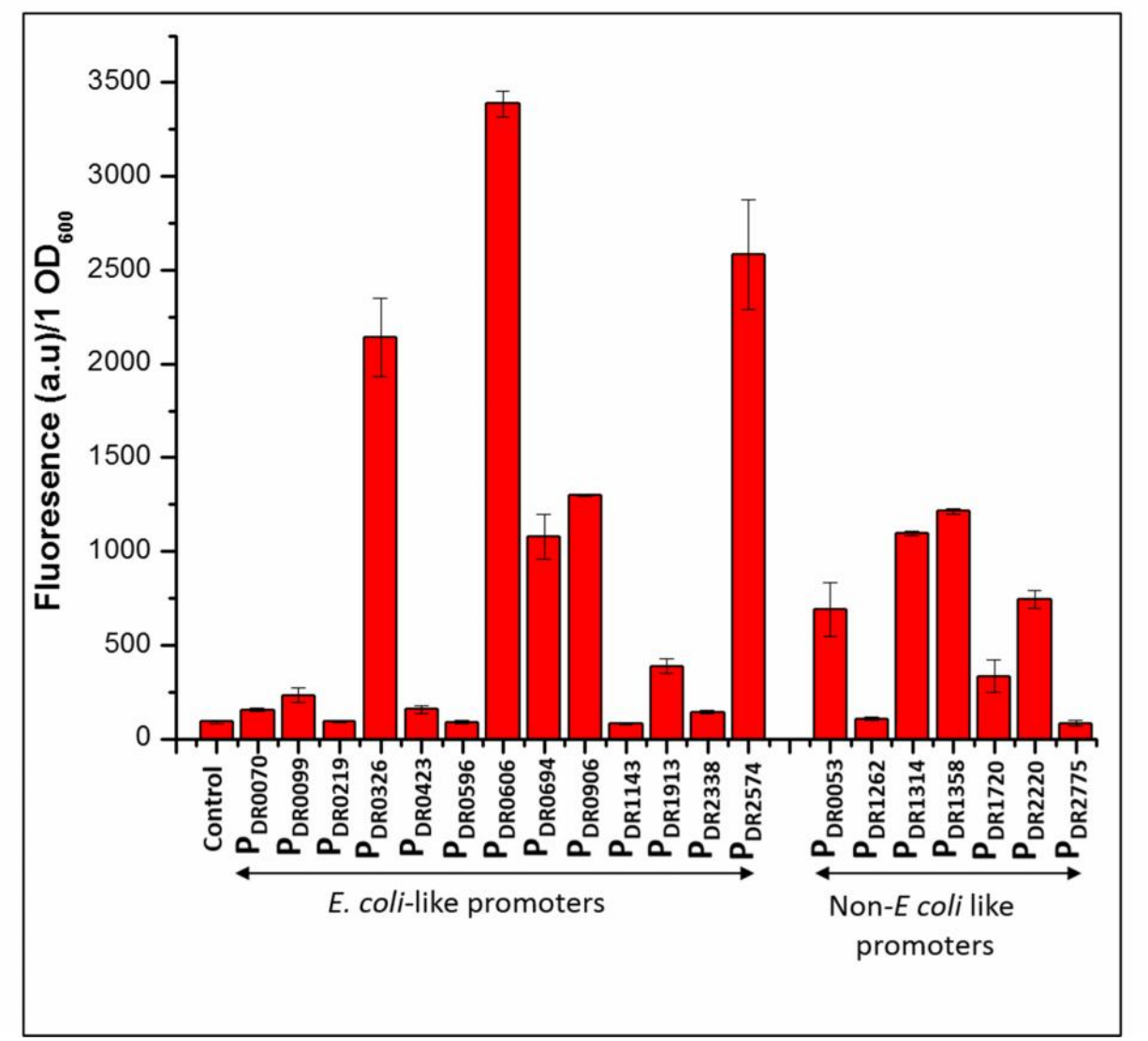

Fig. 4.7. Basal activity of *E. coli*-like and non-*E. coli*-like promoters in *D. radiodurans*. The *D. radiodurans* cells carrying pKG plasmid with different promoters were grown in rich media overnight and the GFP expression was quantified, as described in the legend to Fig. 4.5.

The *ddrB* gene (DR0070) whose promoter also has *E. coli*-like -10, -35 sequences and is synthesized *de novo* during PIR in *Deinococcus* [8], showed very weak GFP fluorescence in *D. radiodurans* (Fig. 4.7). These results indicated that the *E. coli*-like -10, -35 sequences are not essential for promoter activity in *D. radiodurans*. Several Deinococcal promoters (P_{DR0053}, P_{DR1262}, P_{DR1314}, P_{DR1358}, P_{DR1720}, P_{DR2220} and $P_{DR2275)}$ which lacked conserved −10 and −35 consensus

sequences showed very weak or no promoter activity (<200 a. u) in *E. coli* cells (Fig 4.5 and 4.6). But these promoters showed moderate to strong promoter activity in *D. radiodurans* (Fig. 4.7) suggesting that the Deinococcal promoters are different from *E. coli* promoter and unique to *Deinococcus*. The *E. coli* like -10 and -35 consensus sequences are, thus, dispensable in *D. radiodurans*.

4.7 Presence of RDRM lowers promoter activity in *D. radiodurans*

The data shown in the following Figs. 4.8-4.10 revealed that the presence of RDRM upstream of genes was inhibitory, in some way, to promoter activity in *D. radiodurans*. Majority of the RDRM containing Deinococcal promoters showed poor promoter activity (Figs. 4.8, 4.10), except P_{DR0326} (*ddrD*), P_{DR0906} (*gyrB*), P_{DR1913} (*gyrA*) and P_{DR2574} (*ddrO*) (Fig. 4.10) which are required during normal growth. In comparison, all the non-RDRM Deinococcal promoters (P_{DR0053}, P_{DR0606}, P_{DR0694}, P_{DR1314}, P_{DR1358}, P_{DR1720} and P_{DR2220}) showed good promoter activity (Figs. 4.9, 4.10) in *D. radiodurans*. The results clearly indicated that RDRM strongly inhibited promoter activity in *D. radiodurans* during normal growth conditions. Presence/absence of RDRM did not influence expression of corresponding promoters in *E. coli*, where presence/absence of -10 and -35 like sequences determined the level of promoter activity (Fig. 4.7).

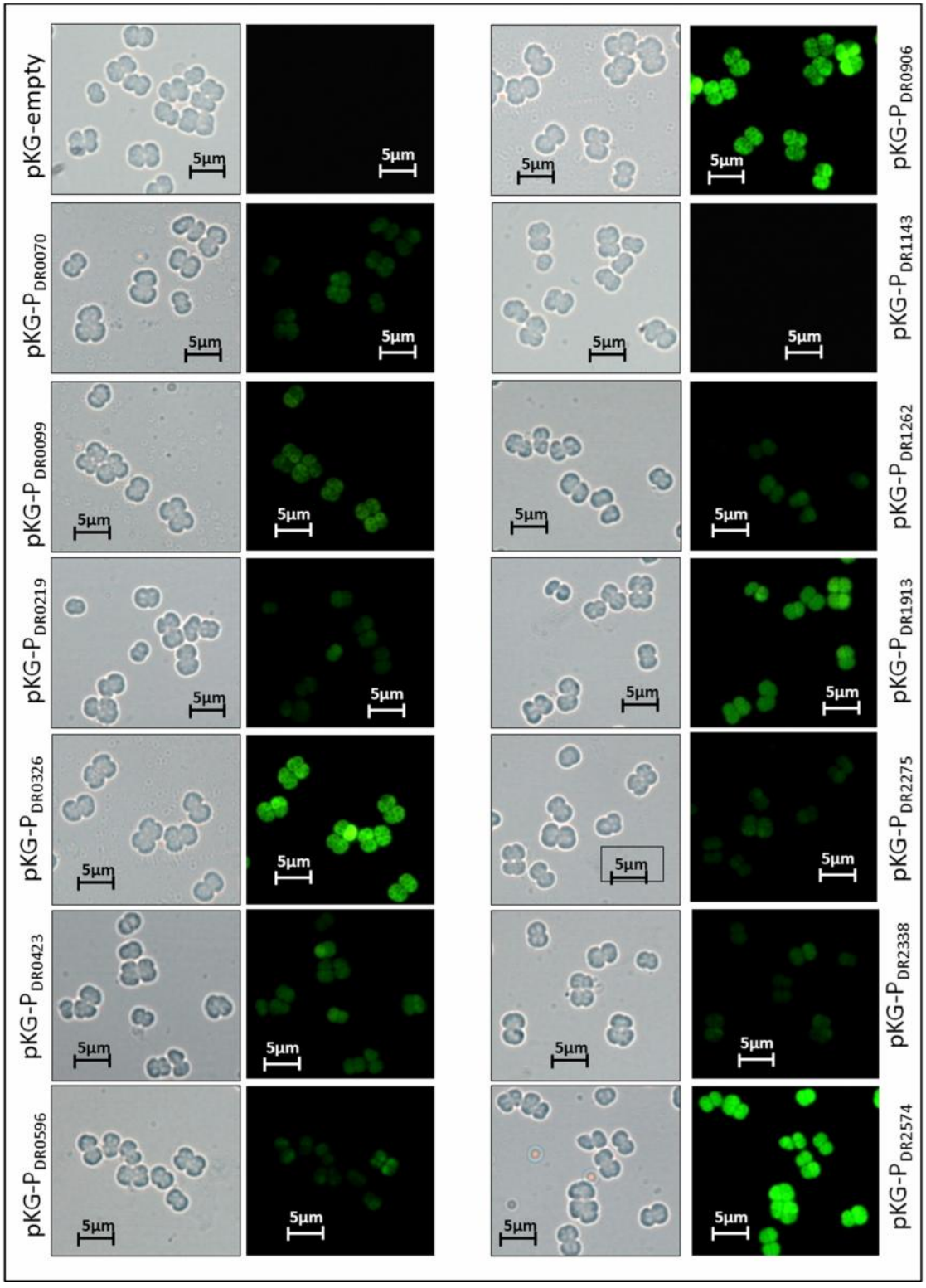

Fig. 4.8. Light and fluorescence microphotographs of recombinant *D. radiodurans* cells carrying various RDRM containing promoters. Overnight grown *D. radiodurans* cells were observed under fluorescence microscope. Other details were same as described in the legend to Fig. 4.4.

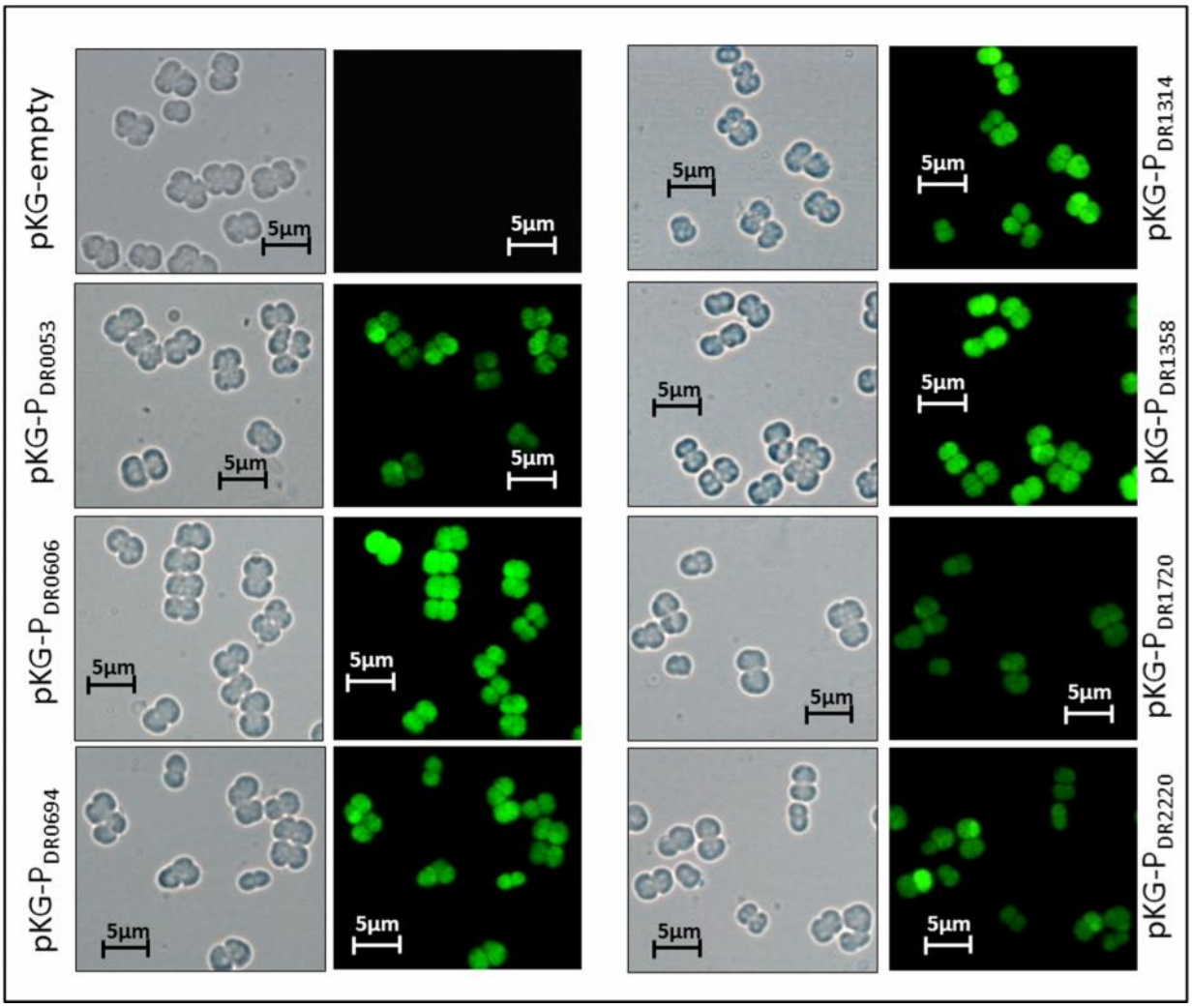

Fig. 4.9. Light and Fluorescence microphotographs of recombinant *D. radiodurans* cells carrying non-RDRM promoters. Overnight grown of *D. radiodurans* cells were observed under fluorescence microscope. Other details were as described in the legend to Fig. 4.4.

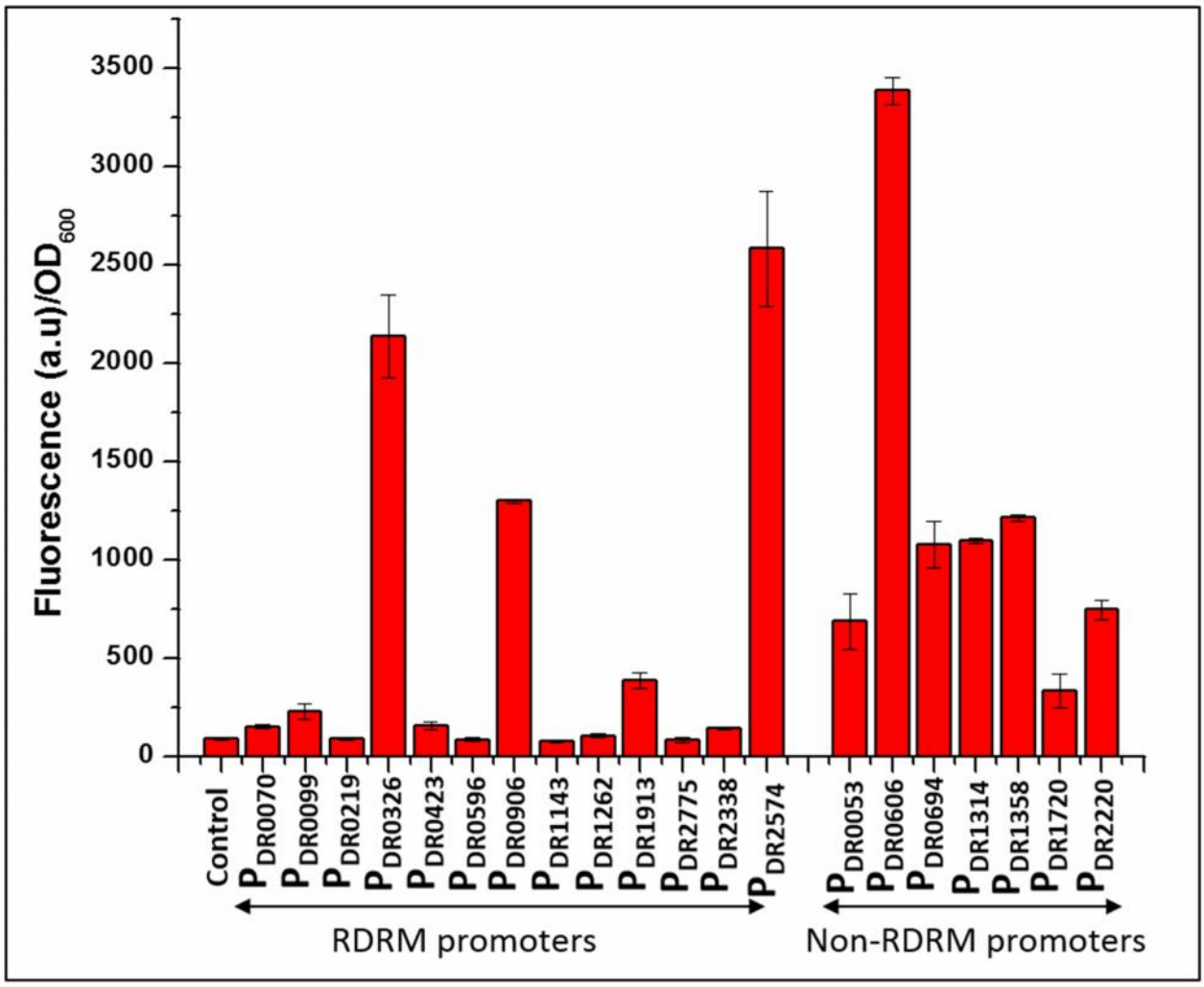

Fig. 4.10. Activity of Deinococcal promoters in *D. radiodurans*. The *D. radiodurans* cells carrying pKG plasmid with different promoters were grown in rich media overnight and the GFP fluorescence was quantified by fluorescence spectrophotometer, as described in the legend to Fig. 4.5.

4.8. All RDRM containing Deinococcal promoters are radiation inducible in *D. radiodurans*

D. radiodurans responds to gamma irradiation by upregulation of several genes involved in DNA repair and oxidative stress alleviation [6, 8, 89, 131]. Based on gamma radiation responsive transcriptome dynamics in *D. radiodurans* and *D. geothermalis*, the palindromic sequence RDRM was predicted to be responsible for upregulation of gene expression during post irradiation

recovery [10]. In the genome of *D. radiodurans*, RDRM sequence is present in the vicinity of 24 genes [10]. Among these, the radiation inducible upregulation of *ssb* and *ddrB* genes has been well established by transcriptome, proteome and promoter level studies [6, 8, 11].

D. radiodurans clones harboring different putative promoters (both RDRM-based and RDRM-lacking) were exposed to 6kGy gamma radiation and promoter activity was monitored (as GFP fluorescence), at different time points during post irradiation recovery (PIR) by fluorescence spectrophotometry (λex: 489nm and λem: 509nm) and also visualized in cells by fluorescence microscopy. The promoters wherein GFP fluorescence was induced >2 fold only were considered to be radiation induced.

All the promoters (P_{DR0070}, P_{DR0099}, P_{DR0219}, P_{DR0326}, P_{DR0423}, P_{DR0596}, P_{DR0906}, P_{DR1143}, P_{DR1262}, P_{DR1913}, P_{DR2275}, P_{DR2338} and P_{DR2574}) which possessed RDRM sequence, showed distinct radiation induction (Fig. 4.11). The promoters P_{DR0070}, P_{DR0099}, P_{DR0596}, P_{DR0906}, P_{DR1143} and P_{DR2574} showed 5-25 fold induction of their activity during PIR (Fig 4.11, 4.12). The highest induction ~25 fold was observed for P_{DR0070} promoter, followed by ~15 fold for P_{DR1143} promoter. Both these promoters displayed very weak promoter activity under normal growth conditions (Fig. 4.10).

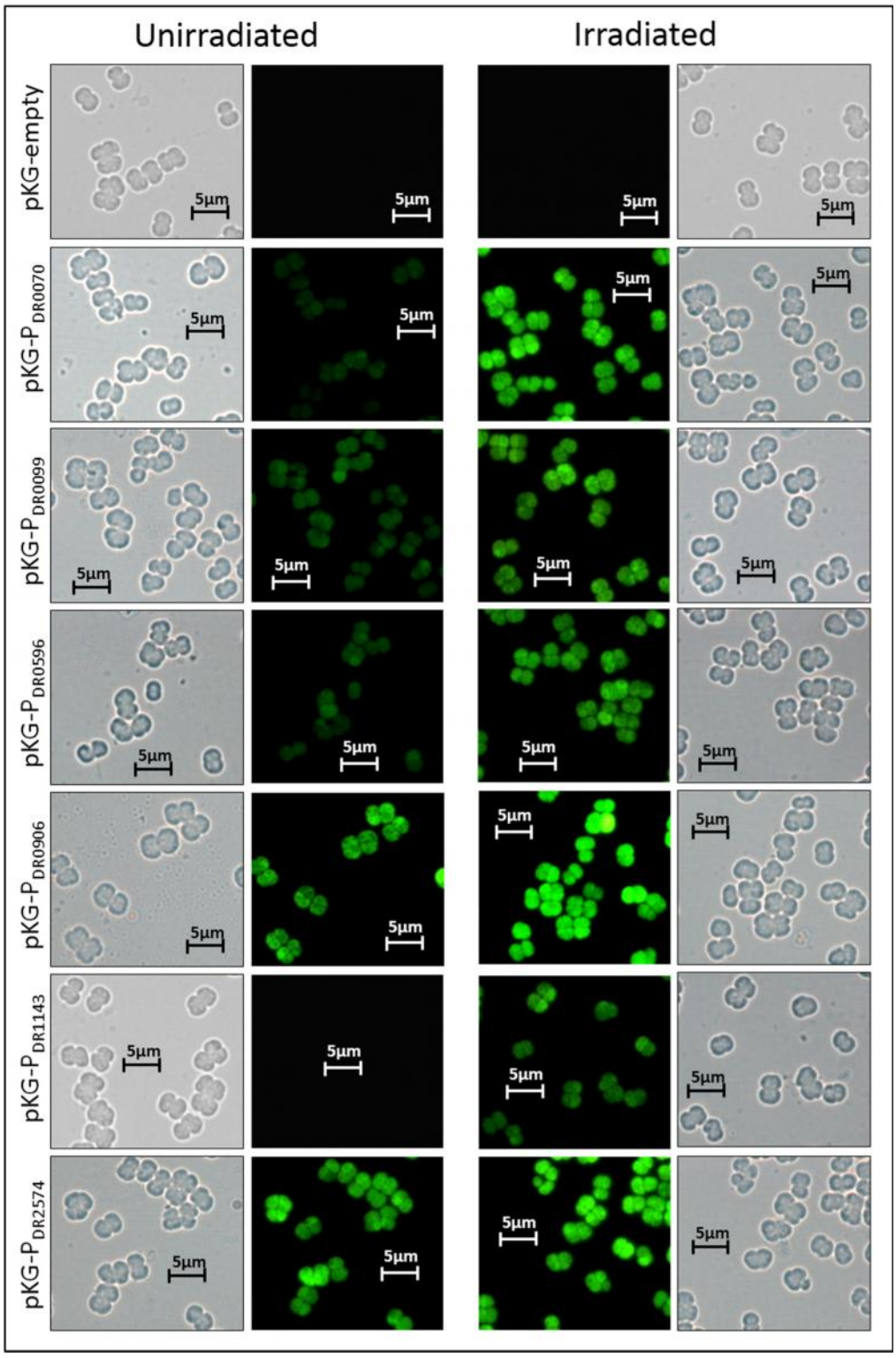

Fig. 4.11. Light and fluorescence microphotographs of recombinant *D. radiodurans* cells carrying strongly radiation inducible promoters at 4h of PIR. Following 6kGy gamma irradiation the *D. radiodurans* cells were observed under fluorescence microscope. Other details were as described in the legend to Fig. 4.4.

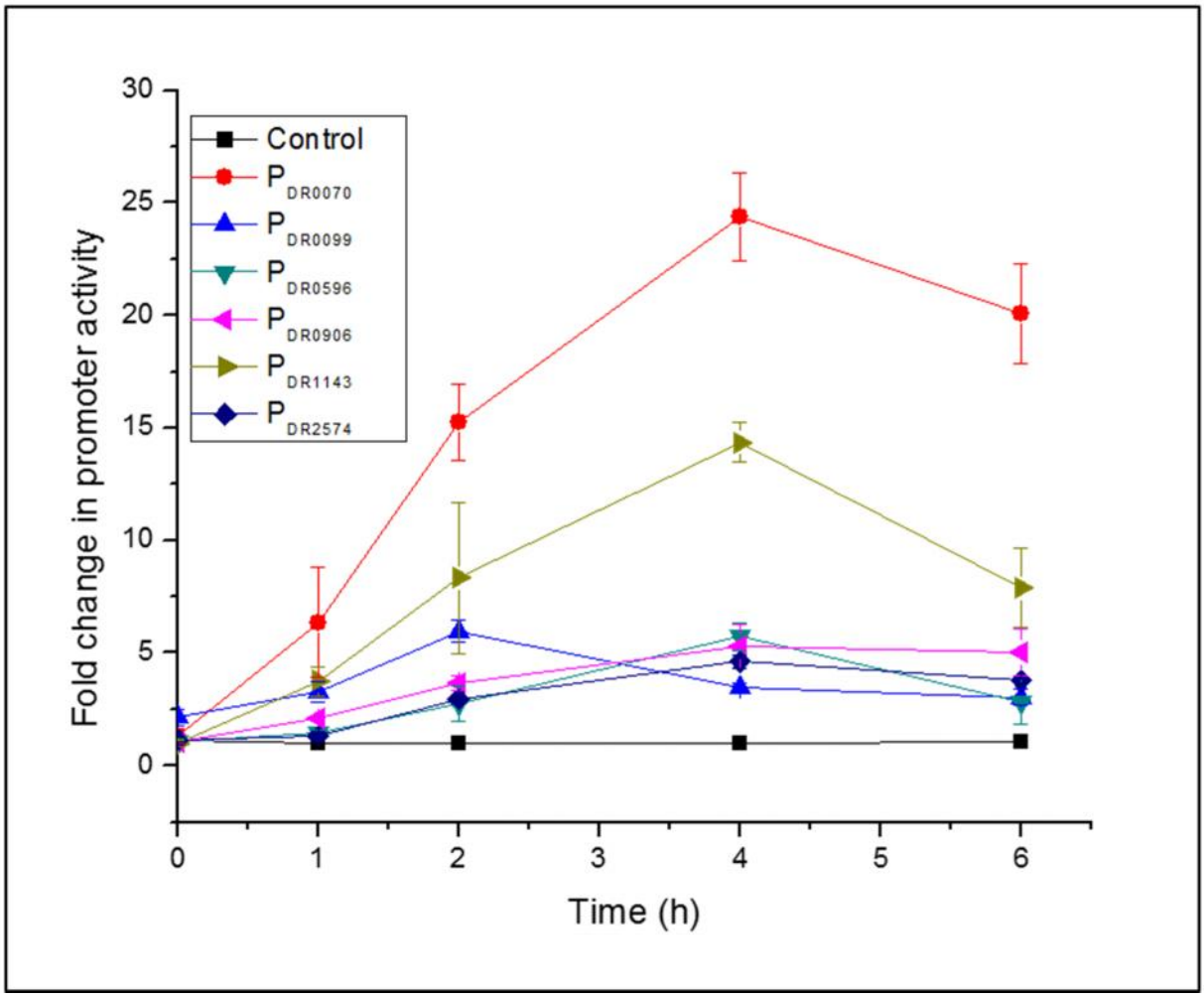

Fig. 4.12. Kinetics of promoter activity of strongly radiation inducible Deinococcal promoters during PIR. Recombinant *D. radiodurans* strains carrying the specified promoters were subjected to 6 kGy ^{60}Co gamma irradiation (GC-220 Gamma Cell, at a dose rate of 5 Gy/min). Fold change in promoter activity was monitored and calculated by quantifying the GFP fluorescence, during 6h of post irradiation recovery (PIR). Other details were as described in the legend to Fig. 4.5.

The other RDRM containing promoters (P_{DR0219}, P_{DR0326}, P_{DR0423}, P_{DR1262}, P_{DR1913}, P_{DR2275} and P_{DR2338}) showed moderate promoter activity under normal growth conditions (Fig. 4.10) and 2-3 fold induction during PIR (Fig. 4.13, 4.14). Almost all the promoters showed highest induction of activity at 4h of PIR which declined later with time (Figs. 4.12, 4.14) as the damaged DNA was repaired and cells started growing again.

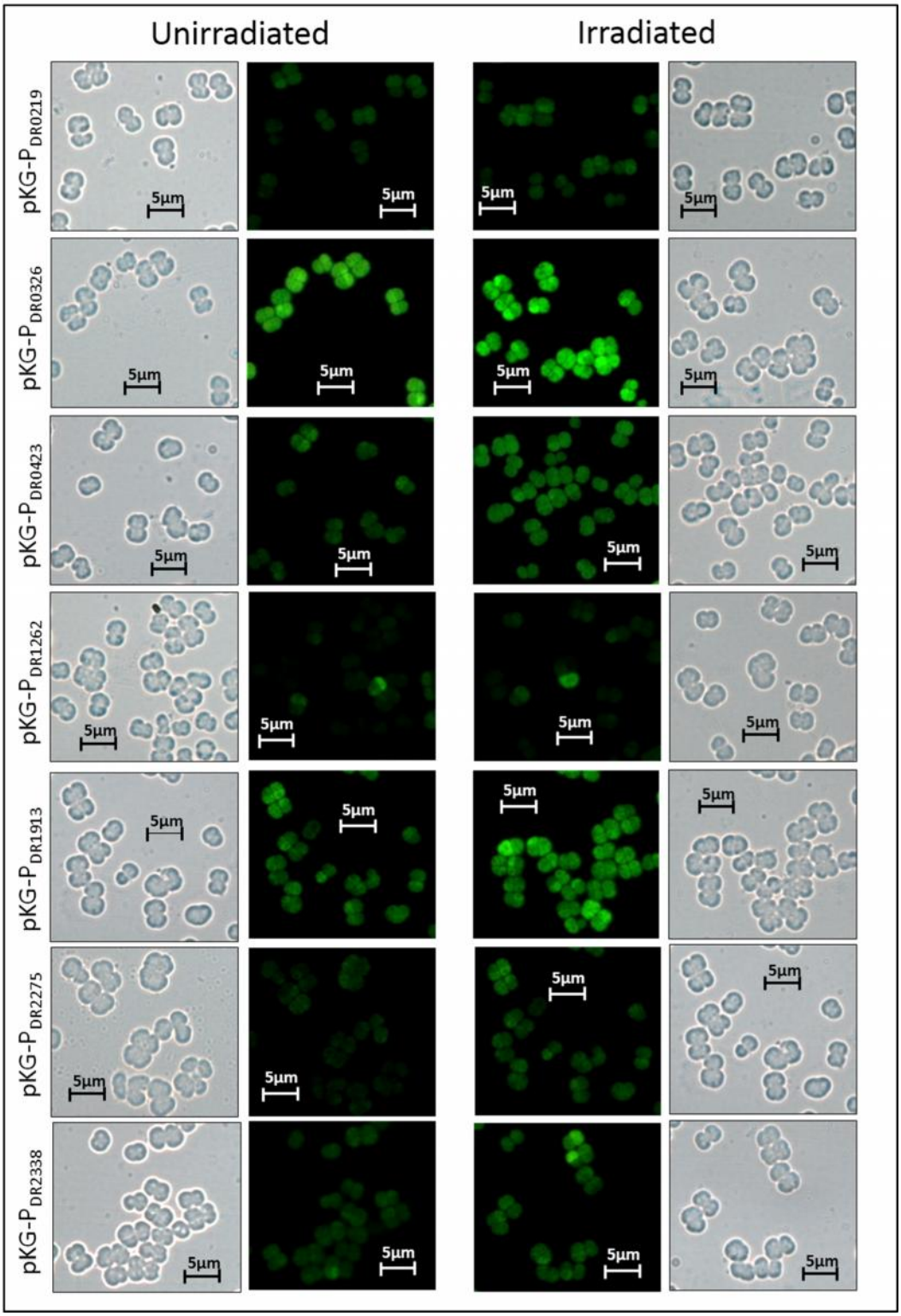

Fig. 4.13. Light and fluorescence microphotographs of recombinant *D. radiodurans* carrying moderately radiation induced promoters at 4h PIR. Following exposure to 6 kGy γ-radiation GFP fluorescence was monitored under fluorescence microscope, as described in the legend to Fig. 4.4.

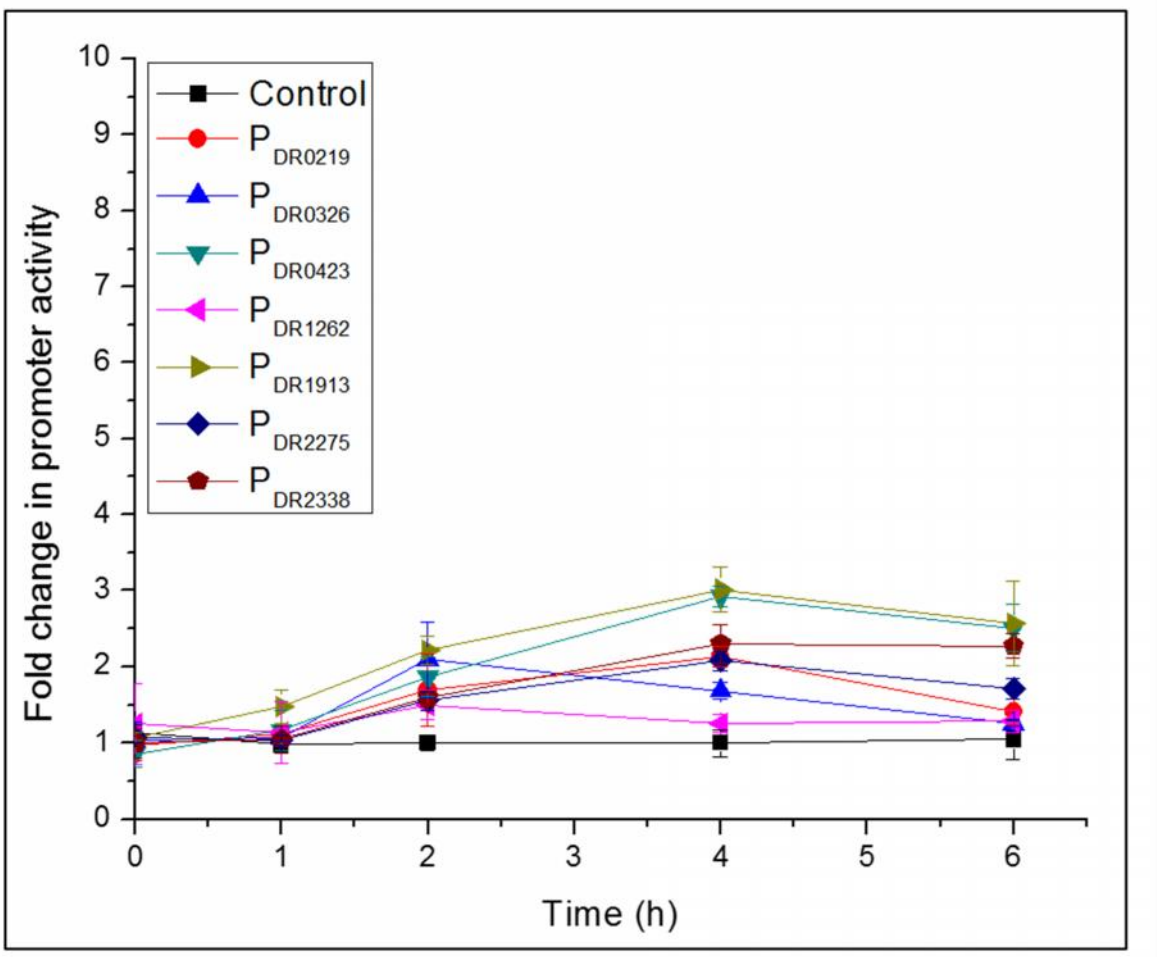

Fig. 4.14. Kinetics of moderately radiation induced Deinococcal promoter activity during PIR. The activity of selected moderately radiation induced promoters was monitored during PIR after 6 kGy γ-radiation. Other details were as described in the legend to Figs. 4.5, 4.12.

The RDRM lacking promoters P_{DR0053}, P_{DR0606}, P_{DR0694}, P_{DR1314}, P_{DR1358} P_{DR1720} and P_{DR2220} showed moderate to high basal promoter activity in normal growth conditions (Figs.4.9, 4.10). The highest activity was observed with P_{DR0606} and lowest was seen with P_{DR1720} promoter. These non-RDRM promoters showed constitutive expression (equivalent to basal level activity) throughout PIR (up to 6h). No significant change in promoter activity was observed with any of these non-RDRM promoters during PIR (Fig. 4.15, 4.16). These results established that RDRM is, in some way, also

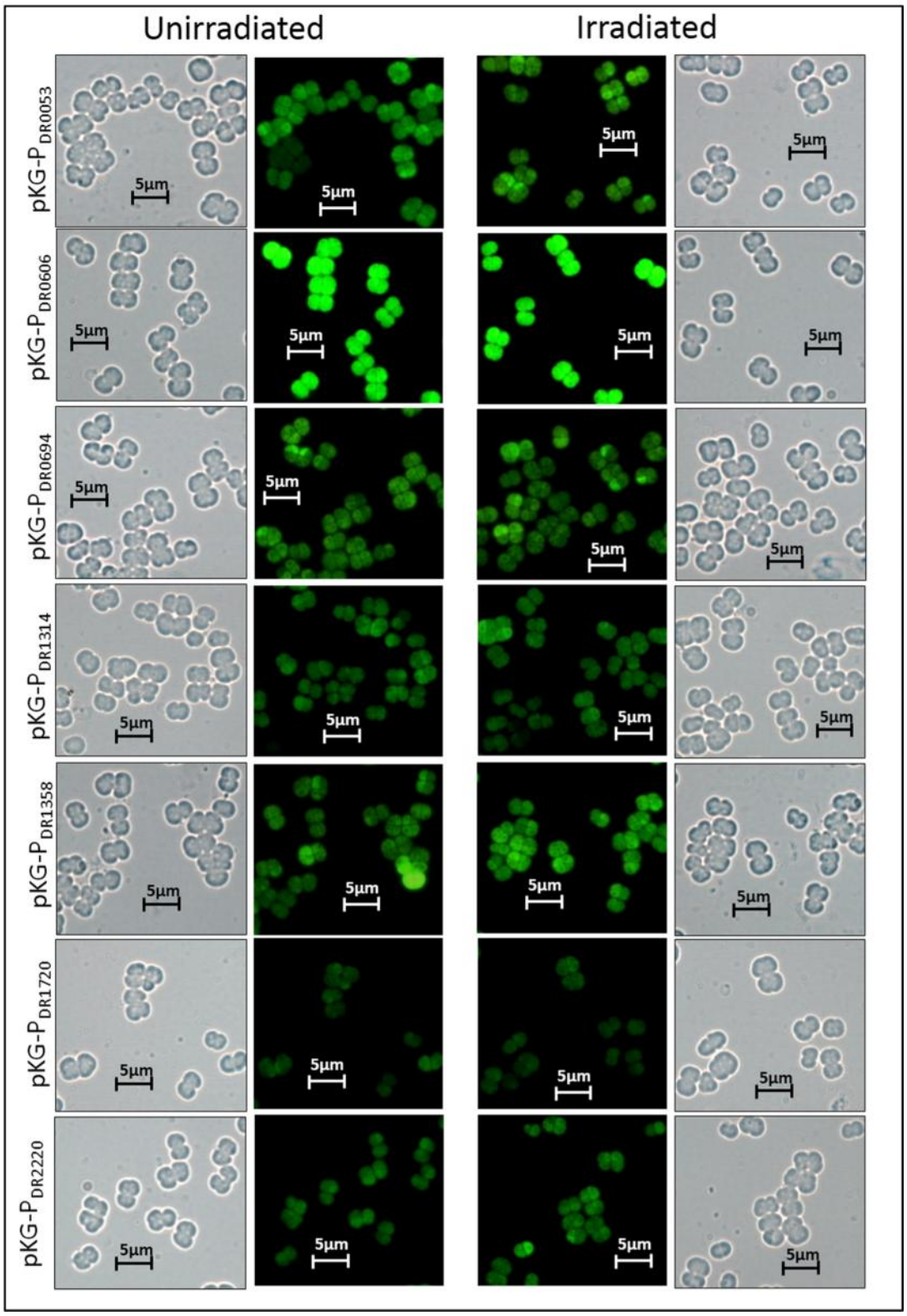

Fig. 4.15. Light and Fluorescence microphotographs of recombinant *D. radiodurans* cells carrying selected non-RDRM Deinococcal promoters. Other details were as described in the legend to Fig. 4.4.

necessary for radiation induction. All non-RDRM promoters selected for this study, except P_{DR0606} and P_{DR1314} have earlier been reported to be radiation inducible [6], but none of them showed radiation induction during PIR (Fig. 4.16). This may be due to different dose and dose rates used in different experiments. Other possibility may be that the cloned promoter fragments may lack other *cis*-regulatory elements which may be required for radiation induction.

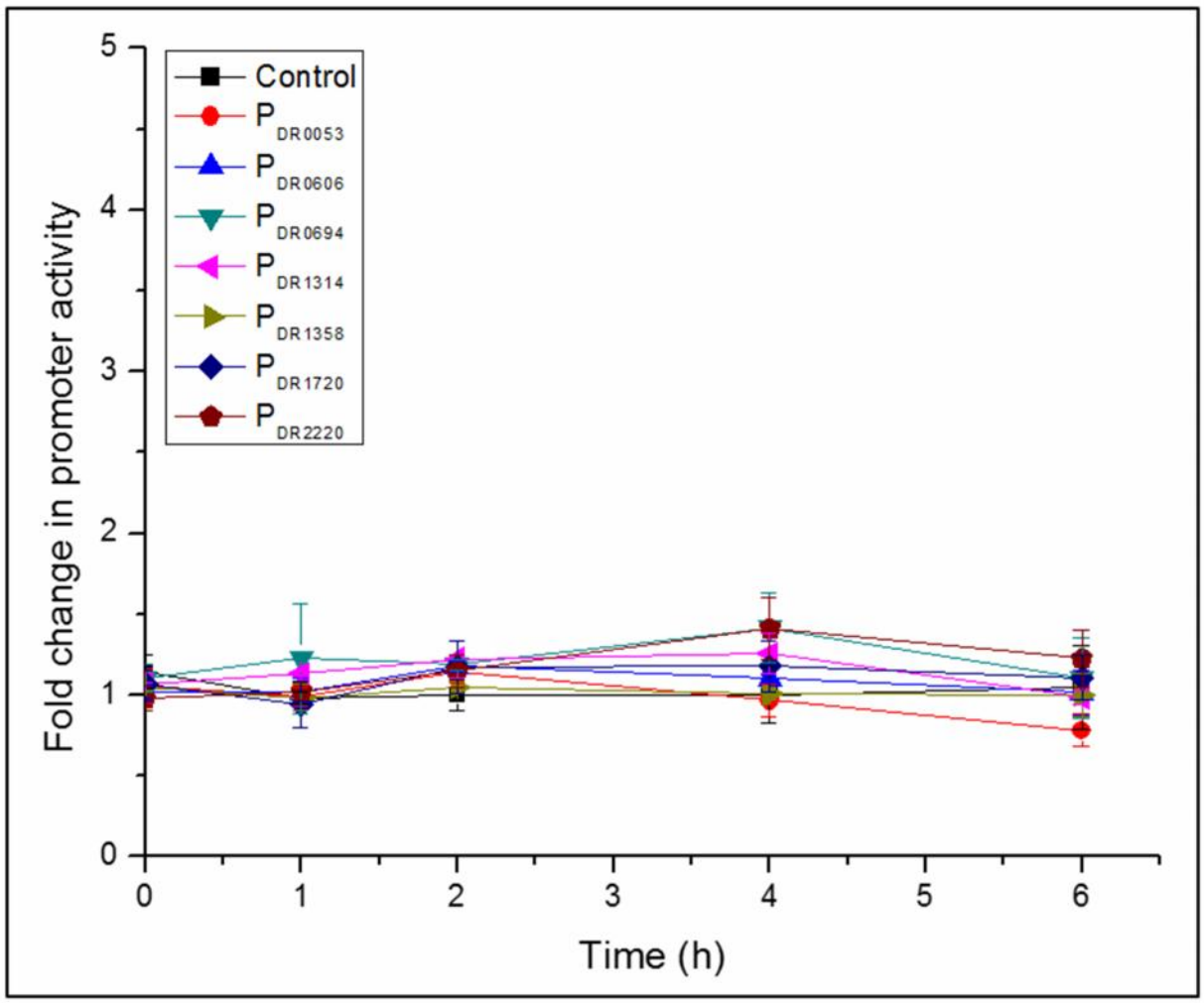

Fig. 4.16. Activity of non-RDRM promoters during PIR of recombinant *D. radiodurans* strains. Fold change in promoter activity was monitored during PIR. Other details were as described in the legend to Figs. 4.5, 4.12.

4.9. Deinococcal promoters are not induced by radiation in the heterologous *E. coli* system

The previous sections described that (a) RDRM reduced the promoter activity in *D. radiodurans*, (b) only RDRM containing promoters were induced in *D. radiodurans* during PIR, while (c) non-RDRM promoters were not induced by radiation. To see whether the effects of RDRM were due to the *cis*-element *per se* or also required other accessory factors, their radiation induction was examined in *E. coli* background where only RDRM was present but other required factors of *D. radiodurans* were expected to be absent. Three highly radiation inducible *D. radiodurans* promoters ($P_{DR0070,}$ P_{DR0906} and $P_{DR1143)}$ harboring *E. coli*-like promoter sequences were tested. Interestingly, while the basal promoter activity of P_{DR0906} was comparable in *E. coli* and *D. radiodurans*, those of P_{DR0070} and P_{DR1143} were significantly higher in *E. coli* than in *D. radiodurans* (Figs. 4.5, 4.7).

Recombinant clones of heterologous bacterium, *E. coli* carrying plasmids pKG- $P_{0070,}$ pKG-P_{0906} or pKG-P_{1143} were subjected to 300Gy of gamma radiation at a cell density of OD_{600nm} = 3. The D_{10} value of *E. coli* cells to gamma radiation varies from 0.27 kGy- 0.72 kGy, depending on the media in which cells are irradiated [133]. The 300 Gy dose is lower than the D_{10} dose. No radiation-responsive induction of GFP fluorescence was observed from any of these gene promoters in *E. coli* up to 6h of PIR (Fig. 4.17). The results suggested that the radiation responsive element RDRM alone is responsible neither for reduction in promoter activity under normal conditions nor for radiation induction of promoter activity. Other radiation-responsive factors present in *D. radiodurans* appeared to be necessary. *E. coli*, which apparently lacked them showed neither repression under normal conditions nor radiation induction of RDRM-based Deinococcal promoters during PIR.

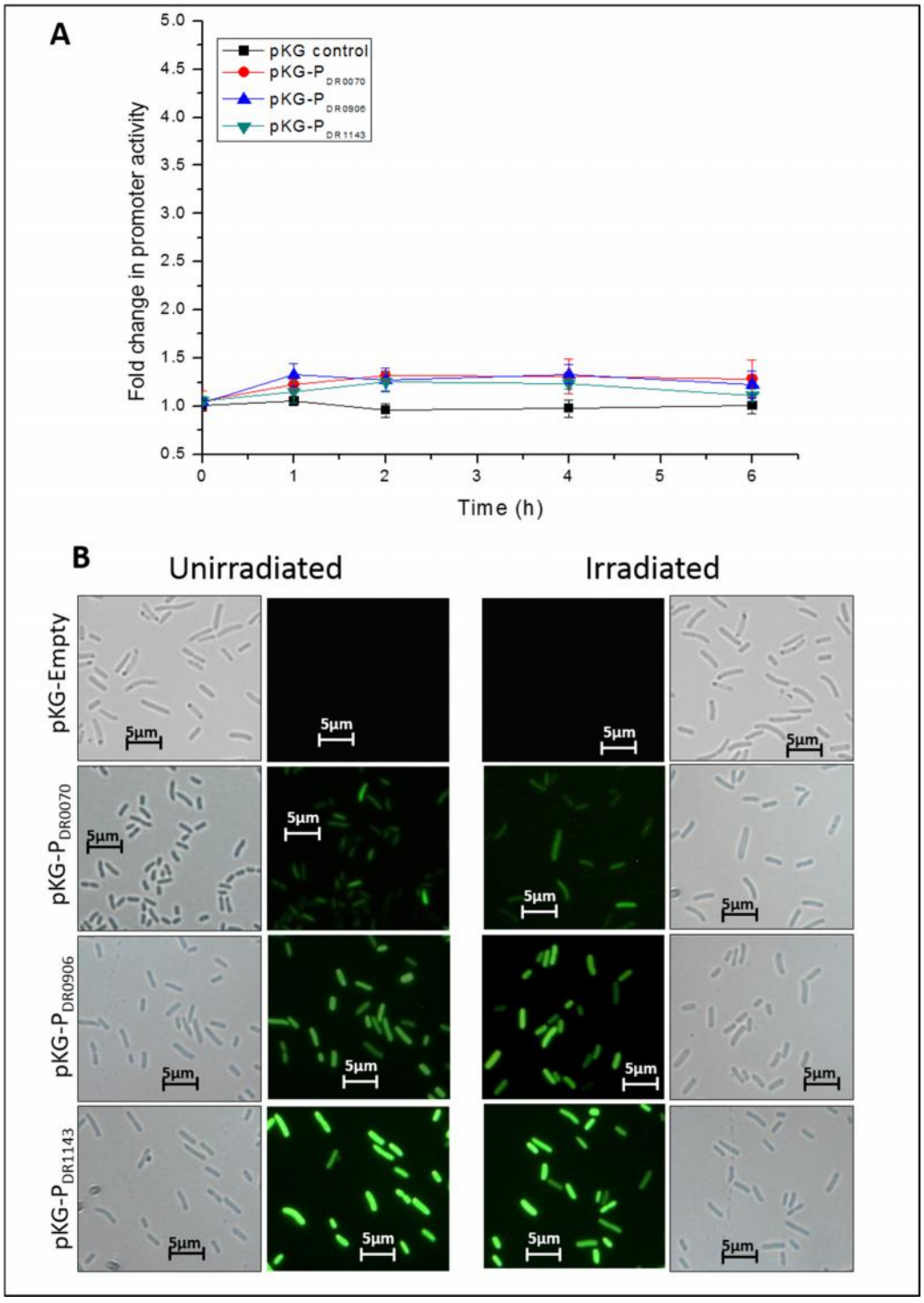

Fig. 4.17. Deinococcal promoter activity during PIR of recombinant *E. coli* strains. (A) The activity of Deinococcal promoters was monitored in *E. coli* by quantifying GFP Fluorescence during PIR following exposure to 300 Gy of γ-radiation. (B) Fluorescence microphotographs of recombinant *E. coli* strains at 4h of PIR. Other details were as described in the legend to Fig. 4.4.

4.10 Deletion of RDRM increases the basal promoter activity of RDRM containing promoters in *D. radiodurans.*

To further elucidate the role of RDRM in gene expression under normal and radiation stress conditions, the RDRM deletion mutants of radiation inducible genes *ddrB* (P_{DR0070}), *gyrB* (P_{DR0906}), P_{DR1143} (hypothetical) and *cinA* (P_{DR2338}) were constructed (Fig. 4.18A). Deletion of RDRM from P_{DR0070}, P_{DR1143} and P_{DR2338} promoters was carried out by PCR using promoter specific forward and reverse primers P0070F/P0070R6, P1143F/P1143R4 and P2338F/P2338R2, respectively (Table 2.2) containing required RDRM deletion. From P_{DR0906} RDRM was deleted by splicing PCR using P0906F1/P0906R3 and P0906F8/P0906R1 primers (Table 2.2) which include RDRM deletion. From all the 4 promoters only 17 bp RDRM was deleted and the remaining sequence was kept intact (Fig. 4.18). When the 17bp RDRM was deleted from P_{DR0070} promoter, the basal activity was significantly reduced, since deletion of RDRM from P_{DR0070} destroyed the transcription start site (TSS) (described in section 5.4**,** chapter-5). In P_{DR0906} promoter RDRM is present at -257 position, far upstream from start codon but very near to -10, -35 sequences. RDRM deletion increased (~1.5 fold) the basal level promoter activity of P_{DR0906}. The RDRM is located at - 45bp position in P_{DR1143} promoter and overlaps with -10 sequence. The deletion of RDRM, which also deleted -10 sequence, resulted in 3-fold increase in basal activity (Fig. 4.18B), which again showed that -10 and -35 sequences are dispensable in *D. radiodurans*. There is an AT rich motif in P_{DR1143} promoter which overlaps with -35 sequence and may be responsible for basal level promoter expression in *D. radiodurans*. The *cinA* (DR2338) gene promoter contains the RDRM at -3 position of translational start site and enters into protein coding region. Both DNA fragments (with and without RDRM) were cloned in pKG vector. The basal level GFP fluorescence was increased ~7 fold upon RDRM deletion. The results demonstrated that RDRM plays the role of a

negative regulatoru element (NRE) under normal growth conditions and its removal induced promoter activity, except when the TSS was compromised.

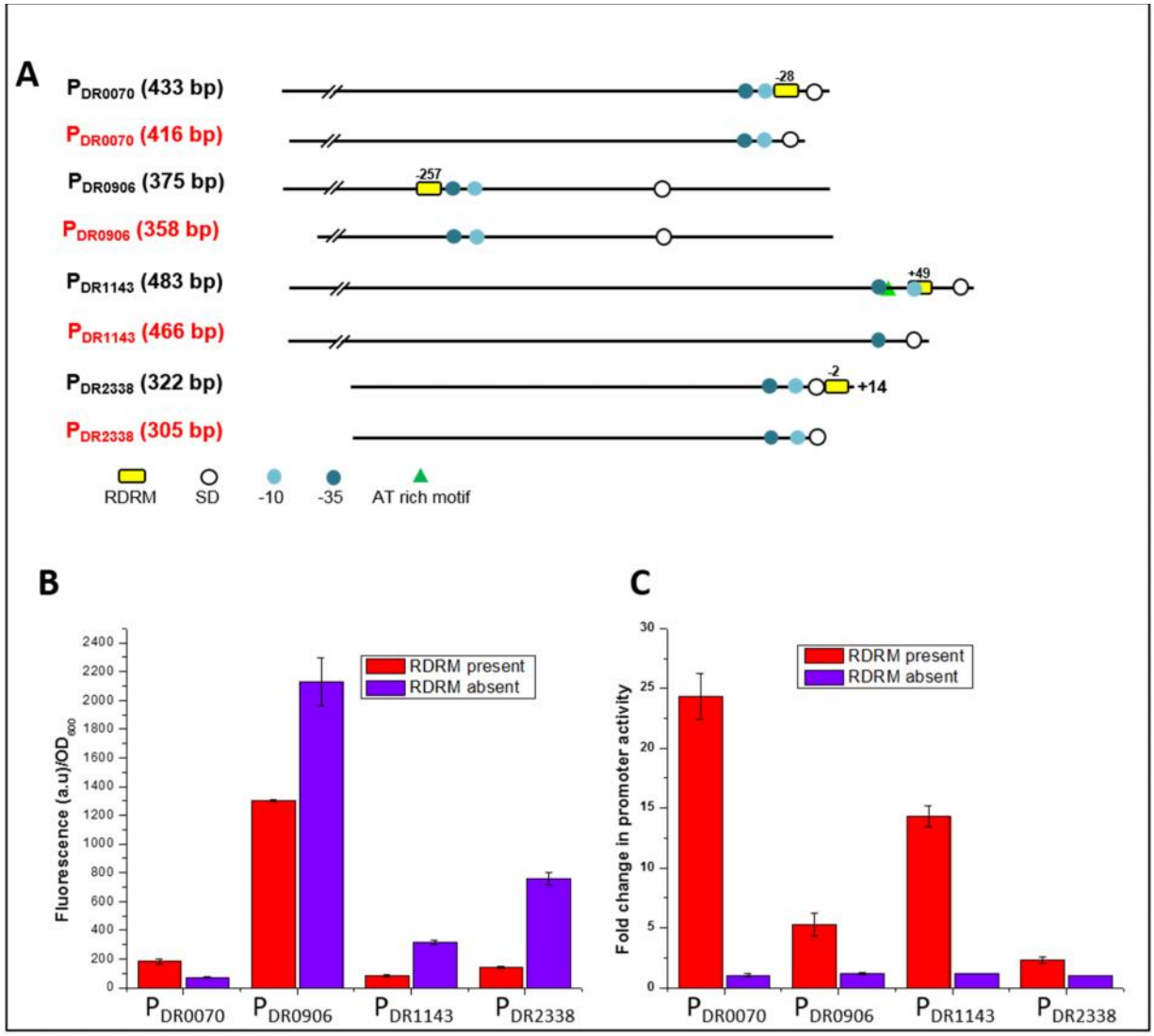

Fig. 4.18. Effect of RDRM deletion mutagenesis on the basal and radiation induced promoter activity in *D. radiodurans*. The RDRM from P_{DR0070}, P_{DR1143} and P_{DR2338} was deleted by PCR using forward primer and RDRM-deleted reverse primer. In P_{DR0906} RDRM was deleted by splicing PCR. (A) Schematic representation of promoters with (wild type) and without (mutant) RDRM sequence. Various elements like SD, -10, -35, RDRM and AT rich motifs are shown with different symbols (B) Basal activity of wild type (with RDRM) or mutant (without RDRM) promoters in *D. radiodurans*. (C) The activity of promoters shown in (A) and (B) at 4h of PIR following exposure of recombinant *D. radiodurans* strains to 6 kGy dose of γ-radiation.

4.11 RDRM deletion abolishes the radiation induction in *D. radiodurans*

The earlier results showed that non-RDRM promoters were not inducible under radiation stress (Figs. 4.15, 4.16). To evaluate if the aforementioned RDRM deletion mutants behaved similarly to non-RDRM promoters, the RDRM deletion mutants along with their wild type parent clones were subjected to 6 kGy gamma irradiation and analyzed for GFP expression during PIR. None of the RDRM deletion mutants of *D. radiodurans* showed gamma radiation responsive induction of GFP expression (Fig. 4.18C), whereas the wild type P_{DR0070}, P_{DR1143}, P_{DR0906} and P_{DR2338} promoters showed 25, 13, 6 fold and 2.5 fold radiation induction, respectively. These results suggest three possibilities: (i) the palindrome like RDRM forms hairpin loop to block the movement of RNA polymerase (RNAP) under normal conditions and is somehow dissolved under radiation stress to enhance the promoter activity. (ii) binding of a positive regulator to the RDRM enhances promoter activity after radiation stress. (iii) a negative regulator binds to RDRM under normal conditions to repress promoter activity and its release after radiation stress increases the promoter activity.

The first possibility can be ruled out since RDRM *per se* does not repress or radiation induce promoter activity in *E. coli*. The second possibility is unlikely, because the radiation induction of promoter activity during PIR is higher than de-repression by RDRM deletion mutant (Fig. 4.18B & C), unless the activator also binds to RDRM after clearance of the repressor. Third possibility seems most likely, since the DdrO repressor has been shown to bind RDRM *in vitro* and is cleaved by radiation-induced PprI protease. A more detailed study with selected promoters is described in the next Chapter to understand the role of RDRM in the radiation inducibility of Deinococcal promoters.

4.12 Discussion

The structure/function relationships in gene promoters of *D. radiodurans* are not yet clear but are distinct from those of *E. coli*, which harbours typical σ^{70} dependent promoters that comprise of conserved hexameric sequences at −10 and −35 position, relative to transcription start site (TSS). RNA sequencing in *D. deserti* has revealed that about 60% of the transcripts are either leaderless or have very short sequences. Most of the Deinococcal gene promoters lack *E. coli*-like -10, -35 sequences, but instead possess AT rich motifs in *D. deserti* [9]. *D. radiodurans* responds to gamma irradiation by upregulation of several genes involved in DNA repair and oxidative stress alleviation [6, 8, 89]. RDRM sequence has been implicated in such upregulation of gene expression during post-irradiation recovery (PIR) [10, 11]. To understand the intricacies of gene regulation at promoter level in *D. radiodurans*, several radiation induced genes were selected based on published transcriptomic, proteomic and mutagenesis data. Among the RDRM containing promoters, BPROM software detected presence of sequences similar to −10 and −35 like consensus sequences, except for P_{DR1262} and P_{DR2275} which harbored only an AT-rich motif (Fig. 4.2). Among non-RDRM promoters, P_{DR0606} showed well conserved and P_{DR0694} poorly conserved −10 and −35 like consensus sequences while P_{DR0053}, P_{DR1314}, P_{DR1358}, P_{DR1720} and P_{DR2220} harbored only an AT-rich motif upstream of the start codon (Fig. 4.2).

The promoters carrying the -10, -35 sequences exhibited various levels of promoter activity in *E. coli* but non-*E. coli-like* promoters did not. This showed that -10 and -35 sequences are necessary for promoter activity in *E. coli*. In contrast, *D. radiodurans* promoter sequences which lack *E. coli*-like -10, -35 consensus sequences express very well in *D. radiodurans*, indicating that *E. coli*-like consensus -10, -35 sequences are dispensable in *D. radiodurans*. Activity of Deinococcal promoters in heterologous host *E. coli* was dependent on (a) presence and (b) similarity to typical

σ^{70} controlled promoter sequences in *E. coli*, but not in *D. radiodurans*. The observed variation in Deinococcal promoter activity in *E. coli* could be attributed to the extent of conservation of -10 and -35 sequence and their location (Fig 4.5) or their regulation due to other DNA sequences present in Deinococcal promoters. *D. radiodurans* appears to utilize either alternate conserved sequences and/or sigma factors, for promoters that lack σ^{70} controlled promoter sequences (Fig. 4.7).

In *D. radiodurans*, except for P_{DR0906} (gyrB), P_{DR1913} (gyrA) and P_{DR2574} (ddrO) which are needed during normal growth, all RDRM containing Deinococcal promoters showed reduced basal promoter activity during normal growth and significant induction in promoter activity following exposure to 6 kGy of gamma rays (Figs. 4.8, 4.10). The variation (2-25 fold) in the activity of different RDRM containing Deinococcal promoters may be due to differences in promoter strength, conservation of RDRM sequence, position of RDRM with respect to TSS etc. GFP fluorescence based gamma radiation induced promoter activity, reported here, correlates well with the transcript level [6, 89] and protein [8] level induction of corresponding RDRM-based genes following gamma irradiation. In contrast, GFP expression by non-RDRM promoters or RDRM-deleted promoters remained unaltered during PIR, as evident from quantitation by fluorescence spectrometry as well as visualization by fluorescence microscopy (Figs.4.15, 4.16, 4.18C).

Radiation responsive gene expression displayed a clear time dependence that fits the autoregulatory nature of radiation response in *D. radiodurans*. The results obtained are consistent with those reported in literature [6, 8, 89]. Transcript or protein level induction in DNA damage repair related genes have been observed in the lag phase of PIR while the transcript or protein levels return back to normal by the time exponential growth resumes. Decline in GFP expression beyond 4h of PIR is clearly indicative of reestablishment of repressor mediated negative regulation

of RDRM promoters. Expectedly, promoters that lacked RDRM did not show significant change in GFP expression (0.94- to 1.4-fold) throughout PIR (Fig.4.14-15) .The gene products of *groESL* at both transcript and protein level, have been routinely used as internal control in *D. radiodurans* since its expression levels do not change in response to radiation stress [6, 8].

The RDRM based regulation of gene expression was tested in heterologous *E. coli*. No reduction in basal promoter activity of RDRM containing promoters was observed in *E. coli*. Irradiating *E. coli* cells carrying RDRM-based P_{DR0070}, P_{DR0906} and P_{DR1143} promoters at 300 Gy produced no change in promoter activity during PIR (fig.4.16). Thus, RDRM *per se* does not repress or induce promoter activity during PIR, in *E. coli*. On the other hand, when RDRM was deleted from P_{DR0906}, P_{DR1143} and P_{DR2338}, increase in basal promoter activity and loss of radiation induction was observed (Fig. 4.18). An exception was the deletion of RDRM from P_{DR0070} which abolished basal activity as well as radiation induction, since it destroyed its TSS. These results suggest possible involvement of a repressor, which binds RDRM to downregulate RDRM-based promoters under normal conditions. Destruction of such repressor post-irradiation can explain derepression of RDRM based promoters during PIR.

Recent studies have revealed that DdrO, a repressor protein, binds to RDRM sequence *in vitro* and represses the promoter activity in *Deinococcus* under normal growth conditions. Upon iradiation an activated protease PprI cleaves the DdrO repressor in to 11 kDa and 3kDa fragments, inducing the gene expression [12, 13]. While *trans*-acting protein factors regulating RDRM based promoters are now known, little is known about the structural and functional relationships of and within the RDRM *cis*-element. Next chapter reports mechanistic aspects of RDRM-based negative/positive regulation of radiation responsive promoters in *D. radiodurans*.

Chapter 5

Regulation of promoter activity under normal and radiation stress conditions in *D. radiodurans*

Understanding the regulation of a promoter activity requires knowledge of the *cis* regulatory elements and *trans* acting factors. The study of promoter activity in presence and absence of these elements under normal and stress conditions reveals the regulatory mechanisms underlying the particular promoter activity. Deletion of the *cis* element RDRM from *D. radiodurans* promoters increased the basal activity of promoter but lost the radiation induction of gene expression during gamma radiation stress (Chapter 4). Recently three elegant studies presented first evidence of transacting proteins involved in the negative regulation of DNA damage regulon (DDR) during normal growth conditions in *D. radiodurans* and *D. deserti* [12, 13, 24]. The transcriptional repressor protein DdrO (DR2574 gene product) was shown to bind RDRM *in vitro* and repress the RDRM-harboring promoters under normal growth conditions, while radiation induction occurred upon repressor clearance by a radiation activated protease PprI (also known as IrrE, DR0167 gene product) which cleaved DdrO [12, 13, 24]. Interestingly, *ddrO* gene also harbors a RDRM-based promoter and, therefore, regulates its own expression [10].

While DdrO dimer required the entire 17bp of RDRM for binding [24], point mutations in the RDRM sequence were shown to relieve negative regulation of the *ddrB* gene [12]. Wang *et.al* showed by *in vitro* study that DdrO binds to 17bp RDRM and one extra base pair on both sides [24]. If so, DdrO should bind the RDRM wherever it is introduced in the promoter sequence, irrespective of its position. Also, if the release of DdrO repressor (derepression) from the promoter alone can cause the induction of the promoter activity, the basal activity of the promoter in the absence of RDRM (where the repressor cannot bind) should reach the same level as radiation induction. But the promoter activity in the absence of RDRM was always less than the radiation induced level of native wild type RDRM in five genes studied (Fig. 4.18, Chapter 4).

Results reported in this chapter evaluate the effect of RDRM sequence, position and orientation on promoter activity. First, the regulation of RDRM-based promoters by DdrO and PprI was ascertained for the cloned promoters. Subsequently effect of introduction of RDRM in to non RDRM promoter was examined. The promoter activity was also assessed in the presence of RDRM in reverse orientation. To understand the RDRM/DdrO repressor interaction, several point and deletion mutations were made in P_{DR0070} and P_{DR0906} promoters. The RDRM was also shifted to different locations around the promoter, in addition to the native one or in absence of the wild type RDRM. The promoter activity of various RDRM containing and non-RDRM promoters were evaluated in the presence or absence of repressor protein DdrO in the heterologous host *E. coli*. A few selected promoters were also assessed for their activity in a *pprI* knockout mutant of *D. radiodurans*. To understand the position effect of RDRM, chimeric fusions of P_{DR0906}-P_{DR0606} promoters were also constructed and their activity was assessed during normal growth and under radiation stress conditions in *D. radiodurans*.

5.1 Interaction of DdrO repressor with RDRM containing promoters in heterologous *E. coli*

DdrO is a transcriptional repressor with helix-turn-helix (HTH) type of DNA binding domain [24]. Recent studies showed that it acts as a repressor by binding to RDRM and downregulates gene expression under normal growth conditions [12, 13, 24]. Upon radiation stress, the activated metallo-protease PprI cleaves the DdrO protein and thereby induces gene expression during PIR [12, 13, 24]. This mode of gene regulation has been demonstrated with *ddrB* gene promoter which harbors RDRM sequence with highest conservation. Since the RDRM in other promoters vary in the sequence conservation, the repression of their downstream genes by DdrO can be different. Indeed, a few RDRM containing gene promoters are tightly regulated with no basal expression

under normal growth conditions [87], while others show significant basal expression even in the presence of RDRM sequence [87]. To assess possible differential repression of cloned promoter activity by DdrO protein, 10 RDRM containing promoters [P_{DR0070} (*ddrB*), P_{DR0099} (*ssb*), P_{DR0219} (*ddrF*), P_{DR0423} (*ddrA*), P_{DR0596} (*ruvB*), P_{DR0906} (*gyrB*), P_{DR1143} (hypothetical), P_{DR1913} (*gyrA*), P_{DR2275} (*uvrB*) and P_{DR2338} (*cinA*)] along with two non-RDRM promoter [P_{DR0606} (*groESL*) and P_{DR0694} (hypothetical)] taken as controls, were tested in the presence of Deinococcal DdrO protein overexpressed in the heterologous host *E. coli*.

For this, the *ddrO* gene was PCR amplified from *D. radiodurans* genomic DNA with ddrO-F/ddrO-R2 primes (Table 2.2) and cloned in pET21a expression vector at NdeI/HindIII restriction sites. This plasmid was transformed into *E. coli* over expression strain [BL21 (DE3) pLysS]. The cloning was confirmed by colony PCR with ddrO-F/ddrO-R2 (Fig.5.1A) and DNA sequencing. DdrO protein expression was induced by adding 1mM IPTG. Appearance of intense CBB stained protein band of correct size (14.3 kDa) (Fig.5.1B) confirmed DdrO protein expression. Maximum protein induction was observed at 3h of IPTG induction (Fig. 5.1B).

The pKG vector carrying above mentioned 10 promoters were transformed in to *E. coli* cells carrying either pET21-empty or pET21-ddrO plasmid. The promoter activity was compared in cells lacking DdrO or having high level of DdrO and analyzed by measuring GFP fluorescence during 3h of IPTG induction. RDRM containing promoters showed varying degree of repression P_{DR0070} (3.87 fold), P_{DR0099} (3.8 fold)), P_{DR0219} (2 fold), P_{DR0423} (1.5 fold), P_{DR0596} (1.8 fold), P_{DR0906} (2.8), P_{DR1143} (7fold), P_{DR1913} (2 fold), P_{DR2275} (1.7fold) and P_{DR2338} (1.5 fold) of their promoter activity in the presence of DdrO protein. The non-RDRM promoters of P_{DR0606} and P_{DR0694} showed

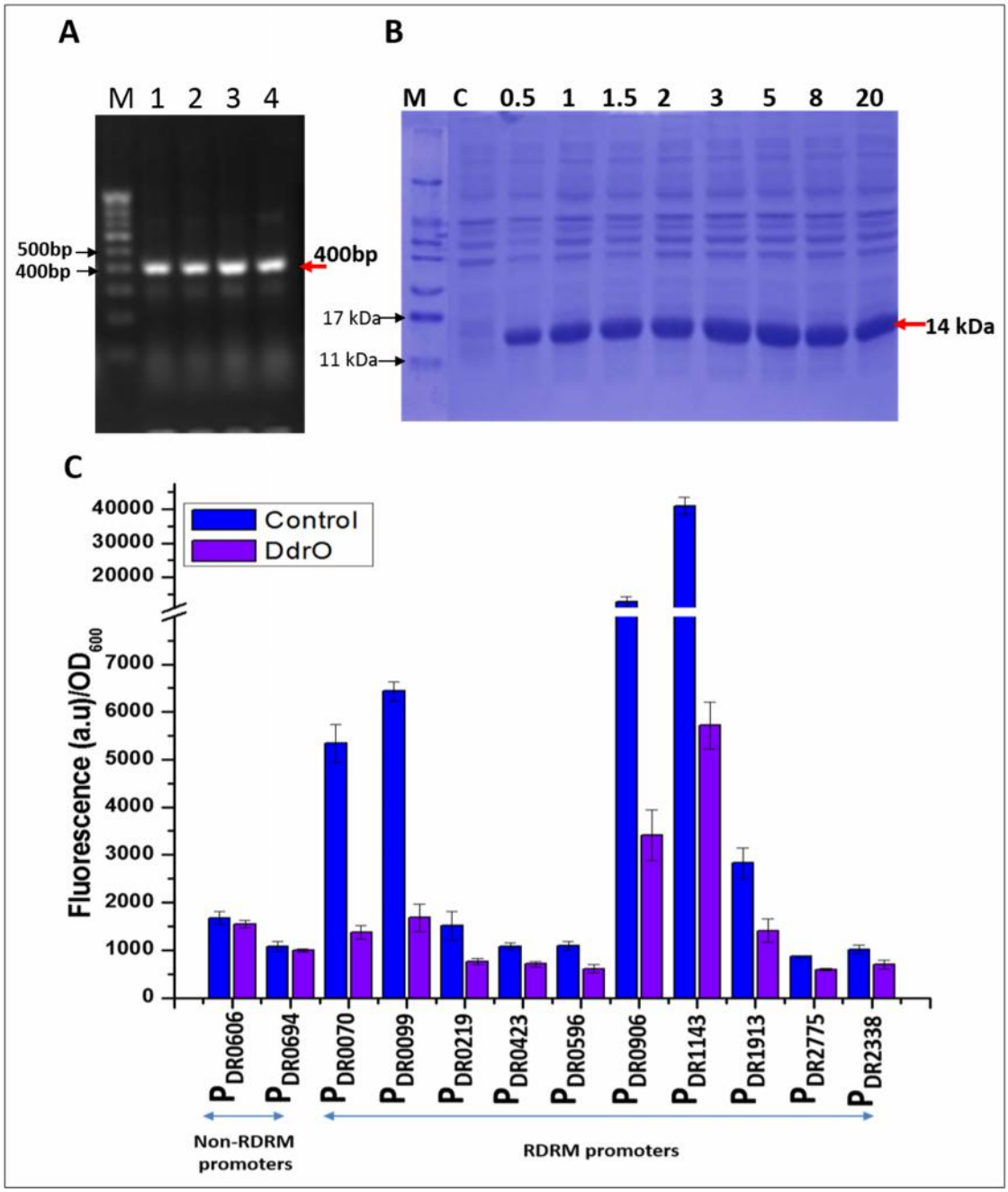

Fig. 5.1. Cloning of *ddrO* gene in pET21 and evaluation of DdrO protein effect on various Deinococcal promoters in heterologous *E. coli*. (A) Colony PCR of *E. coli* clones carrying pET21-ddrO plasmid with ddrO-F/ddrO-R2 primers. Lanes: 100bp DNA marker and 1-4 PCR products of different colonies. (B) Over expression of DdrO protein in *E. coli* cells. Lanes, M-protein marker, C protein from control cells (pET21-empty) and other lanes contained protein extracts of *E. coli* carrying pET21-ddrO taken at specified time points (h) during IPTG induction. (C) Deinococcal promoters with/without RDRM were cloned in pKG vector and transformed into *E. coli* (BL21a) cells carrying either pET21-empty (control) or pET21-ddrO. The promoter activity (GFP expression) was quantified in live cells after IPTG induction of DdrO protein.

no change in promoter activity in the absence or presence of DdrO (Fig. 5.1C). The results demonstrate DdrO-based repression of all cloned RDRM promoters in *E. coli* which is in good agreement with the *in vitro* results, wherein DdrO was shown to bind at RDRM sequence [24]. The results reconfirm that DdrO repression occurs through its binding to RDRM, with the degree of repression varying with RDRM sequence and possibly other regulatory elements present in the cloned promoter.

5.2 PprI alone cannot induce Deinococcal promoter activity in heterologous *E. coli*

The PprI protein is a general switch responsible for activation of several genes during PIR [23]. A recent study revealed that PprI is a metalloprotease which cleaves DdrO repressor protein (which binds to RDRM *in vitro*), during PIR in *D. radiodurans* [13]. To assess the effect of PprI protein *per se* on the activity of Deinococcal promoters, if any, the *pprI* gene was PCR amplified with pprI-F/pprI-R primers (Table 2.2) and cloned in pET21a expression vector at NdeI/HindIII restriction site. Cloning was confirmed by colony PCR with pprI-F/pprI-R primes (Fig. 5.2A) and DNA sequencing, while overexpression in *E. coli* was confirmed by resolving PprI protein in IPTG induced cells by PAGE (Fig. 5.2B). The pKG plasmids carrying cloned RDRM-based promoters P_{DR0070}, P_{DR0099}, P_{DR0219}, P_{DR0423}, P_{DR0596}, P_{DR0906}, P_{DR1143}, P_{DR1913}, P_{DR2275} and P_{DR2338}, or non-RDRM promoters P_{DR0606}, P_{DR0694}, were transformed into *E. coli* BL21 cells carrying either plasmid pET21-*pprI* which expresses PprI or pET21-empty which does not express PprI.

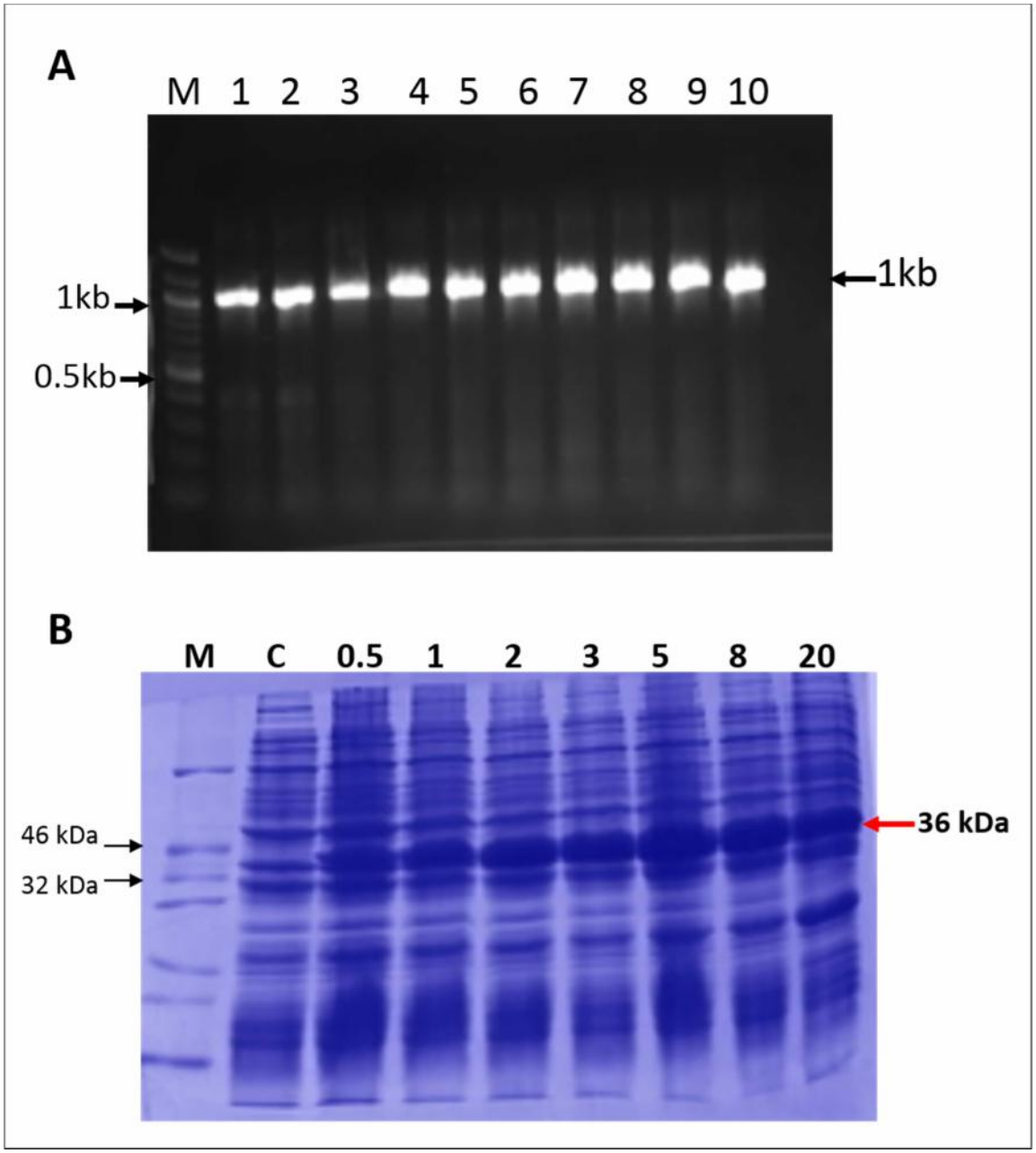

Fig. 5.2. Cloning of *pprI* gene in pET21 and its expression in *E. coli*. (A) Colony PCR of *E. coli* cells transformed with pET21-pprI plasmid construct with pprI-F/pprI-R primers. Lanes M-100bp DNA marker, lanes 1-10 contain PCR amplicons of various colonies. (B) The *pprI* gene was PCR amplified from genomic DNA of *D. radiodurans* with pprI-F/pprI-R primers and cloned in pET21a vector at NdeI/HindIII restriction sites. Protein was over expressed by IPTG induction and whole cell protein was extracted from samples withdrawn at different time points and resolved on 12% PAGE gel. Lanes represent M- protein markers, C-control, while other lanes contained protein samples taken at different time points (in hours) during IPTG induction. The 36 kDa PprI protein is marked with a red arrow.

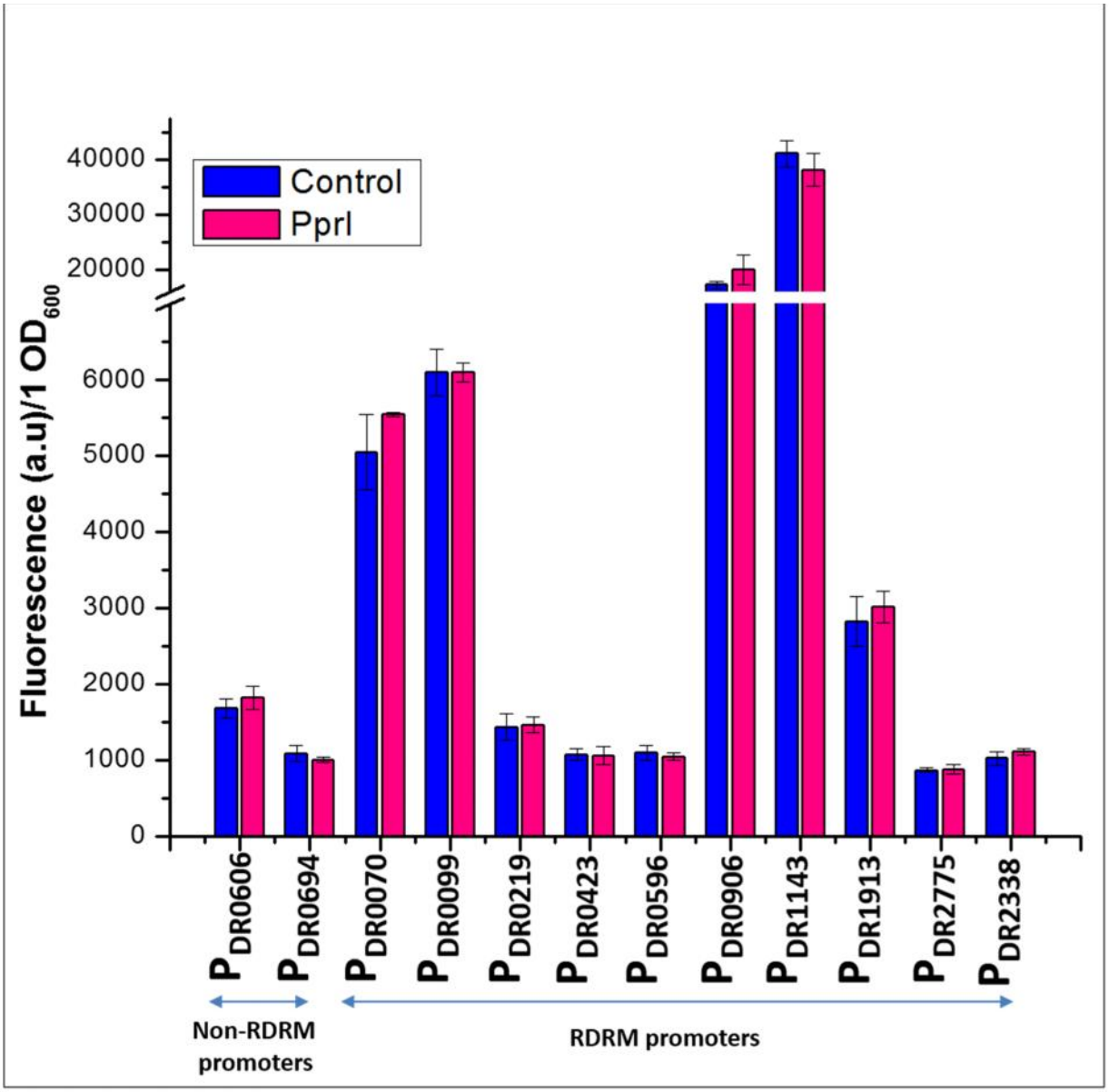

Fig. 5.3. Effect of PprI *per se* on promoter activity. The pKG plasmids with different cloned Deinococcal promoters were co-transformed into *E. coli* (BL21) cells harboring either pET21a-empty or pET21-pprI plasmid. The promoter activity was monitored by quantifying the GFP expression at 3h of PprI induction with IPTG.

Effect of overexpression of Deinococcal PprI protein on promoters with/without RDRM was evaluated in *E. coli*, which does not possess either the DdrO or PprI regulatory proteins. No significant change in promoter activity of either RDRM containing or non-RDRM promoters was observed with PprI protein in *E. coli* (Fig. 5.3). This *in vivo* result suggests that PprI *per se* is not an activator and cannot induce the promoter activity on its own. The results indirectly support the PprI effect through its protease activity which cleaves DdrO repressor during PIR and switches on the induction of several RDRM-based promoters of genes encoding DNA repair proteins.

5.3 No radiation induction of Deinococcal promoters is seen in *pprI* knockout mutant of *D. radiodurans*

The *pprI* gene of *D. radiodurans* is a metalloprotease that cleaves DdrO repressor, thereby, upregulating expression of RDRM-based promoters during PIR. However, proteomic investigations have revealed that the gene also controls several non-RDRM promoters [23]. Over-expression of PprI protein alone did not affect activity of RDRM containing promoters or non-RDRM promoters in *E. coli* (Fig. 5.3). But this may be because other regulatory proteins required were absent in *E. coli*. To investigate the effect of absence of PprI on promoter activity in *D. radiodurans*, where all other regulatory factors would be present, a *pprI* knockout mutant of *D. radiodurans* was constructed as described earlier [87] and is shown in Fig. 5.4. In brief, the spectinomycin resistance cassette (Spc^R) was excised by restriction digestion with HindIII/EcoRI from p13840 plasmid, blunted and blunt end ligated to SmaI restriction digested pBluescript plasmid to generate the pBS-spc construct (Fig. 5.4A). The upstream and downstream sequences (~500 bp) of Deinococcal *pprI* (DR0167) gene were PCR amplified using pprI-up-F/pprI-upR and

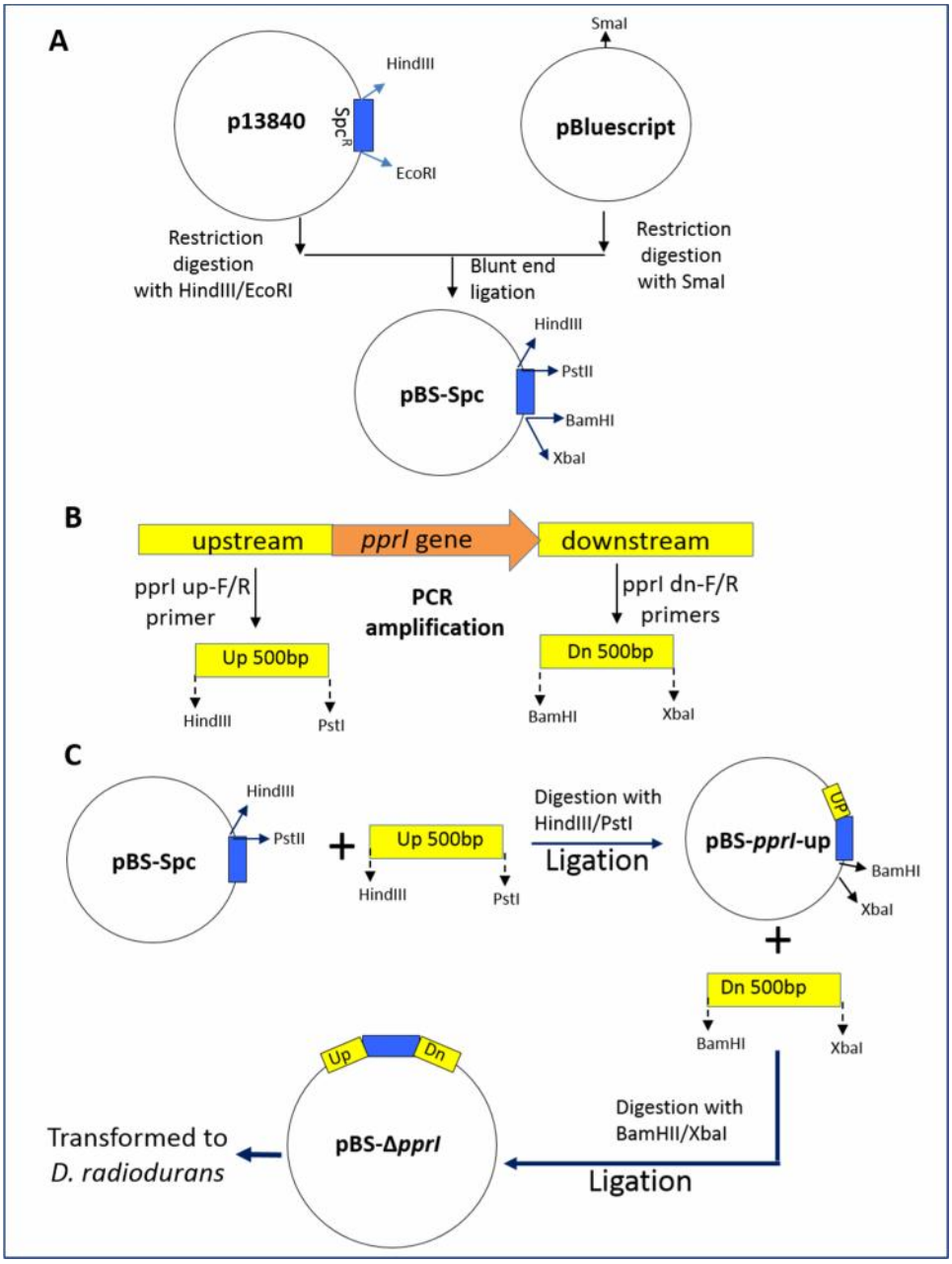

Fig. 5.4.Construction of *pprI* knockout mutant of *D. radiodurans*. (A) The schematic diagram represents the cloning of spc^R (spectinomycin) cassette which was eluted by restriction digestion (EcoRI/HindIII) of p13840 plasmid and its clone in pBluescrpt plasmid by blunt end ligation at SmaI site. (B) Shematic representation of *pprI* gene along with upstream and downstream regions. The upstream and downstream 500bp of *pprI* gene was PCR amplified using pprI-up-F/pprI-up-R and pprI-dn-F/pprI-dn-R primers, respectively. (C) Construction of pBS-ΔpprI. Plasmid pBS-Spc and *pprI* upstream fragment were digersted with HindIII/PstI and ligated to get pBS-pprI-up plasmid. Plasmid pBS-pprI-up and *pprI* downstream fragment were digeted with BamHI/XbaI and ligated to get final construct pBS-Δ*pprI*. This was transformed into *D. radiodurans*.

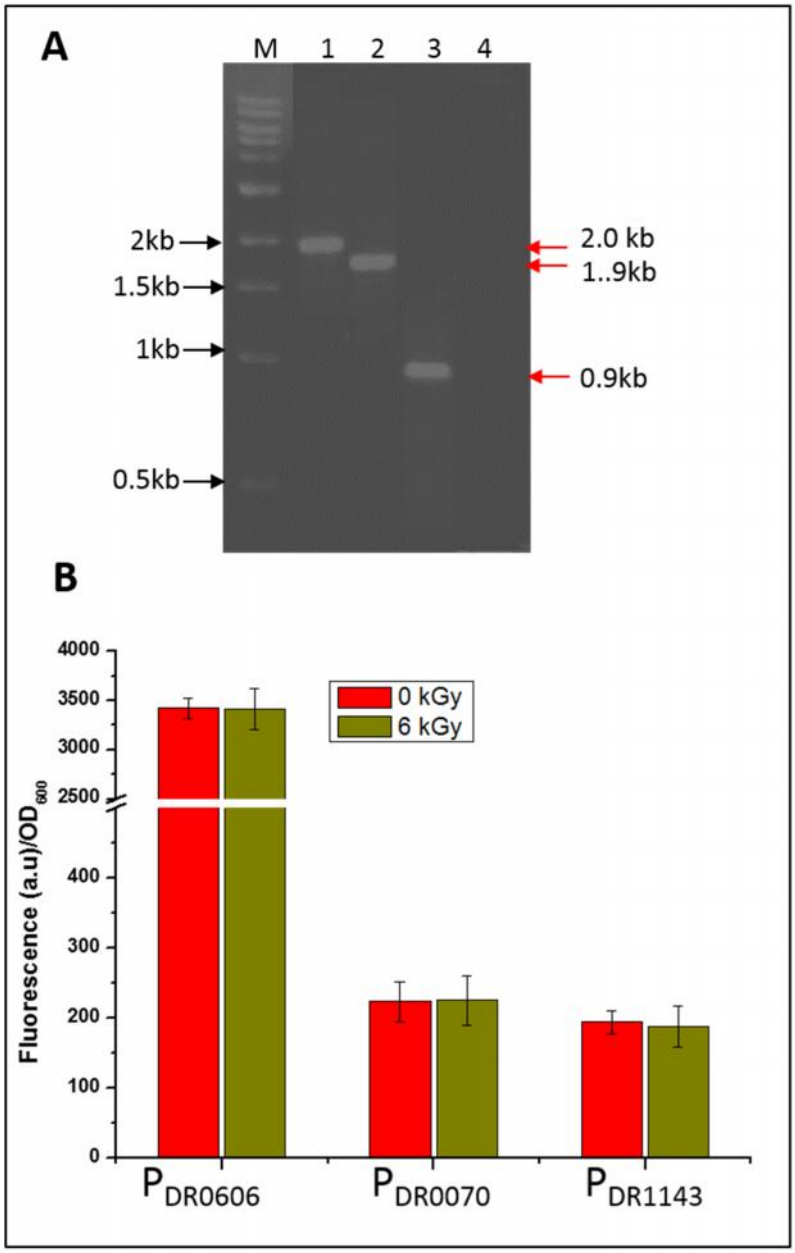

Fig. 5.5. Confirmation of *pprI* knockout mutant and assessment promoter activity in *pprI* mutant of *D. radiodurans*. (A) *pprI* knockout mutant was confirmed by PCR with pprI-up-F/pprI-dn-R primers to confirm replacement of *pprI* gene with Spc^R. Lane M: 1kb DNA marker, lane 1: wild type with ~2kb size band (500bp up+1kb *pprI* gene+500bp down region), lane 2: mutant with ~1.9kb size band (500bp up+900bp Spc^R+500bp down region). PCR amplicons of wild type and mutant with *pprI* gene specific primers pprI-F/pprI-R, lane 3 ~1kb band of *pprI* gene in wild type and lane 4: no band in mutant confirming deletion of *pprI* gene. (B) The pKG harboring RDRM promoters P_{DR0070} and P_{DR1143} and non-RDRM promoter P_{DR0606} were transformed into *D. radiodurans pprI* knockout mutant and the promoter activity was quantified at 4h of PIR.

pprI-dn-F/pprI-dn-R primers (Table 2.2), respectively. The pprI-up and pprI- dn DNA fragments were digested with HindIII/PstI and BamHI/XbaI restriction enzymes respectively and cloned in identical sites in pBS-spc on either side of spc^R cassette, to generate plasmid pBS-ΔpprI (Fig. 5.4B). Cloning of correct sequences was confirmed by colony PCR with pprI-up-F/ pprI-dn-R and DNA sequencing. The pBS-ΔpprI construct was transformed into *D. radiodurans* competent cells and selected on the TGY agar plate containing spectinomycin (100 µg/mL). Positive transformants were subjected to 15 serial passages to obtain homozygous *pprI* deletion mutant. The mutation was ascertained by PCR amplification either using (a) pprI-up-F/pprI-dn-R primers to confirm replacement of *pprI* gene with Spc or (b) pprI-F/pprI-R primers to ascertain absence of *pprI* gene from *pprI* knockout mutant (Fig. 5.5A). The *pprI* knockout mutant of *D. radiodurans* grew at rates comparable to the wild type strain under normal growth conditions.

Two highly inducible RDRM containing promoters (P_{DR0070}, P_{DR1143}) and one strong non RDRM promoter (P_{DR0606}) were tested in *pprI* knockout background of *D. radiodurans* by quantifying the GFP fluorescence under normal growth and radiation stress conditions. As expected, no change in basal promoter activity was observed but the radiation induction was completely abolished (Fig. 5.5B). The results demonstrate that PprI protein is essential for radiation induction of RDRM-based promoters. These data substantiate the DdrO/PprI model of radiation regulation at RDRM, proposed earlier based on *in vitro* studies [13], and further establish it *in vivo*.

5.4 Mutations in P_{DR0070} promoter

One of the top five radiation responsive genes of *D. radiodurans* which showed highest fold change during PIR is the *ddrB* (DR0070) gene [89]. Following gamma irradiation, transcription of *ddrB* gene is known to be induced several fold, [6, 89] and its protein is synthesized *de novo* [8,

125]). In the present study, the *ddrB* promoter showed very weak activity under normal growth conditions but showed time dependent increase following gamma irradiation, ~25 fold increase at 4h of PIR, suggesting its temporal regulation. RDRM present upstream of *ddrB* gene is highly conserved in its sequence and location among all the sequenced Deinococcal genomes and has the highest score 5.24 (score calculated based on positional weight matrix) in both *D. radiodurans* and *D. geothermalis* [10].

The *ddrB* gene promoter was chosen to systematically investigate the role of RDRM sequence in radiation-responsive temporal regulation of *ddrB* gene. RDRM is located immediately downstream of -10 sequence in this promoter. The site directed mutagenesis approach was employed to reconstruct *ddrB* promoter ($P_{DR0070\text{-}1}$) with a series of RDRM sequence mutants wherein either (a) most conserved bases of RDRM were mutated (G5A, C13T) ($P_{DR0070\text{-}2}$), (b) 5 bases from 5' end of RDRM were deleted ($P_{DR0070\text{-}3}$), (c) RDRM sequence was completely deleted ($P_{DR0070\text{-}4}$) or (d) RDRM was replaced with random DNA sequence of the same length (5'-GTTCGGGCTCTTGCGAA-3') ($P_{DR0070\text{-}5}$) (Fig. 5.6A). The aforesaid five promoter mutants were constructed by amplifying the mutated fragments by PCR using single forward primer and different reverse primers carrying specified mutations (P0070F/R3, P0070F/R4, P0070F/R5, P0070F/R6 and P0070F/R8 primer pairs respectively) (Table 2.2). Except for the specific mutations in RDRM sequence, rest of the promoter sequence was kept identical in all the promoter mutants, for easy interpretation of the role of specific bases in determining GFP fluorescence. All the promoter mutants, except for $P_{DR0070\text{-}3}$, displayed very low GFP expression (Fluorescence < 200 AU/A_{600}) similar to native *ddrB* promoter ($P_{DR0070\text{-}1}$) and empty vector control (Fig. 5.6B). Significantly high basal GFP expression (8 fold more than $P_{DR0070\text{-}1}$) was observed in cells carrying P_{DR0070}-3 (Fig. 5.6B). The data suggest that (a) *ddrB* promoter is negatively regulated by presence

of RDRM cis-element under normal conditions and (b) $P_{DR0070\text{-}3}$, where 5 bases were deleted from 5' end of RDRM sequence, exhibited high basal promoter activity, probably due to inability of mutated RDRM to bind to DdrO repressor. Further, the ability of *ddrB* promoter mutants to drive gamma radiation inducible GFP expression was also assessed. Compared to wild type *ddrB* promoter ($P_{DR0070\text{-}1}$), gamma radiation inducible GFP expression by P_{DR0070}-2 decreased 2 fold while the other 3 mutants did not display any significant change in GFP expression throughout PIR (Fig. 5.6C). Absolute GFP fluorescence quantitation revealed that GFP expression by $P_{DR0070\text{-}2}$ and $P_{DR0070\text{-}3}$ was nearly similar (Fluorescence ~ 2000 a.u./A_{600} = 1) at 4h PIR (Fig. 5.6D) but radiation induction was 2 fold less than $P_{DR0070\text{-}1}$ (Fluorescence ~ 4000 a.u./A_{600} = 1) (Fig. 5.6C). In comparison mutants $P_{DR0070\text{-}4}$ and $P_{DR0070\text{-}5}$ showed GFP fluorescence close to the irradiated empty vector control (Fluorescence < 100 a.u./A_{600} = 1) (Fig. 5.6D). Thus, mutation in the most conserved bases of RDRM (G5A, C13T) decreased gamma radiation responsive gene expression although basal level of gene expression remained unaffected. Further, GFP expression by $P_{DR0070\text{-}2}$ during PIR was similar to the mutant that lacked 5 bases ($P_{DR0070\text{-}3}$). It indicates possible involvement of an inducer/activator protein that binds to RDRM at the same binding site as that of repressor after its degradation by PprI, but specifically needs the most conserved bases (G5 and C13) for optimal binding. Location of TSS is not known in *ddrB* promoter of *D. radiodurans* but is likely to be within the RDRM which is just upstream of ATG start codon, as is case in the *D. deserti ddrB* gene, since (a) deletion of initial 5 bases did not decrease GFP fluorescence but (b) deletion of entire RDRM sequence or its replacement with non-specific sequence completely abolished basal as well as radiation inducible gene expression. Generally promoters are present upstream of transcription start site (TSS). To understand why above RDRM variants $P_{DR0070\text{-}4}$ and

$P_{DR0070-5}$ did not show any activity, the transcription start site (TSS) of *ddrB* gene was mapped by primer extension method.

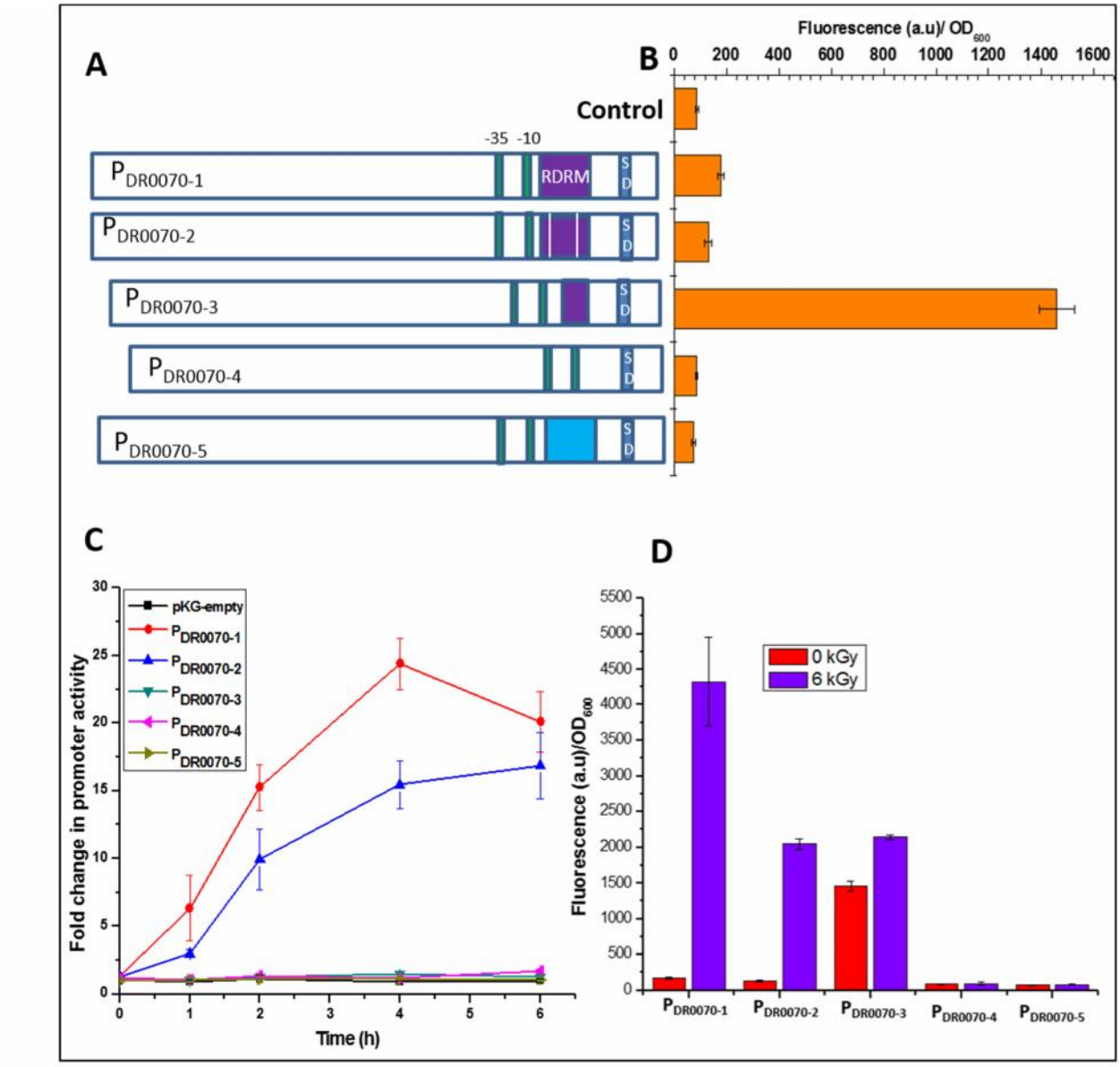

Fig. 5.6. Analysis of P_{DR0070} promoter mutants in *D. radiodurans* (A) Schematic diagram represents various mutants made in P_{DR0070} promoter, in $P_{DR0070-5}$ the RDRM was replaced with a non-specific sequence of same length (light blue), (B) Basal promoter activity of mutants under normal growth condition. (C) Kinetics of promoter activity of mutants shown in (A) during PIR up to 6h. (D) Comparison of basal and radiation induced promoter activity at 4h of PIR.

To find exact location of promoter of any gene finding TSS is very important. Generally TSS lies within 100-200bp upstream sequence from translation start site in bacteria. Whole RNA sequencing of *D. deserti* revealed that around 60% of its genes are leaderless or have very short upstream sequences [9]. TSS of *ddrB* gene was mapped by primer extension analysis as described in Material and Methods (Chapter 2). TSS of DR0070 was found to be G at -15th position from translation start codon. It is present within the 3' region of RDRM (Fig. 5.7). This is due to disruption of its TSS which lies within the RDRM, the above mentioned mutants did not show any activity. Since TSS is part of RDRM in P_{DR0070} promoter and deletion of RDRM disturbed the TSS, no further mutations were made in this promoter.

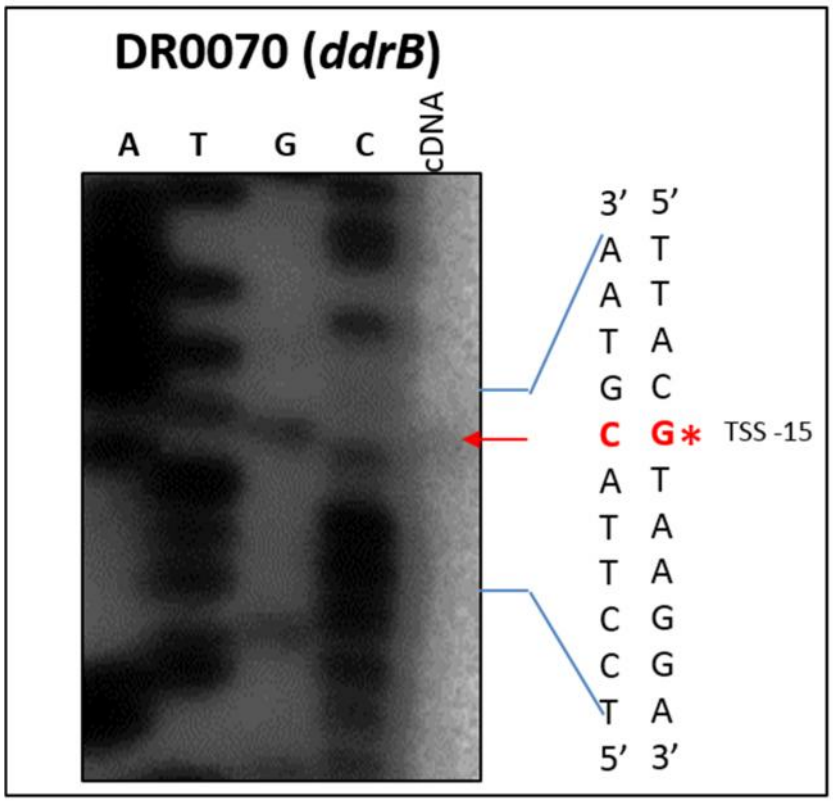

Fig. 5.7. Transcription start site (TSS) mapping of *ddrB* gene. TSS was mapped by primer extension method using [γ-^{32}P] labeled reverse primer P0070Rseq. The arrow indicates cDNA band on the gel. The position of TSS of *ddrB* is indicated with asterisk in red color, the number shows its position with respect to ATG start codon.

5.5 Mutagenesis in P_{DR0906} promoter

The P_{DR0906} promoter is highly active under normal growth conditions in *D. radiodurans* as well as in the heterologous host *E. coli* (Figs. 4.7-4.8, Chapter 4). This promoter harbors *E. coli* -10, -35 like consensus sequences and shows high basal activity and 6 fold induction during PIR in *D. radiodurans* (Fig. 4.2, Chapter 4). The RDRM is positioned in this promoter just 5bp upstream of the -35 sequence and -257bp upstream of +1 ATG codon. To understand the role of RDRM in promoter activity of P_{DR0906} several mutations were constructed systematically (Fig. 5.9). TSS in this promoter is not known. Hence the transcription start site (TSS) of this promoter (P_{DR0906}) was first mapped by primer extension method (Fig. 5.8). The TSS was found to be located C at -200 position from translation start codon and 11bp downstream of -10 like sequence (Fig.5.8).

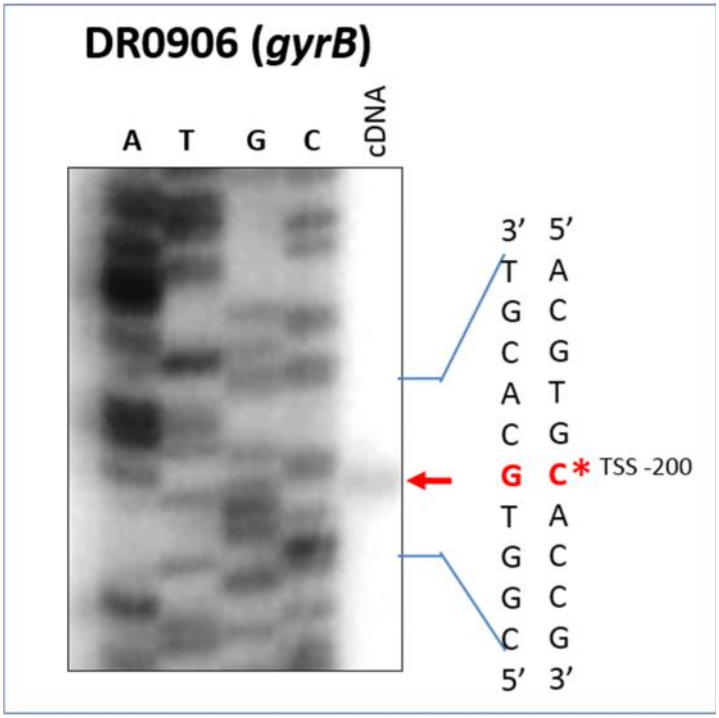

Fig. 5.8. Mapping of TSS of *gyrB* gene. The TSS mapping of *gyrB* (DR0906) gene was done by primer extension method using [γ-^{32}P] labeled primer P0906Rseq3. The arrow indicates cDNA band on the gel. The position of TSS of *gyrB* is indicated with a red asterisk, the number shows its position with respect to ATG start codon.

The different mutations constructed in the native promoter $P_{DR0906\text{-}1}$ were: complete RDRM deletion ($P_{DR0906\text{-}2}$), reverse orientation of RDRM ($P_{DR0906\text{-}3}$), point mutation of two highly conserved bases (G5A, C13T) from RDRM ($P_{DR0906\text{-}4}$), 5bp deletion from 5'end ($P_{DR0906\text{-}5}$) or from 3' end ($P_{DR0906\text{-}6}$) of RDRM individually, duplication of RDRM ($P_{DR0906\text{-}7}$), shifting of RDRM to extreme upstream position ($P_{DR0906\text{-}8}$), deletion of RDRM with complete upstream region excluding ($P_{DR0906\text{-}9}$) or including ($P_{DR0906\text{-}10}$) the -10, -35 like sequences (Fig. 5.9). The complete RDRM deletion ($P_{DR0906\text{-}2}$) caused marginal increase in promoter activity but completely abolished the radiation induction. Similar results were obtained when the whole upstream region (141bp) including RDRM ($P_{DR0906\text{-}9}$) was deleted from $P_{DR0906\text{-}1}$ promoter. Drastic reduction of basal level as well as radiation induction of promoter activity was seen when whole upstream region (173bp) including -10,-35 sequences up to TSS was deleted ($P_{DR0906\text{-}10}$). This is expected of such deletion of 173bp, which results in loss of core promoter preventing initiation of transcription by RNA polymerase.

The other mutations in RDRM were created by splicing PCR using specific primers carrying desired mutations. The point mutations in two highly conserved bases of RDRM at 5^{th} and 13^{th} positions to G5A and C13T were carried out using P0906F1/P0906R4a and P0906F10/P0906R1 primers (Table 2.2). In this mutation basal promoter activity was increased by 2 fold but radiation induction was drastically reduced from 6 to 1.5 fold (Fig. 5.11). This again confirmed that these two bases are very important for DdrO/RDRM interaction and repression as well as radiation induction of promoter activity. The 5 bases form 5' end were deleted ($P_{DR0906\text{-}5}$) by using P0906F1/P0906R5 and P0906F11/P0906R1 primers (Table 2.2). The 5 bases form 3' end were deleted ($P_{DR0906\text{-}5}$) by using P0906F1/P0906R6 and P0906F12/P0906R1 primers (Table 2.2). Deletion of 5 bases from 5' end of the RDRM ($P_{DR0906\text{-}5}$) slightly increased the basal activity but

the radiation induction was completely lost. However deletion of five bases from the 3' end of RDRM in $P_{DR0906\text{-}6}$ resulted in a 5-fold increase in basal expression but only a minor radiation induction was seen during PIR (Fig. 5.11). The results suggest that for this promoter the 5 bases from the 3' end of RDRM are more critical for the negative regulation as well as radiation induction from RDRM in this promoter, which has RDRM just upstream of the core promoter.

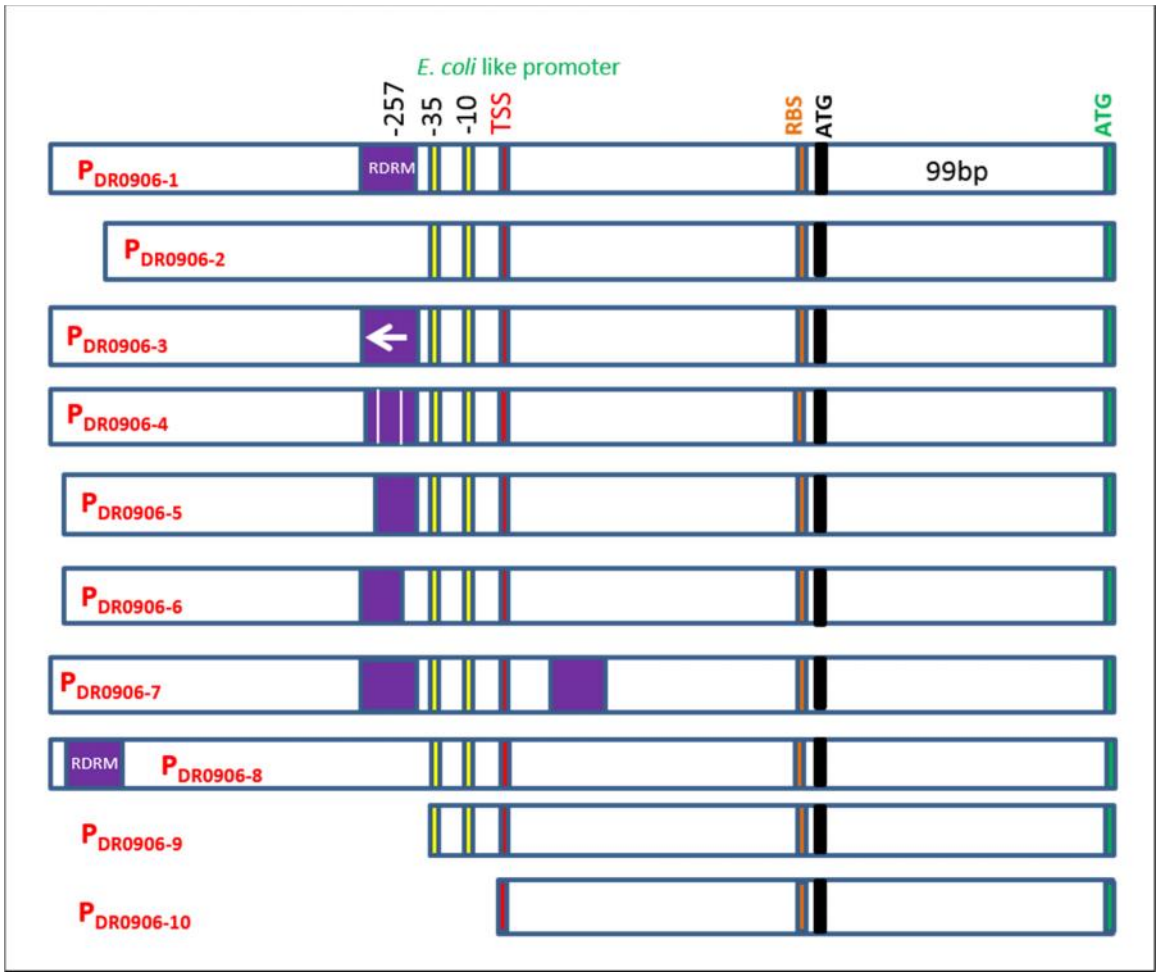

Fig. 5.9. Various mutants of P_{DR0906} promoter. The schematic diagram of different P_{DR0906} mutants constructed by splicing PCR ($P_{DR0906\text{-}2}$ to $P_{DR0906\text{-}7}$) and other variants ($P_{DR0906\text{-}1}$, $P_{DR0906\text{-}8}$ to $P_{DR0906\text{-}10}$) by normal PCR using respective primers as describe in the section 5.6. There is another ATG (black bar) was observed with RBS sequence at -100 bp upstream to annotated ATG (Green bar).

5.5.1 RDRM reversal causes stronger repression of P_{DR0906} promoter activity in *D. radiodurans*

As mentioned earlier, the RDRM is a degenerate palindrome [10]. Generally palindromic sequences, like restriction endonuclease sites, work in both orientations. To assess the activity of RDRM in reverse orientation, the 17bp RDRM was cloned in reverse orientation at the same location as in the native wild type promoter, keeping all the other P_{DR0906} promoter features intact ($P_{DR0906\text{-}3}$). The cloning was done by splicing PCR using P0906F1/P0906R4 and P0906F9/P0906R1 primers (Table 2.2). The basal activity of this mutant was reduced 4 fold. During PIR, following 6 kGy of gamma irradiation, the activity of promoter with reverse oriented RDRM was however induced 10 fold as against 5 fold induction of the native promoter (Fig. 5.11). This suggests that in reverse orientation, RDRM acts as a more stringent negative regulatory *cis*-element and further enhances radiation induction. It is possible that in reverse orientation RDRM may control any upstream genes which are transcribed in opposite direction to that of DR0906 (*gyrB*) gene. In silico analysis showed three hypothetical genes DR0905, DR0904 and DR0903 in opposite orientation to DR0906, of which DR0905 sequence overlapped with DR0906 (Fig. 5.10).

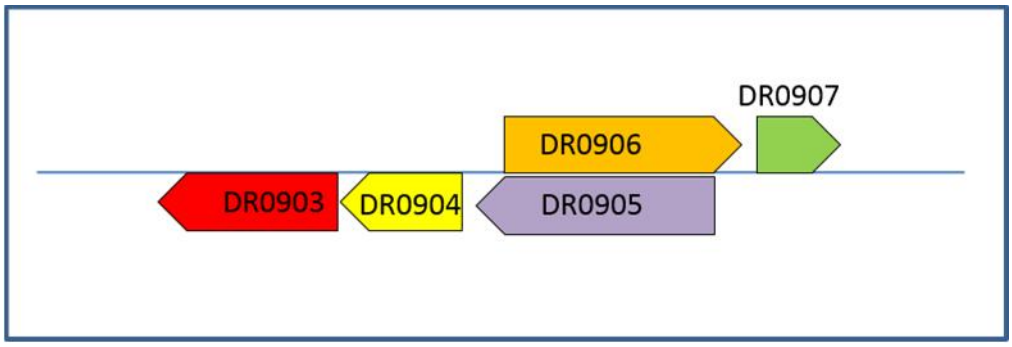

Fig. 5.10. Location of DR0906 gene in the genome of *D. radiodurans*. Schematic diagram shows the genes in the vicinity of DR0906 and their orientation.

No radiation induction was found at transcriptional level or protein level for DR0904 and DR0903 genes, but 3 fold radiation induction of DR0905 has been reported at transcriptional level [6, 8] and the promoter for DR0905 is located far downstream from the RDRM sequence. This shows that the RDRM is not regulating the upstream genes in reverse orientation.

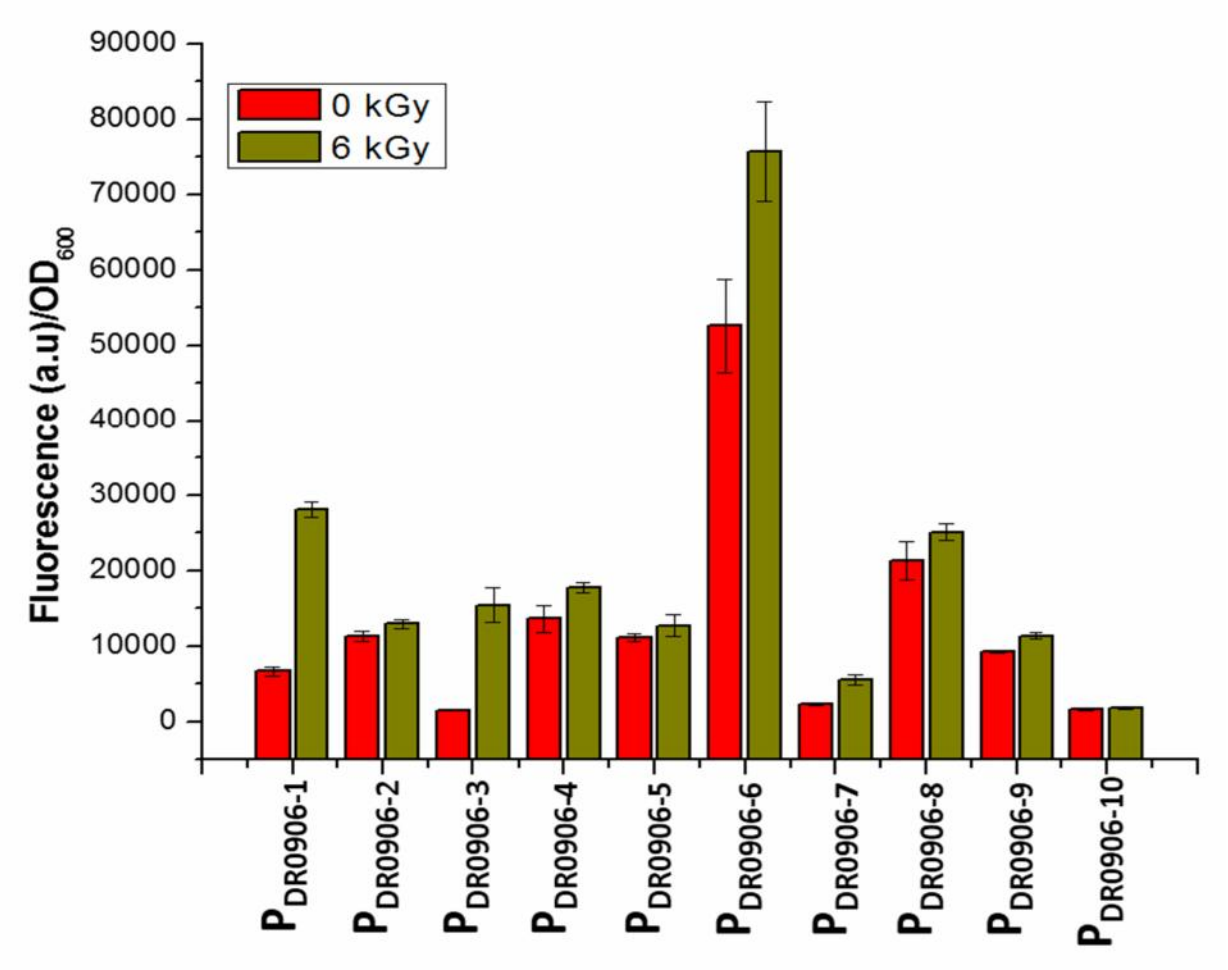

Fig. 5.11. Assessment of promoter activity of P_{DR0906} mutants. The *D. radiodurans* clones carrying pKG with different P_{DR0906} promoter variants were exposed to 6 kGy gamma radiation and the promoter activity was measured by quantifying the GFP fluorescence at 4h of PIR.

5.5.2 Additive effect of double RDRM introduction

DdrO protein requires complete 17bp RDRM to bind and repress the promoter activity [12]. If this is true, then DdrO should bind wherever RDRM is located in the genome. If there are more than one RDRM the repression may increase further. To examine possible additive effect of RDRM on promoter activity, one more identical RDRM was cloned in P_{DR0906} at -179 position, downstream of the core promoter, in addition to the native one at -257 position ($P_{DR0906\text{-}7}$). The extra RDRM was introduced by splicing PCR using P0906F1/P0906R7 and P0906F13/P0906R1 primer pairs (Table 2.2). As expected, it showed additive repression of basal promoter activity (Fig. 5.11), but similar fold induction in promoter activity during PIR. The results indicated that DdrO binds to RDRM and represses promoter activity even if it is located downstream of the core promoter. But it cannot induce promoter activity post-irradiation when it is downstream of core promoter. The effect of RDRM on gene expression, therefore seems to be context specific.

5.5.3 Assessment of promoter activity of P_{DR0906} mutants in the presence of DdrO protein in heterologous *E. coli* cells

To determine the activity of P_{DR0906} promoter variants in the presence of DdrO protein in *E. coli*, the pKG vector with all the P_{DR0906} promoter variants were individually transformed into *E. coli* cells carrying pET2-ddrO (which would express DdrO in *E. coli*) or pET2-empty (DdrO absent) as explained in the previous section 5.3. The promoter activity was monitored during 3h of IPTG induction of DdrO expression by quantifying the GFP fluorescence. Repression was observed in the promoter construct with reverse RDRM or with double RDRM sequence, as was also seen with the native promoter in the wild type *D. radiodurans* under normal growth conditions. In all other

variants no repression was observed compared to control. The results showing that DdrO protein repress promoter activity of RDRM promoter variants in a similar way in both organisms (Fig. 5.12).

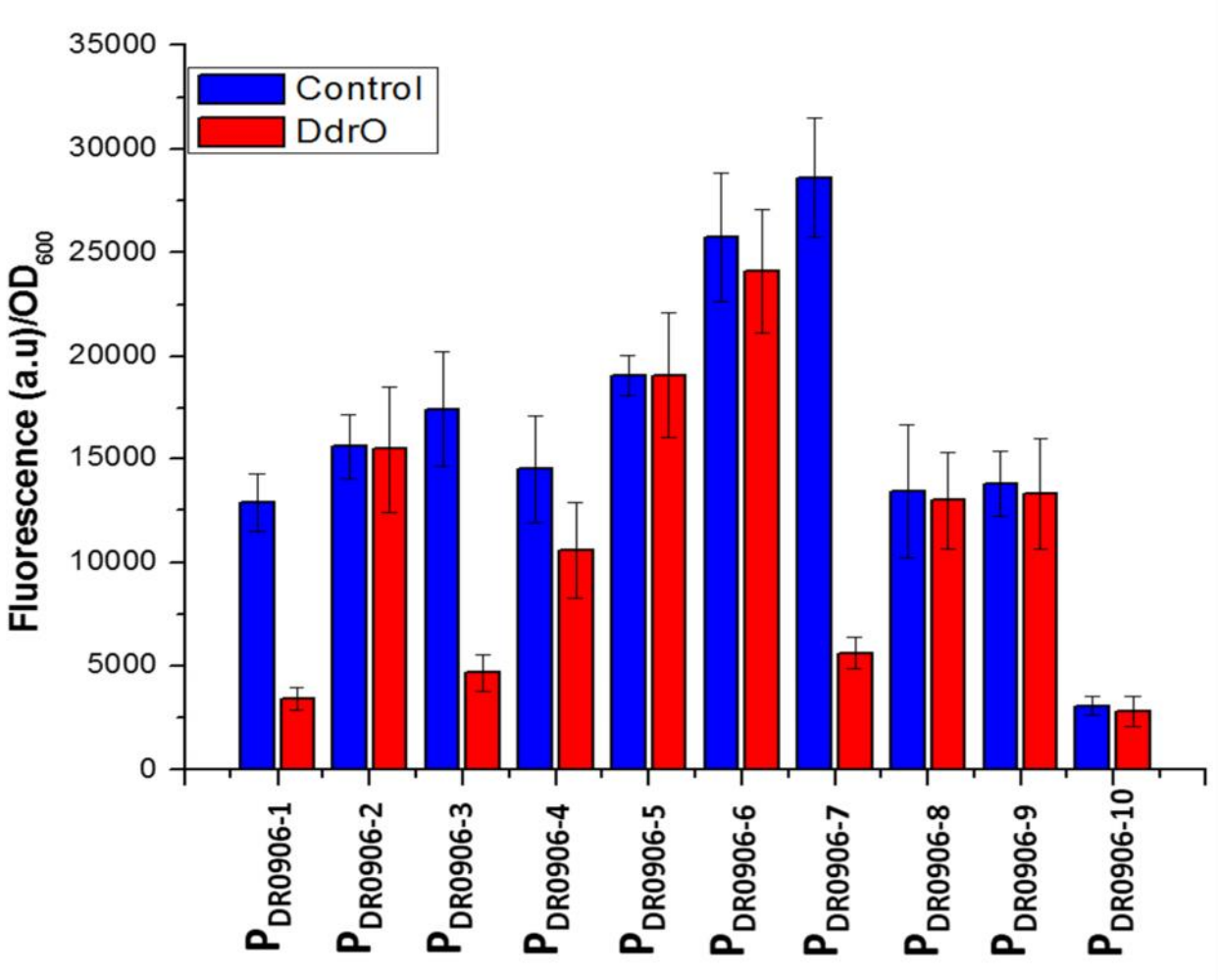

Fig. 5.12. Analysis of activity of various P_{DR0906} promoter mutants in presence of DdrO in heterologous *E. coli*. The P_{DR0906} mutants cloned in pKG plasmid were transformed into recombinant *E. coli* carrying pET2-empty or pET2-ddrO. DdrO protein expression was induced by IPTG and the promoter activity was monitored by quantifying the GFP fluorescence.

5.6 Promoter proximity of RDRM is crucial for induction of gene expression following irradiation

The P_{DR0906} promoter showed additive repression when RDRM sequence was duplicated in P_{DR0906} promoter but failed to cause additive induction in PIR (Fig.5.11). These results indicated that radiation induction by RDRM is context specific or involves another positive regulator during PIR. To investigate if RDRM alone is responsible for radiation inducible promoter activity following repressor clearance or involves another activator protein that binds to RDRM or other sequences near RDRM, the RDRM sequence of P_{DR0906} was introduced in to a non-RDRM promoter P_{DR0606} (Fig. 5.13A) close to its TSS and its effect on gene expression in unirradited and irradiated cells was tested. The native non-RDRM P*groESL* gene promoter-reporter construct ($P_{DR0606\text{-}1}$) displays high GFP expression but no radiation induction (Fig. 5.13B and C). Introduction of RDRM (from *gyrB* promoter) at -14 position in *groESL* ($P_{DR0606\text{-}2}$) (Fig. 5.13A) resulted in about 5 fold repression of promoter activity under normal growth conditions (Fig. 5.13B). However, this RDRM-inserted promoter-reporter construct neither displayed radiation-induced promoter activity nor restored basal promoter activity upon gamma irradiation (Fig.5.13C). Thus, introduction of RDRM in the non-RDRM promoter repressed gene expression, but its mere presence was not sufficient for radiation induction.

To evaluate whether RDRM effect is context specific or not, the RDRM was grafted close and upstream of the promoter. The TSS and -10, -35 like consensus sequences are known for *groESL* gene promoter [15]. To mimic the P_{DR0906} promoter structure in P_{DR0606}, the RDRM sequence from *gyrB* promoter was introduced just 5 bases upstream of -35/-10 sequence in *groESL* promoter ($P_{DR0606\text{-}3}$), as it was in its native *gyrB* promoter. $P_{DR0606\text{-}3}$ promoter-reporter variant (Fig. 5.13A) repressed GFP fluorescence by 2.2 fold under normal growth conditions (Fig.5.13B) and

expression levels of native *groESL* gene promoter ($P_{DR0606-1}$) were achieved upon irradiation (Fig. 5.13C). Next the promoter sequence upstream to -35 sequence from *gyrB* promoter including RDRM was grafted immediately upstream of -35 sequences of *groESL* gene promoter to get a chimeric promoter ($P_{DR0606-4}$) (Fig. 5.13A). The basal as well as radiation inducible GFP fluorescence by $P_{DR0606-4}$ remained similar to GFP fluorescence by $P_{DR0606-3}$ (Fig. 5.13B and C).

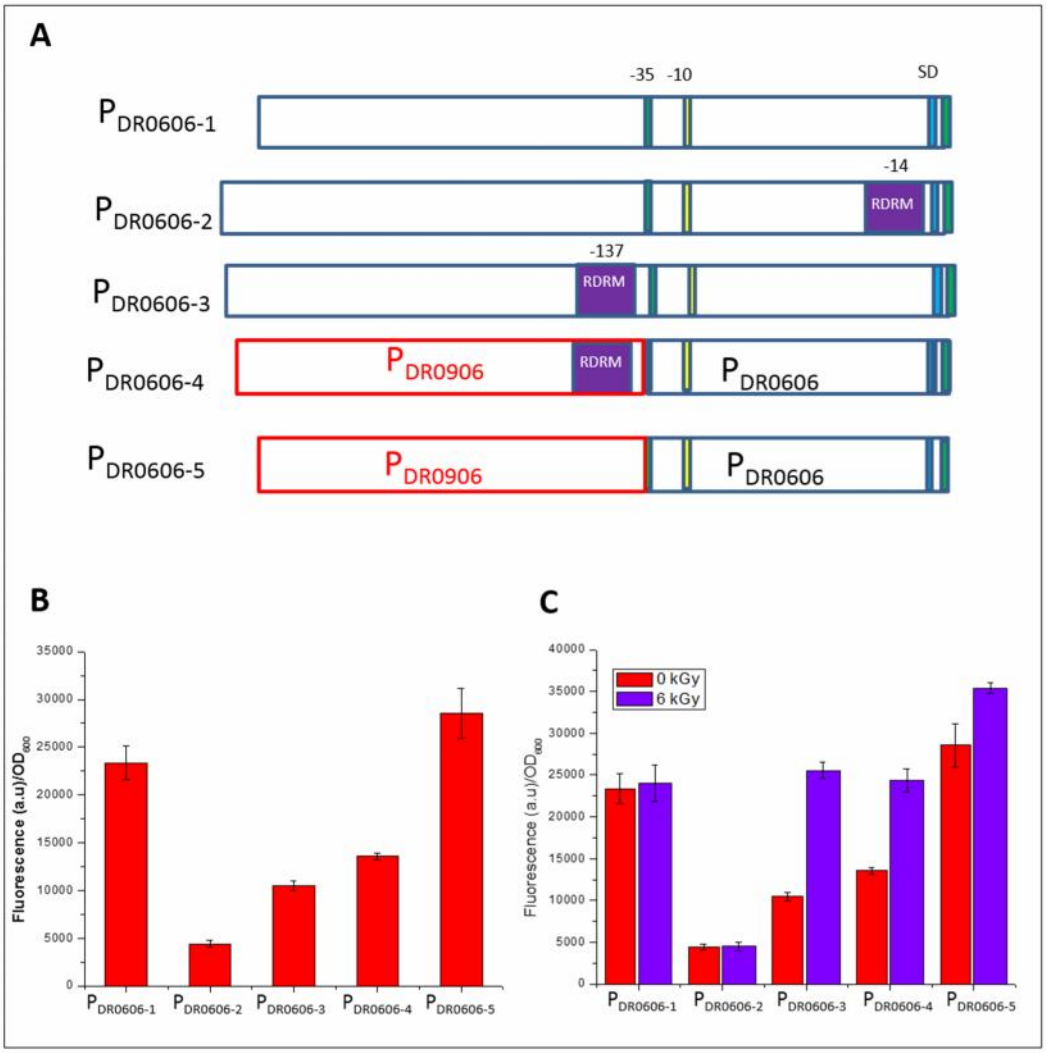

Fig. 5.13. Construction of P_{DR0606} mutants containing RDRM grafts at different locations and analysis of their activity in *D. radiodurans*. (A) Schematic representation of structure of various P_{DR0606} promoter variants. (B) The basal level activity of *D. radiodurans* clones carrying P_{DR0606} promoter variants, during normal growth conditions. (C) The activity of P_{DR0606} promoter variants at 4h of PIR following 6 kGy gamma radiation.

When RDRM sequence from chimeric promoter $P_{DR0606\text{-}4}$ was deleted keeping all other sequence unchanged to obtain promoter construct $P_{DR0606\text{-}5}$ (Fig.5.13A), the basal as well as radiation inducible GFP fluorescence levels were similar to those observed for native *groESL* gene promoter ($P_{DR0606\text{-}1}$) (Fig. 5.13B and C). These results revealed two important aspects of RDRM mediated gene regulation (a) radiation induction, which relies on cleavage of repressor DdrO bound to RDRM sequence, requires close proximity of RDRM and the core promoter, and (b) radiation induction appears to result in restoration of maximal de-repressed promoter activity upon repressor clearance.

5.7 Discussion

Bacteria are constantly exposed to fluctuating environmental stresses, irrespective of their natural habitat. In response to stress, bacteria alter the pattern of gene expression, especially for those genes whose products are required to combat the deleterious effects of environmental insult [15]. The stress responsive genes are under tight repressed condition in normal growth conditions. As the bacteria experience the stress, these genes get up-regulated either by releasing the repressor from operator or binding the enhancer molecule/transcription factor to the promoter. Negative regulation of gene expression by an autoregulatory repressor under normal growth conditions is relatively common in bacteria. [134]. Some of the extensively studied examples of autoregulatory repressors include LexA, OxyR and SoxS involved in stress responses or LacI, AraC, GalS and TrpR repressors involved in metabolism [134]. *D. radiodurans* exploits this negative autoregulatory circuit to mount a prompt response to substantial DNA damage caused by gamma rays. RDRM-DdrO interaction was reported earlier *in vitro* for RDRM promoters [24]. The *in vivo* over expression of DdrO in presence of RDRM promoters in heterologous *E. coli* resulted in strong

repression of promoter activity (Fig. 5.1C), which substantiated the earlier reported *in vitro* results. No change in promoter activity was observed *in vivo* either in PprI over expressed strain of *E. coli* cells (Fig. 5.3) or *pprI* knockout mutant of *D. radiodurans* which were carrying Deinococcal promoters (Fig. 5.5B), suggesting thereby that PprI alone does not affect the promoter activity, rather it activates gene expression by cleaving the repressor protein during PIR.

With details of interactions between *cis*-regulatory element RDRM, autoregulatory repressor DdrO and DdrO-specific protease PprI known from the published literature [12, 13, 24] and results obtained by RDRM mutation analyses in this study, it is possible to dissect functional aspects of RDRM-based promoters in *D. radiodurans in vivo*. The core promoter comprising of -10 like AT rich motif and TSS is present in majority of the Deinococcal promoters [9] and is necessary for transcription. Two highly conserved bases of RDRM are very important for radiation induction. But these may not interfere with RDRM-repressor interaction, because when these bases were mutated in the *ddrB* (P_{DR0070}) and *gyrB* (P_{DR0906}) promoters the radiation induction of promoter activity came down but no change in basal activity was observed. Deletion of 5 bases from RDRM increased basal promoter activity but lost radiation induction in both *ddrB* (P_{DR0070}) and *gyrB* (P_{DR0906}) promoters. It is interesting that 5 bases from 5'end in P_{DR0070} are as important as 5 bases of the 3' end in P_{DR0906}. While in P_{DR0070} RDRM is located downstream of core promoter, in P_{DR0906} RDRM is located upstream of core promoter. These data, therefore, suggest that RDRM bases (and DdrO bound there) facing the RNA polymerase, interact with it stronger and may play a greater role in stalling RNA polymerase at the core promoter, than the bases at the opposite end. Upon clearance of DdrO from RDRM by PprI post irradiation the stalled RNA polymerase takes off to result in far enhanced transcription than what is achieved in the absence of DdrO/RDRM interaction in mutants carrying modified or deleted RDRM.

RDRM works as negative regulator when introduced in to a non-RDRM promoter ($P_{DR0606-2}$) (Fig. 5.12) and causes additive repression when it is duplicated in *gyrB* ($P_{DR0906-7}$) promoter, but it does not restore the wild type promoter activity (Fig. 5.10) indicating the additional RDRM acts only negatively, both in normal and radiation stress conditions, when it is present away from the core promoter. No radiation induction is observed also when the RDRM is shifted far upstream from core promoter in ($P_{DR0906-8}$) (Fig. 5.10). The results thus demonstrate that positioning of RDRM in close proximity of promoter is necessary for maximal induction of DNA damage responsive genes in *D. radiodurans* recovering from DNA damage inflicted by gamma irradiation.

Repression of RDRM-based promoters under normal growth conditions could be explained on the basis of 'interference with transcription' model. When RDRM overlaps with core promoter or is located in the close vicinity, upstream or downstream of the core promoter, binding of DdrO repressor would prohibit binding and/or movement of RNA polymerase resulting in maximal repression. However, either deletion of RDRM sequence or mutations in the RDRM sequence, that diminish repressor binding at RDRM, lead to only a small incremental increase in promoter activity (which is much less than the radiation induced promoter activity that follows DdrO clearance upon irradiation) and abolishes radiation induction. Relocating RDRM far downstream from the core promoter retains repression due to transcriptional interference but abolishes radiation induction. On the other hand DdrO/RDRM interaction happening far upstream of core promoter does not interfere with transcription and has no effect on promoter activity, both in case of a grafted promoter or in case of a natural promoter. Thus, presence of RDRM and its proximity to the core

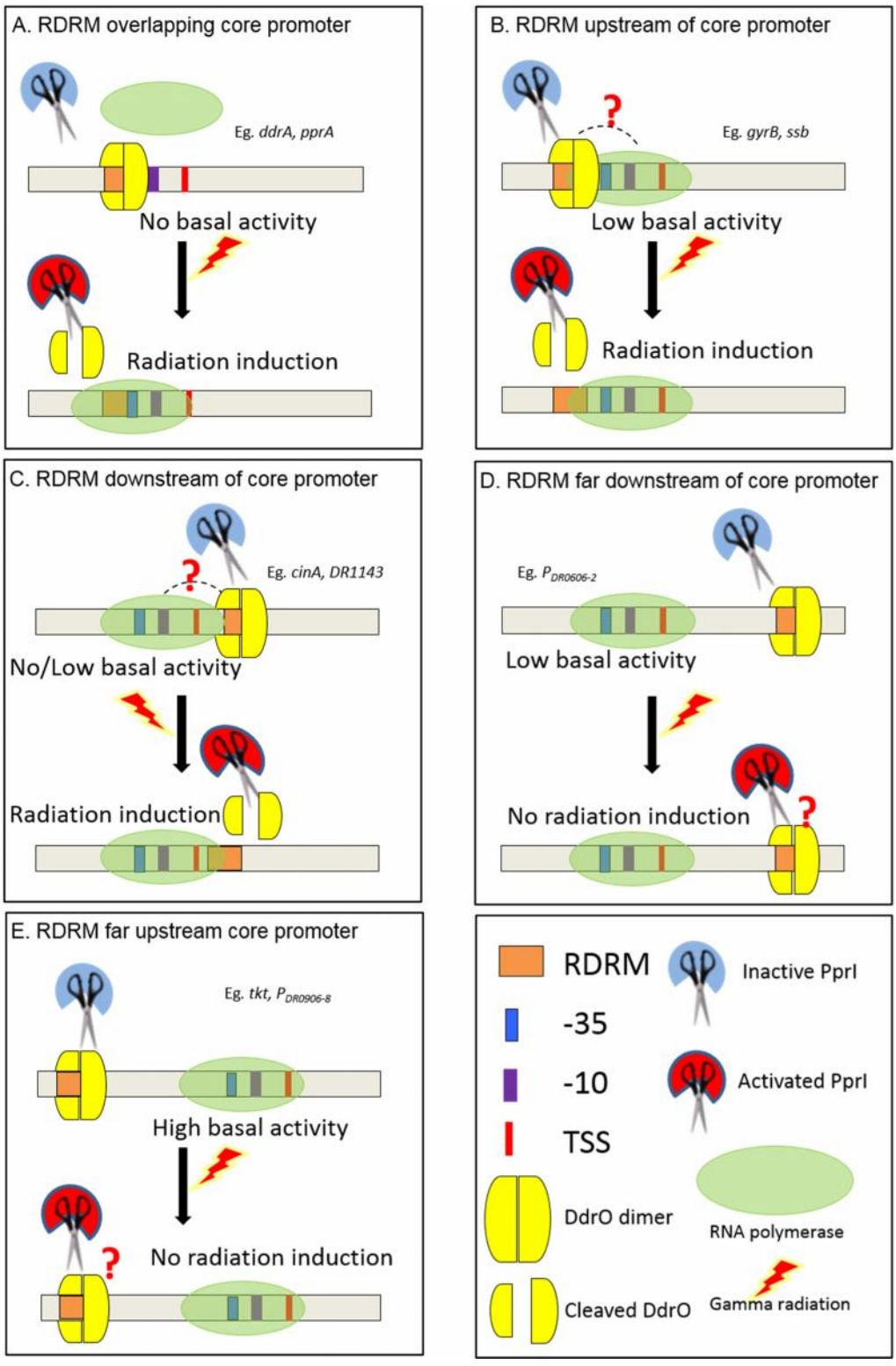

Fig. 5.14. Model of Gene regulation by RDRM in *D. radiodurans*. Different molecules involved in regulation under normal and radiation stress conditions are shown with different colors. The possible interactions between DdrO bound to RDRM and RNA polymerase are shown with question mark.

promoter are essential prerequisites for induction of gene expression following irradiation. Interpretation of this observation requires explanation beyond ‘transcription interference’ model.

Dependence of radiation induction on close proximity of RDRM to core promoter and RDRM-DdrO interactions can be explained by the assumption that *DdrO bound to RDRM physically interacts with RNA polymerase*. When RDRM is present right in the core promoter region, it would interfere with RNA polymerase binding and transcription initiation itself resulting in near zero transcription. When present in close proximity (6-29 bp upstream or downstrean) of the core promoter, such interaction *may stall RNA polymerase and prevent initiation of transcription*. Further enhancement in gene expression would then occur following repressor clearance by irradiation-activated PprI protease. Relocating RDRM far downstream of core promoter would not cause stalling of RNA polymerase but may interrupt elongation resulting in (a) repression of promoter activity that depends upon a chance interaction of RNA polymerase with core promoter and the strength of promoter, and (b) complete loss of radiation induction. RDRM located too far upstream of core promoter cannot stall transcriptional machinery through DdrO and does not exercise any control on regulation of RDRM-based promoters. The results obtained in this study, thus better fit ‘stalled transcription model’ than ‘transcription interference model’ and are summarized in Fig. 5.14. The *stalled transcription model* needs to be substantiated by protein-protein interaction studies in future.

Chapter 6

Summary and Conclusions

Summary

The salient findings of the present work are as follows:

- A gene encoding the green fluorescent protein (*gfp-mut2*) was cloned in the pre-existing *Deinococcus-E. coli* shuttle vector pRAD1 to generate a GFP-based promoter probe vector. However, the vector exhibited leaky GFP fluorescence even in the absence of a promoter.
- The desired shuttle vector was generated by following improvisations :
    1. A *rho*-independent transcription terminator (*term116*) from *D. radiodurans* was introduced upstream of the *gfp* gene in pRAD-gfp to prevent leaky expression.
    2. The antibiotic resistance genes Cm^R and Amp^R were replaced with a single kanamycin resistance marker (Kan^R), which functions in both *E. coli* and *D. radiodurans*.
    3. A new multiple cloning site (MCS) with eight unique restriction sites was inserted.
    4. A 386bp sequence, harbouring an *E. coli* promoter-like sequence, and a 305bp sequence (with no known function) present downstream of Kan^R cassette were removed from pKG to eliminate leaky GFP expression.
- The final GFP-based promoter probe shuttle vector pKG was validated as follows :
    1. It could be restriction digested at each of the 8 restriction sites present in its MCS.
    2. It displayed no background GFP fluorescence in *E. coli* or *D. radiodurans* in the absence of a cloned promoter.
    3. It expressed the strong Deinococcal P_{groESL} (P_{DR0606}) promoter constitutively in *E. coli* and in *D. radiodurans*.
    4. It showed the expected radiation induction of Deinococcal P_{ssb} promoter during PIR in *D. radiodurans* but not in *E. coli*.

- Based on the available transcriptomic (microarray, real time PCR) and proteomic data, mutation phenotypes and *in silico* analysis, 20 *D. radiodurans* genes were selected for promoter study.
- Genes selected for promoter analysis included radiation-induced genes (with or without RDRM and/or *E. coli*-like -10, -35 hexameric sequences), and a couple of radiation-insensitive genes as well.
- About 300-500bp upstream DNA sequence of all the 20 selected genes was PCR amplified and individually cloned in the MCS of pKG vector. The cloning was confirmed by colony PCR, restriction digestion to release the cloned insert, and finally by DNA sequencing.
- The Deinococcal promoters harbouring *E. coli* like -10, -35 consensus sequences expressed well in *E. coli*, while promoters which lacked these showed weak or no GFP fluorescence. Such hexameric sequences were dispensable for gene expression in *D. radiodurans*.
- *D. radiodurans* promoters containing RDRM sequences showed low or no basal promoter activity while promoters which lacked RDRM showed high basal activity in *D. radiodurans*. Expression of these genes in *E. coli* was not influenced by the presence/absence of RDRM.
- RDRM thus appeared to play a role as a negative regulatory element in *D. radiodurans*, but not in *E. coli*. RDRM deletion increased the basal promoter activity but abolished radiation induction in *D. radiodurans*.
- No radiation induction of Deinococcal promoters was observed in irradiated *E. coli* cells.
- All RDRM containing promoters were repressed in *E. coli* expressing Deinococcal DdrO protein. Non-RDRM Deinococcal promoters were unaffected by presence of DdrO in *E. coli*.
- Overexpression of PprI protein alone did not alter promoter activity of RDRM/non-RDRM promoters in *E. coli*. Basal promoter activity of Deinococcal promoters did not change, but

their radiation induction was abolished in *pprI* deletion mutant (*ΔpprI*) of *D. radiodurans*. This clearly shows that PprI protein is essential for radiation induction of Deinococcal promoters in *D. radiodurans*. DdrO/PprI model of regulation of RDRM containing promoters thus stands vindicated for Deinococcal RDRM-based promoters *in vivo*.

- Mutational dissection of the Deinococcal P_{DR0070} promoter in *D. radiodurans*, wherein the core promoter lies immediately downstream of RDRM, revealed the following :
    1. Mutations (G5A and C13T) in two highly conserved bases of RDRM produced no change in promoter activity, but lowered the radiation induction.
    2. Deletion of 5 bases from 5' end of RDRM resulted in high basal promoter activity.
    3. Deletion or complete replacement RDRM with a random sequence of same length abolished both the basal promoter activity as well as its radiation induction.
- Different promoter variants were constructed in P_{DR0906}, wherein RDRM lies immediately upstream of core promoter, such as complete RDRM deletion, deletion of 141bp and 173bp upstream sequence, mutation of two conserved bases at 5^{th} and 13^{th} position of RDRM, 5base deletion either from 5' end or 3' end of RDRM, RDRM reversal, duplication of RDRM, and shifting the position of RDRM to far upstream of the core promoter sequence. These revealed the following information :
    1. RDRM reversal further repressed promoter activity but had no effect on radiation induction. Thus DdrO can bind and repress promoter activity in both RDRM orientations.
    2. Deletion of 5 bases from 3' end of RDRM enhanced basal promoter activity far more than the deletion of 5 bases from 5' end. Combined with similar deletions in P_{DR0070} promoter, the data suggest that the 5 bases closer to the core promoter are very important for repression.

3. RDRM duplication caused additive repression but retained normal radiation induction. Shifting RDRM far upstream moderately increased the basal activity but lost the radiation induction. Thus, the repressive as well as radiation induction effects of RDRM appear to be context specific or position-dependent.

- Introduction of RDRM from the most radiation-induced promoter P_{DR0906} in to a non-RDRM promoter P_{DR0606} had following effects:
    1. Introduction at -14 position, far downstream of core promoter, resulted in loss of basal promoter activity but showed no radiation induction of gene expression.
    2. Introduction at 5 bases upstream of -35 sequence in P_{DR0606}, which mimicked its location in PDR0906, caused strong repression of basal promoter activity. The original promoter activity was restored upon radiation induction.
- RDRM grafting experiments in to non-RDRM promoters clearly revealed that RDRM interferes with transcription even when it is present downstream of TSS, but radiation induction requires its presence in close proximity of the core promoter.
- In agreement with this, most RDRM-based Deinococcal promoters harbor RDRM in close proximity of the core promoter.
- Deletion of RDRM causes a smaller derepression of basal promoter activity than what is observed upon irradiation. Thus location, orientation, sequence and proximity of RDRM to core promoter and its interaction with DdrO determine the magnitude of radiation induction.
- Grafting of whole P_{DR0906} upstream region without RDRM in to P_{DR0606} had no effect on promoter activity under normal conditions or during PIR. This highlights importance of RDRM/DdrO interaction *per se* in repression and radiation induction and rules out possible interaction of another activator in close vicinity of RDRM.

Conclusions

- Presence of *E coli*-like -10, -35 consensus sequences are necessary for Deinococcal promoters to be active in *E. coli*, but these sequences are dispensable in *D. radiodurans*.
- RDRM based repression of promoters operates through its interaction with DdrO. RDRM proximity to core promoter completely stalls transcription while RDRM present far downstream can also cause transcriptional interference. When present far upstream of core promoter RDRM does not exert a repressive effect.
- Context specificity of RDRM for radiation induction is explained by sudden release of transcriptional stalling upon clearance of DdrO bound to RDRM (when present close to core promoter) by PprI protease post-irradiation. Such effect is not seen if RDRM is located far away from the core promoter.

Future directions

The present work has generated important leads for both basic and applied research on Deinococcal radiation-responsive promoters. These are:

1. Physical interaction of DdrO, bound to RDRM, with transcriptional machinery needs to be demonstrated experimentally, both *in vitro* and *in vivo*, to establish the need for core promoter proximity of RDRM for radiation induction.
2. Mechanisms underlying radiation induction of certain non-RDRM promoters needs to be elucidated.
3. Selected radiation-responsive superior Deinococcal promoters can be recruited for bioremediation of metals and organic wastes in high radiation environment of nuclear waste.

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Appendix

pKG vector sequence

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Promoter sequences

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P0906-5

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P0906-6

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P0906-7

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P0906-8

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P0906-9

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P0906-10

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P0906-11

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P0906-12

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P0906-13

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P0906-14

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P0906-15

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P1143-1

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P1143-2

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P1262

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P1314

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P1358

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P1720

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P1913

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TGCC

P2220

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P2275

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P2338-1

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CA

P2338-2

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P2574

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