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G-quadruplex forming structural motifs in the genome of *Deinococcus radiodurans* and their regulatory roles in promoter functions

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Abstract *Deinococcus radiodurans* displays compromised radioresistance in the presence of guanine quadruplex (G4)-binding drugs (G4 drugs). Genome-wide scanning showed islands of guanine runs (G-motif) in the upstream regions of coding sequences as well as in the structural regions of many genes, indicating a role for G4 DNA in the regulation of genome functions in this bacterium. G-motifs present upstream to some of the DNA damage-responsive genes like *lexA*, *pprI*, *recF*, *recQ*, *mutL* and *radA* were synthesized, and the formation of G4 DNA structures was probed in vitro. The G-motifs present at the 67th position upstream to *recQ* and at the 121st position upstream to *mutL* produced parallel and mixed G4 DNA structures, respectively. Expression of β-galactosidase under *recQ* and *mutL* promoters containing respective G-motifs was inhibited by G4 drugs under normal growth conditions in *D. radiodurans*. However, when such cells were exposed to γ radiation, *mutL* promoter activity was stimulated while *recQ* promoter activity was inhibited in the presence of G4 drugs. Deletion of the G-motif from the *recQ* promoter could relax it from G4 drug repression. *D. radiodurans* cells treated with G4 drug showed reduction in *recQ* expression and γ radiation resistance, indicating an involvement of G4 DNA in the radioresistance of this bacterium. These results suggest that G-motifs from *D. radiodurans* genome form different types of G4 DNA structures at least in vitro, and the *recQ* and *mutL* promoters seem to be differentially regulated at the levels of G4 DNA structures.

Keywords *Deinococcus* · DNA repair · G4 DNA · Promoter activity · Radioresistance

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Introduction

Deinococcus radiodurans, a Gram-positive bacterium, is best characterized for its extraordinary resistance to the DNA-damaging agents including radiation and desiccation. An efficient DNA double-strand break (DSB) repair (Cox and Battista 2005; Misra et al. 2013) and the extraordinary tolerance to oxidative stress (Daly et al. 2010; Tian and Hua 2010; Slade and Radman 2011) are attributed to the extreme phenotypes of this bacterium. The cytogenetic features of this bacterium are also interesting as it contains a multipartite genome system comprised of chromosome I, chromosome II and plasmids. Its GC-rich genome has numerous islands of guanine runs intercepted by one or two other bases (hereafter referred to as G-motif(s)) (White et al. 1999). Genome-wide scanning indicated the possible involvement of these G-motifs in the formation of guanine quadruplex DNA (G4 DNA) structures. A guanine quadruplex can be formed in both DNA and RNA and can exist in different molecular forms. Depending on the polarity of the strands in which the G4 structures are formed, the planar geometry of the G4 DNA will be parallel, anti-parallel, or mixed (Maizels and Gray 2013) and is distinguishable by circular dichroism (CD) analysis. It is also known that the regulation of gene expression and genome structure maintenance by G4 DNA is dependent upon the position of these elements in the genome. For example, G4 DNA located in the

proximity of a promoter can downregulate gene expression while its presence at the far distance in the same strand or anywhere in the opposite strands could upregulate gene expression (Kennedy et al. 1992; Cogoi et al. 2006; Du et al. 2008). It is known that monovalent cations such as Na^+ and K^+ can stabilize G4 structures by interacting in the central channels between each pair of tetrads (Burge et al. 2006). Natural products like telomestatin identified from *Streptomyces anulatus* (Shin-ya et al. 2001) and porphyrins such as *N*-methyl mesoporphyrin IX (NMM) and 5, 10, 15, 20-tetrakis-(*N*-methyl-4-pyridyl) porphyrin (TmPyP4) (Ren and Chaires 1999; Smith and Johnson 2010) can bind to G4 DNA and have been used as probes for ascertaining guanine quartet formation. These agents could inhibit telomerase activity in a mammalian system (Yamamoto et al. 2014). In *D. radiodurans*, G-motifs are found on both strands of the chromosome and at different positions upstream to putative promoters. Recently, it was shown that G4 DNA-binding drug (G4 drug) like NMM could repress transcription of many genes in *D. radiodurans* (Beaume et al. 2013). Therefore, the molecular mechanisms underlying the effect of G4 drugs would be worth investigating in this bacterium.

Here, we report the structural characterization of G-motifs located upstream to the promoter regions of a few DNA repair genes and demonstrate the involvement of G4 DNA in the regulation of *recQ* (DR_1289) and *mutL* (DR_1696) promoter functions. Interestingly, the G-motif located at the 67th position upstream to the coding sequences of RecQ (G4-recQ) and at the 121st position upstream to MutL (G4-mutL) produced parallel and mixed G4 geometry, respectively, in the presence of K^+ ions. G4 drugs produced differential effects on respective G-motifs in the *recQ* and *mutL* promoters and promoters' activity in response to γ radiation. These results suggest that G-motifs in the *D. radiodurans* genome could form different G4 DNA structures, which in turn are likely to play important roles in differential regulation of promoter activities under different growth conditions.

Materials and methods

Bacterial strains and plasmids

D. radiodurans R1 (ATCC13939), a gift from Professor J. Ortner, Germany (Schaefer et al. 2000), was maintained in TGY (0.5 % Bacto Tryptone, 0.3 % Bacto Yeast Extract and 0.1 % glucose) medium at 32 °C. *Escherichia coli* strain DH5α (Life Technologies, Bengaluru, India) was used for maintaining cloned genes on plasmids while *E. coli* BL21 (DE3) pLysS (Life Technologies, Bengaluru, India) was used for expression of recombinant protein. *E. coli* and *D. radiodurans* cells containing pET28a+ (Novagen Inc., Madison, USA) and its derivatives, and pRADZ3 (Meima and Lidstrom 2000) and its derivatives were grown in LB broth or TGY broth with appropriate antibiotics, with shaking at 180 rpm. All recombinant techniques used in this study were as described earlier (Sambrook and Russell 2001).

Identification of putative G-motifs in *D. radiodurans* genome

Putative G-motif-forming sequences in the *D. radiodurans* genome (accession numbers AE00513, AE001825, AE001826 and AE001827) were searched as described earlier (Rawal et al. 2006). The formation of G-motifs within 500 bp upstream of some DNA repair genes as well as those located within the coding sequences of genes was analysed in silico using the QGRS Mapper (http://bioinformatics.ramapo.edu/QGRS/index.php). Some of these G-motifs (Table 1) were synthesized on a 0.2-μmole scale and purified by HPLC followed by polyacrylamide gel electrophoresis (PAGE) (Misra et al. 1998). The concentration was estimated using absorbance at 260 nm with appropriate molar extinction coefficients as determined by employing the oligo analyzer from IDT (http://biophysics.idtdna.com/UVSpectrum.html).

Cloning of upstream sequences into a promoter probe vector and analysis

Approximately 400 bp upstream regions containing G4-recQ and G4-mutL motifs were PCR amplified from genomic DNA of *D. radiodurans*, using forward primer RecQup (5′CGGGATCCCGAGA CCGGGCACGAG3′) and reverse primer RecQdn (5′GGACTAGTCATCTCCCCAGGATAG3′) for *recQ* promoter sequences (P_{recQ}); and PMLF1 (5′CGGGATCCGGGATGAGGTTTCGCTG 3′) and PMLR1 (5′GGACTAGTCATTCTGTTGTGAGC3′) primers for a 418-bp *mutL* promoter region (P_{mutL}). Similarly, 445-bp non-promoter sequences (between 595093 and 595928 complement, regions of DR_0583, accession number NC_001263) in the *D. radiodurans* genome were also PCR amplified using forward primer RTH583F (5′CGGAGATCTGCGTACTGGGTATTGAGGATGT3′) and reverse primer RTH583R (5′GGACTAGTGTGAAGCAGACCGCAAAGTCA3′). These PCR products were cloned at *Bgl*II and *Spe*I sites immediately upstream to the *lacZ* gene in pRADZ3. The cloning of P_{recQ} and P_{mutL} promoters in place of the existing P_{lac} promoter in pRADZ3 was confirmed by sequencing, and the resultant plasmids were named pRZ3PrecQ and pRZ3PmutL, respectively. These plasmids were transformed into wild-type *D. radiodurans* and *E. coli* as required. Expression of the *lacZ* gene on these plasmids was checked by either spotting recombinant cells on LB/TYG agar plates containing 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or by measuring β-galactosidase activity in toluene-

Table 1 Summary of guanine runs located upstream/downstream to DNA repair genes in the genome of *D. radiodurans*. These sequences were commercially synthesized, purified and then used for circular dichroism (CD) analysis

Gene name and ORFs	Size (base)	Sequences with guanine runs in 5′ → 3′ polarity	Position in genome[a]	QGRS prediction	CD analysis[b]	
					λ_{min}	λ_{max}
pprI DR_0167	24	GGGGTAAGGGGCGGGGGGATGGGG	169914 (+36)	Parallel	239	262
mutL DR_1696	24	GGGGATAGGGGTTTGGGGTTGGGG	1718634 (−121)	Mixed	242	268/292
lexA DR_A0074	19	GGGAAGGGAGGGAAACGGG	80527 (−287)	Parallel	241	262
recQ DR_1289	17	GGGGGGGCCGGGCGGGG	1295975 (−67)	Parallel	240	260
recF DR_1089	18	GGGGTGGGGTGGCCGGG	1098076 (−46)	Parallel	240	262
radA1 DR_1105	23	GGGGTGGGGCGGGGGCGGTGGGG	1112442 (−492)	Parallel	240	260
dnaA DR_0002	24	GGGCGCGGGTGGGGGGTCGTTGGG	3017 (+251)	Parallel	240	262
Control oligonucleotides						
Sequence identity	Size (base)	Sequences in 5′ → 3′ polarity	Mutation/controls	QGRS prediction	CD analysis[b]	
					λ_{min}	λ_{max}
lexAm1	19	GGGAAAGGAGGGAAACGGG	6G → A	Destabilizing	240	255/276
lexAm2	19	GGGATGGGAGGGAAACGGG	5A → T	Parallel	240	262
recQm	17	GGGGGGGCCAGGCGGGG	10G → A	Mixed	240	269
AvG4	16	GGGTGGGTTGGGTGGG	Positive control	Parallel	240	262
HS	16	TCCTGCATCTTCAGGC	Negative control	No G4 structure	242	271

[a] Position in genome (accession nos. AE00513 and AE001825) indicates as annotated and values within parentheses indicate upstream (−) or downstream (+) positions in the respective promoter regions of respective genes

[b] CD analysis results showing $\lambda_{max}/\lambda_{min}$ are from a reproducible representative experiment and data shown in Fig. S1 in the Supplementary Material

permeabilized cells as described earlier (Meima et al. 2001). In vivo G4 DNA formation and stabilization of G4 structures were determined by measuring β-galactosidase activity in the presence/absence of G4 drugs like NMM (Frontier Scientific, UT, USA) and 5, 10, 15, 20-tetrakis-(*N*-methyl-4-pyridyl) porphyrin (TMPyP4) (Sigma-Aldrich, St. Louis, USA).

Circular dichroism spectroscopy and thermal stability studies

CD spectroscopy and thermal stability studies were carried out using the purified G4-recQ motif as described earlier (Borgognoone et al. 2010). In brief, different concentrations (4–10 μM) of DNA samples were heated at 95 °C and slowly cooled to 25 °C in a buffer containing 10 mM Tris–HCl pH 7.4 with/without KCl. KCl (1 or 100 mM) was added during annealing and incubated at 37 °C for 30 min in the absence or presence of different concentrations (1, 2, 3, 4 and 5 μM) of NMM as required before CD spectra were recorded on a spectrophotometer (JASCO Model: PTC-423S, JASCO Analytical Company, Tokyo, Japan). The spectra were measured in the wavelength range from 220 to 320 nm using a quartz cuvette with 1.0-mm path length with a response time of 2 s at a 100-nm/min scanning speed. The reported CD spectrum of each sample represents the average of three scans taken at 25 °C. Contributions from buffers, salts and NMM to CD characteristics were subtracted appropriately by using the software supplied with the spectrometer, and all the experiments were repeated at least three independent times.

For melting studies, 10 μM of the G4-recQ motif DNA was annealed in a buffer containing 1 mM KCl, 99 mM LiCl and 10 mM Li cacodylate, pH 7.2. The sample was divided into two sets. One set was incubated with NMM, and the other was used as control. Thermal stability of DNA in the presence and absence of NMM was monitored at 262 nm at a heating rate of 1 °C/min. The melting temperatures (T_m) were determined by sigmoidal curve fit using the Boltzmann function in Origin 8.0 (OriginLab Corporation, Northampton, USA).

Gel electrophoresis

G4 DNA formation was also analysed by native PAGE as described earlier (Li et al. 2013). In brief, 2 μM each of G4 sequence was incubated in the absence or presence of different concentrations (5 to 100 mM) of KCl in buffer containing 25 mM Tris–HCl, pH 8.0, 10 % (*v*/*v*) glycerol, 1 mM DTT, 5 mM $MgCl_2$ and 0.5 mM EDTA at 25 °C for 30 min and the products were analysed on 15 % polyacrylamide gel as described in Li et al. (2013). The gels were stained with SYBR Green 1, and DNA bands were visualized under UV and documented using a gel documentation system (SYNGENE, Cambridge, UK).

Cell survival studies

Effect of NMM on γ radiation resistance was essentially monitored as described earlier (Misra et al. 2006). In brief, *D. radiodurans* R1 cells grown in TGY medium at 32 °C were

washed and suspended in sterile phosphate-buffered saline (PBS) and exposed to 6.5 kGy γ radiation at a dose rate of 3.87 kGy/h (Gamma Cell 5000, ^{60}Co., Board of Radiation and Isotopes Technology, DAE, Mumbai, India) in the presence or absence of 50 nM NMM. Cells treated with or without NMM were processed in parallel as controls. Different dilutions of cultures treated with NMM were spotted on a TGY agar plate supplemented with 50 nM NMM, while NMM untreated cells were spotted on TGY agar without NMM. Growth was monitored after 40 h of incubation at 32 °C.

RNA isolation and transcription studies

D. radiodurans cells were grown in TGY broth at 32 °C to late log phase and were separately treated with 50 nM NMM and 4 μM TmPyP4. Total RNA was prepared from both untreated and treated cells using protocols described earlier (Chen et al. 2008) and suitably modified (Rajpurohit and Misra 2010). In brief, 10 μg total RNA was annealed with 10 μg random hexamer primers in 10 μl of reaction mixture at 70 °C for 10 min and cDNA synthesis was carried out with SuperScript III Reverse Transcriptase (GE Healthcare, Bengaluru, India) in the presence of 0.5 mM deoxynucleotide triphosphates (dNTP) at 42 °C overnight. The reaction was terminated with 10 μl of 0.5 M EDTA and 10 μl of 1 M NaOH followed by heating at 65 °C for 20 min. To this, 30 μl 1 M HEPES buffer pH 7.0 was added, and free dNTPs were removed using a G-25 column (GE Healthcare, Piscataway, USA). PCR amplification was carried out using standard protocols as described earlier (Chen et al. 2008) using qrtF (5′TCAGCGTGCTCGCCGAGC3′) and qrtR (5′CGTTTCCTGGTAGTAGCCCT3′) primers for the *recQ* gene; gapF (5′-GAAGGGGCCTCCAAGCACAT-3′) and gapR (5′-TTGTACTTGCCGTTCGCGGCT-3′) primers for the *dr1343* gene encoding glyceraldehyde 3-phosphate dehydrogenase (Gap) and total cDNA mixture as templates. Products were visualized with ethidium bromide on an agarose gel, and the band intensities were quantified densitometrically. Ratios of *recQ* to *gap* were plotted with SD ($n = 3$) using GraphPad Prism software (GraphPad Software, Inc., La Jolla, USA).

Data presented without statistical analysis are illustrative of typical experiments, where variations among the replicates were less than 15 %. All experiments were carried out at least three times. Student's *t* test was applied to find out significance in the difference of the results.

Results

Guanine repeats upstream of some DNA repair genes form G4 structures

The *D. radiodurans* genome showed numerous islands of G-motif expected to form putative G4 DNA structures. The G-motifs located within 500 bp upstream of the coding sequence of DNA repair proteins like LexA, PprI, RecF, RecQ, MutL and RadA1 were subjected to online structure prediction using QGRS Mapper as described in the methods. The software predicted different geometries of G4 DNA structures (Table 1). These G-motifs were subjected to CD analysis and checked for gel mobility in the presence of K^+ ions. CD analysis confirmed theoretical predictions, and almost all the sequences showed λ_{min} at ~240 nm and λ_{max} at ~262 nm, which are the characteristics of a parallel G4 DNA structure, except G4-mutL which produced λ_{min} at ~240 nm but λ_{max} at 269 and 292 nm (Table 1). A single nucleotide base change of G6 → A/T in the G4-lexA motif resulted in the loss of CD ellipticity of the respective G4 DNA geometry, while there was no change in the ellipticity when A5 in G4-lexA was mutated to 'T'. Similarly, a G10 → A mutation on the G4-recQ sequence shifted its CD λ_{max} from 262 to 269 nm while a *mutL* G15 → A change resulted in the conversion of a mixed structure to a parallel geometry (Table 1 and Fig. S1 in the Supplementary Material). Polyacrylamide gel analysis of these G-motifs showed the formation of both intramolecular and intermolecular G4 species in the presence of higher concentration of KCl, which migrated differently during electrophoresis (Fig. 1a). The electrophoretic pattern of these G-motifs when annealed in the presence of KCl was found to be

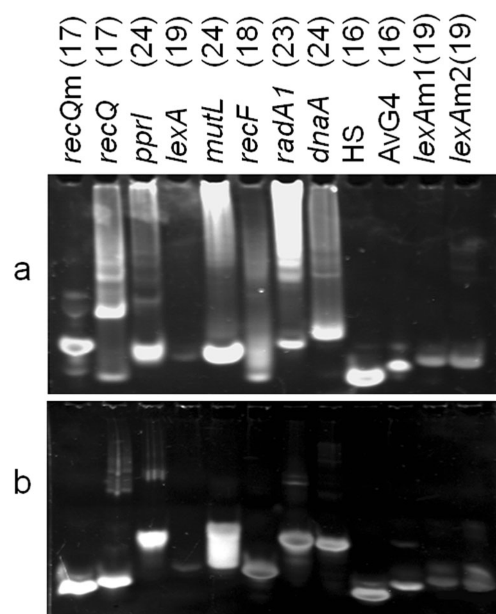

Fig. 1 Electrophoretic mobility assay of different size guanine motifs for the formation of putative G4 DNA structures. The guanine motifs located upstream to different DNA repair genes in *D. radiodurans* were synthesized and checked for the G4 DNA structure formation by CD analysis as given in Table 1 and Fig. S1 in the Supplementary Material. These sequences were heated at 95 °C and brought to room temperature in the presence (**a**) and absence (**b**) of 25 mM KCl. They were analysed by non-denaturing PAGE, and gels were stained with SYBR Green and observed under UV. *Values given within parenthesis* are representing the size of the respective oligonucleotide in base pairs

different than that in the absence of KCl (Fig. 1b), indicating the formation of G4 DNA structures in the presence of K^+. Earlier, intramolecular and intermolecular G4 DNA structures have been distinguished using native polyacrylamide gel electrophoresis (Li et al. 2013). Although the formation of quadruplex structures in the genome of *D. radiodurans* is still to be conclusively proven, the G-motifs located in the genome of *D. radiodurans* when checked in vitro showed CD spectral and electrophoretic characteristics typical of G4 DNA structures.

NMM, a G4-binding drug stabilizes the G4-recQ structure in solution

The G4-recQ motif in the presence of K^+ produced CD spectra with ellipticity λ_{min} at ~240° and λ_{max} at ~262°, which are typical to parallel G4 DNA structures (Fig. 2a). When the G4-recQ motif was annealed in the presence of K^+ ions followed by incubation with increasing concentrations of NMM, the positive ellipticity in the CD spectra increased as compared to the control (Fig. 2b), indicating the stabilization of the parallel G4 structure by NMM. To further probe the effect of NMM on G4 structure stability, the temperature required for the 50 % denaturation (T_m) of G4 DNA incubated with or without NMM was estimated. The T_m of G4 DNA incubated with NMM was higher than that of the control with a ΔT_m of 26.2 ± 0.6 °C (Fig. 2c). The G4-recQ motif when annealed in the presence of K^+ and incubated with NMM showed a faster mobility band on the native polyacrylamide gel as compared to the unfolded G4-recQ motif and G4 DNA stabilized by K^+ ions (Fig. 2d), indicating a stabilization of the intramolecular G4 structures in the presence of NMM. These results suggest that the G4-recQ motif can form a parallel G4 DNA structure in vitro.

recQ and *mutL* promoter activity is regulated by G4 DNA in *D. radiodurans*

In order to ascertain the functional significance of G4 DNA structures in promoter activity regulation in *D. radiodurans*, different promoters were fused to the *lacZ* gene in the pRADZ3 shuttle vector (Fig. 3). The expression plasmids pRZ3PrecQ, pRZ3PQΔG4, pRZ3PmutL and pRADZ3 expressing *lacZ* under different promoters as well as pRZ3ΔPlac without P_{lac} (Fig. 3) were transformed into *D. radiodurans*. The expression of β-galactosidase was monitored in the presence or absence of NMM and TmPyP4. We observed that cells harbouring pRADZ3, pRZ3PrecQ and pRZ3PmutL expressed different levels of β-galactosidase in *D. radiodurans*. However, expression of β-galactosidase was not observed when the indigenous promoter of pRADZ3 was replaced with a non-promoter sequence (Fig. 4). Cells harbouring pRADZ3, upon treatment with NMM and TmPyP4, showed an insignificant difference in the levels of β-galactosidase as compared to the untreated control (Fig. 4a). However, when the cells harbouring pRZ3PrecQ and pRZ3PmutL plasmids were

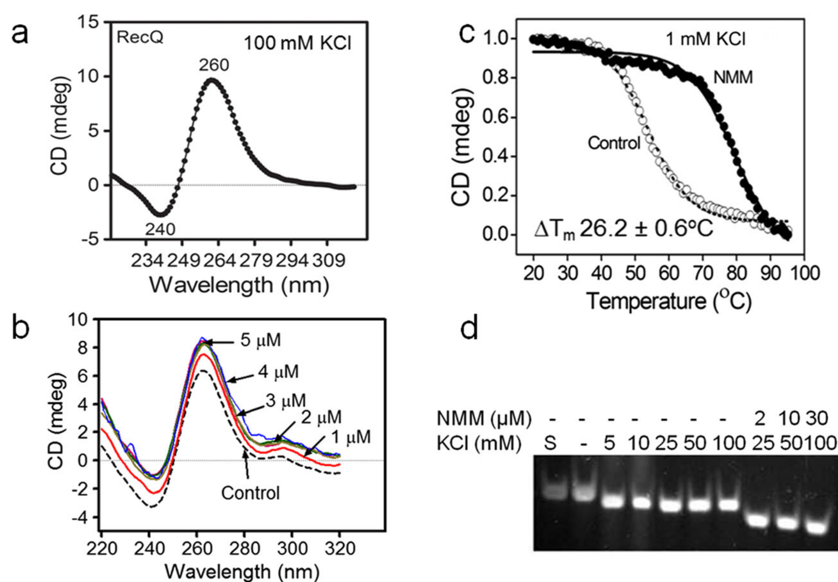

Fig. 2 Characterization of the guanine quartet structure formation of the guanine motif in the *recQ* promoter region. The G4-recQ motif in the genome of *D. radiodurans* was commercially synthesized and annealed in the presence of 100 mM KCl. This DNA was incubated in the absence (**a**) and presence (**b**) of increasing concentrations (1 μM^{-5} μM) of NMM and CD spectra were recorded. K^+ pre-incubated G-motif DNA samples were incubated with and without 2 μM NMM and thermal stability was checked (**c**). Two micromolars of G-motif DNA (S) was first annealed either in the absence (*1*) or presence of 5, 10, 25, 50 and 100 mM KCl. Samples annealed with 25, 50 and 100 mM KCl were also incubated with 2, 10 and 30 μM NMM, respectively, for 30 min. Samples were analysed on the non-denaturing PAGE and stained with SYBR Green and observed under UV (**d**)

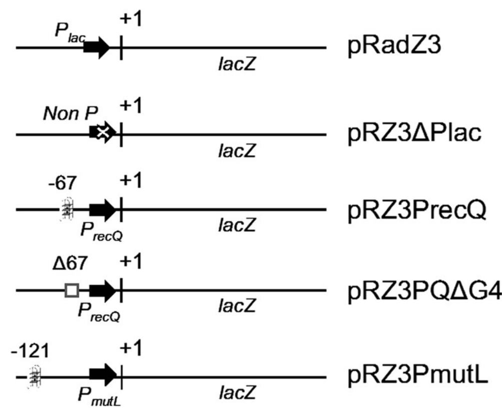

Fig. 3. Schematic representation of different expression plasmids constructed for probing effects of G4 drugs on promoter activities. The pRZ3Δplac, pRZ3PrecQ and pRZ3PmutL plasmids were constructed by replacing the *lac* promoter (P_{lac}) with 445-bp non-promoter (*Non-P*) sequences as described in 'Materials and methods', with 400 bp upstream to *recQ* (P_{recQ}) and with 418 bp upstream to *mutL* (P_{mutL}), respectively, in pRADZ3. Similarly, the G-motif of the P_{recQ} promoter was replaced with a random sequence (5′CGCAAGCTTGCGGCCGC3′) to yield the pRZ3PQΔG4 plasmid

treated with NMM and TmPyP4, the expression of β-galactosidase was significantly inhibited in *D. radiodurans* (Fig. 4b, c) while a very little effect was seen in case of *E. coli* (Fig. 4d). Interestingly, the deletion of the G-motif upstream of *recQ* abolished G4 drug effects on the β-galactosidase expression and no significant difference was observed in both *E. coli* (Fig. 5a) and *D. radiodurans* (Fig. 5b). These results suggested that P_{recQ} and P_{mutL} promoters of *D. radiodurans* are functional in both species, and their activity seems to be regulated at the levels of the G4 DNA structure.

G4 drugs affect *recQ* expression and γ radiation response in *D. radiodurans*

Regulation of *recQ* expression under G4 DNA was directly measured in *D. radiodurans*. The cells treated with G4 drugs were monitored for *recQ* transcription by reverse transcriptase-PCR and normalized with the levels of glyceraldehyde phosphate dehydrogenase (*gap*) transcripts as an internal control. The cells treated with NMM and TmPyP4 showed ~5-fold downregulation of *recQ* transcription as compared to the control (Fig. 6a, b). *D. radiodurans* treated with NMM showed noticeable growth inhibition under normal growth conditions, which increased further when these cells were exposed to γ radiation (Fig. 6c). The combined effect of NMM and γ radiation on *D. radiodurans*'s survival was stronger than the individual effects of these agents. These results indicate that NMM affects the γ radiation resistance in *D. radiodurans*. Quite interestingly, the G4 drugs showed differential effects on β-galactosidase expression in *D. radiodurans* cells harbouring pRZ3PrecQ and pRZ3PmutL plasmids when exposed to γ radiation. The expression of β-galactosidase was reduced in the case of pRZ3PrecQ (Fig. 7a) while it was increased by ~3-fold and 1.5-fold in the cells harbouring pRZ3PmutL upon treatment

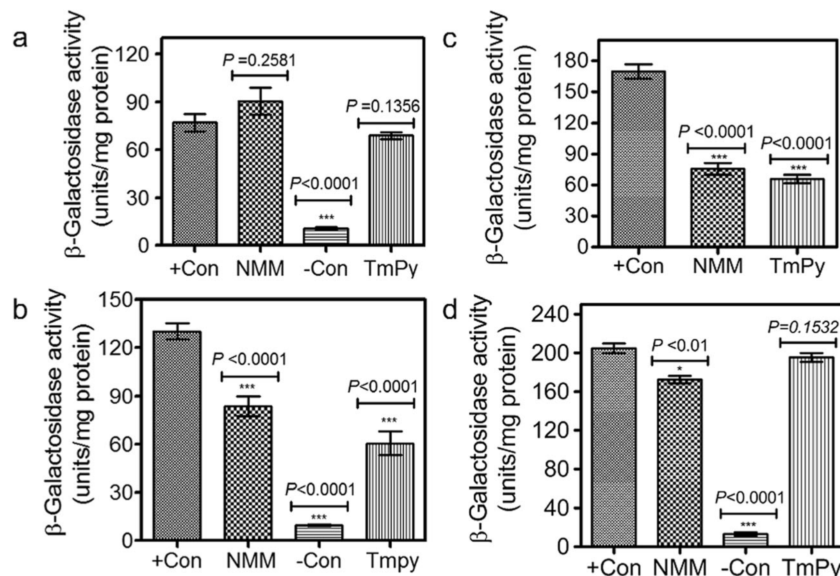

Fig. 4 Effects of G4-binding drugs on *recQ* and *mutL* promoter activity. *D. radiodurans* cells harbouring pRADZ3 (**a**), pRZ3PrecQ (**b**) and pRZ3PmutL (**c**) and *E. coli* cells harbouring pRZ3PrecQ (**d**) were grown in the absence (+Con) and presence of NMM (NMM) and TmPyP4 (TmPy) and the expression of β-galactosidase activity was monitored. Cells harbouring pRZ3ΔPlac plasmid was used as a negative control (−*Con*) for reporter gene expression. Enzyme activity (units/mg protein) is shown here as mean ± SD ($n = 9$), and the significance of the possible difference was analysed using Student's *t* test and *P* values obtained at 95 % confidence intervals are given. Single asterik (*) denotes the *P* values are less than 0.05 and three asteriks denote the *P* values are less than 0.001

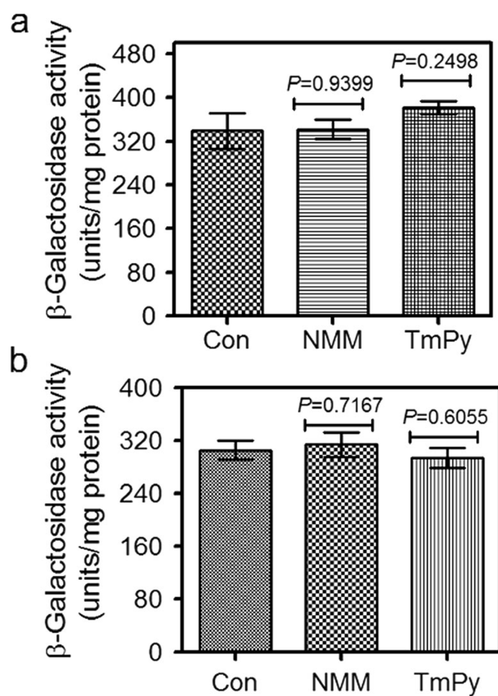

Fig. 5 Role of the G4 structure in the regulation of *recQ* promoter activity. The plasmid pRZ3PQΔG4 was transferred to *E. coli* (**a**) and *D. radiodurans* (**b**) cells, and the expression of β-galactosidase activity in the untreated (*Con*) and cells treated with NMM (*NMM*) and TmPyP4 (*TmPy*) was measured. Enzyme activity (units/mg protein) is shown here as mean ± SD ($n = 9$) and the significance of the possible difference was analysed using Student's *t* test and *P* values obtained at 95 % confidence intervals are given

with NMM and TmPyP4, respectively (Fig. 7b). These results suggest a role for G4 DNA structures in the regulation of promoter function in *D. radiodurans*.

Discussion

The *D. radiodurans* genome is GC rich with islands of G-motifs, which are located at different positions upstream to genes involved in DNA metabolism (White et al. 1999). As we mentioned earlier, this bacterium displays extraordinary resistance to various DNA-damaging agents (Cox and Battista 2005; Misra et al. 2013). The role of G4 DNA in differential regulation of gene expression in a position-specific manner has also been demonstrated in mammalian systems (Kennedy et al. 1992; Cogoi et al. 2006; Du et al. 2008). Therefore, the assumption that G4 DNA may have a role in conferring γ radiation resistant to this bacterium through the control of gene expression is not farfetched. Earlier, we have reported that *D. radiodurans* treated with NMM and TmPyP4 becomes sensitive to γ radiation (Beaume et al. 2013). Here, we provide the molecular basis to G4 drug effects on γ radiation resistance in this bacterium. We have brought forth some evidence to suggest that the *D. radiodurans* genome contains G-motifs that could form geometrically different G4 DNA structures in vitro, which can differentially regulate gene expression as shown at least in case of *recQ* and *mutL*. These sequences when annealed in the presence of K^+ could produce different CD spectra. G4-recQ showed λ_{min} at ~240 nm and λ_{max} at 262 nm, but G4-mutL showed a single λ_{min} at ~240 nm but two λ_{maxima} at 269 and 292 nm. We also found

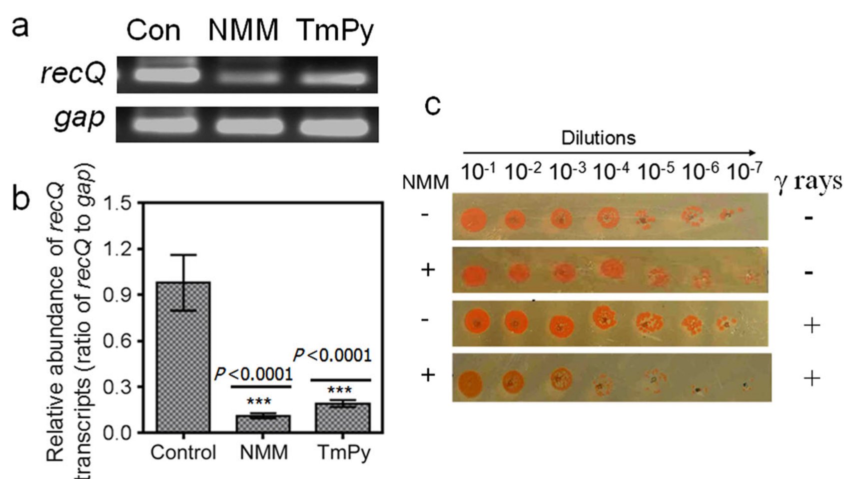

Fig. 6 Role of the G4 DNA structure in the *recQ* promoter on γ radiation response in *D. radiodurans*. *D. radiodurans* wild-type cells were treated with NMM (*NMM*) and TmPyP4 (*TmPy*) and levels of *recQ* transcripts (*recQ*) were determined by reverse transcriptase-PCR and normalized with transcripts of the glyceraldehyde phosphate dehydrogenase gene (*gap*) as an internal control. PCR products were separated on agarose gel (**a**), and ethidium bromide-stained DNA band intensity was measured densitometrically and ratios of *recQ* to *gap* were plotted (**b**). Wild-type cells were grown in the presence and absence of NMM. Different dilutions were spotted on a TGY agar plate and exposed with 6.5 kGy γ radiation. Plates were incubated, and growth was recorded after 30 h (**c**). Data shown in **a** and **c** are the representative data of respective reproducible experiments repeated three times. Data shown in **b** are means ± SD ($n = 3$) and the significance of differences were analysed using Student's *t* test and *P* values obtained at 95 % confidence intervals are given. Three asteriks denote the *P* values are less than 0.001

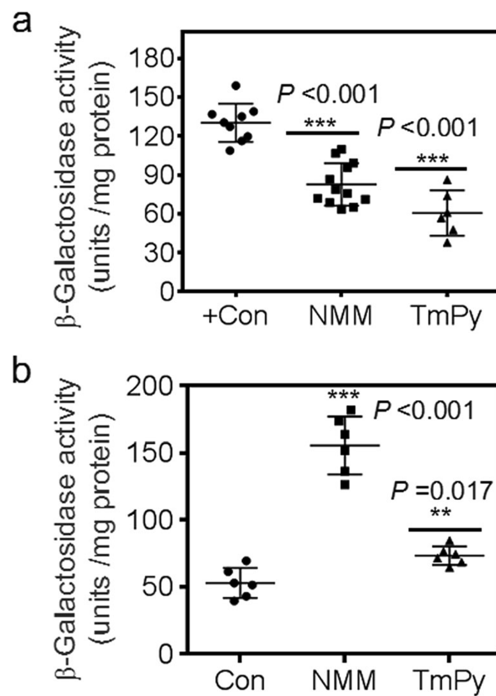

Fig. 7 Effects of G4 DNA-binding drugs on promoter activities in response to γ radiation. *D. radiodurans* cells harbouring pRZ3PrecQ (**a**) and pRZ3PmutL (**b**) were treated with 6 kGy γ radiation in the absence (*Con*) and presence of NMM (*NMM*) and TmPyP4 (*TmPy*) and the expression of β-galactosidase activity was measured. Data shown are the means ± SD with varying numbers of repeats (n = 6–12) and P values obtained using Student's t test at 95 % confidence intervals indicate the significance of possible differences. Two asteriks denote the P values are less than 0.01 and three asteriks denotes the P values are less

that G-motifs present upstream to many DNA repair genes form different types of G4 DNA structure in vitro in the presence of cations. Such CD characteristics of G-motifs in the presence of K^+ ions and other chemical probes have been attributed to the formation of a planar geometry of parallel and mixed G4 DNA structures (Giraldo et al. 1994; Bennett et al. 2000). PAGE analysis of G-motifs in the presence of KCl revealed both slow- and fast-migrating species, representing intermolecular and intramolecular G4 DNA species, respectively (Li et al. 2013). Such sequences, when analysed in the absence of K^+, produced entirely different electrophoretic mobility patterns, compared to those in the presence of KCl. A role of cations such as K^+ and Li^+ ions in the formation of G4 DNA structures resulting in differential migration on polyacrylamide gels has been demonstrated (Burge et al. 2006). When we checked the thermal stability of G4-recQ in the presence or absence of NMM, the ΔT_m values were very high. The effect of NMM on CD spectral change of the G-motif preincubated with KCl has been shown to be due to specific binding of NMM to G4 DNA and stabilization of the intramolecular G4 DNA structures (Ren and Chaires 1999; Smith and Johnson 2010). This suggests that NMM acts as a structural probe for G4 DNA and that the G4-recQ motif produces an intramolecular parallel G4 DNA structure.

Since the G-motifs present upstream of the *recQ* and *mutL* produced CD spectra corresponding to the parallel and mixed type structures, respectively, the possibility of these contributing differently to the regulation of gene expression was studied. Interestingly, *lacZ* reporter gene expression under *recQ* and *mutL* promoters containing respective G4-motifs behaved differently. The molecular mechanisms underlying the differential effects of G4 drugs in the regulation of *recQ* and *mutL* promoter activity are not clear yet. However, it may be noted that the G4-motifs located upstream and in the putative regulatory region of *recQ* and *mutL* are different at least on two aspects: (1) the position is 67 nucleotide upstream in *recQ* while it is 121 nucleotide upstream in the case of *mutL*, and (2) G4-recQ forms a parallel while G4-mutL produces a mixed G4 structural geometry (Fig. S1 in the Supplementary Material). Therefore, it might be speculated that these two distinct differences contribute to the observed differential effects of G4 drugs on the regulation of these two promoter's activities. Earlier, the position and polarity effects of G4 structures on promoter activity have been reported in human cell lines (Du et al. 2008). Future studies can provide insights into the status of G4 DNA in vivo and the mechanism(s) that regulate differential effects of G4 drugs on the activity of *recQ* and *mutL* promoters in *D. radiodurans* under normal and γ stressed growth conditions. From the available results, it is important to emphasize that the GC-rich genome of *D. radiodurans* produces G4 DNA structures in vitro, which can differentially regulate the expression of DNA repair genes, and thus appears to play a role in the radioresistance of this bacterium.

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Conflict of interest The authors declare that they have no competing interests.

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G-quadruplex forming structural motifs in the genome of *Deinococcus radiodurans* and their regulatory roles in promoter functions

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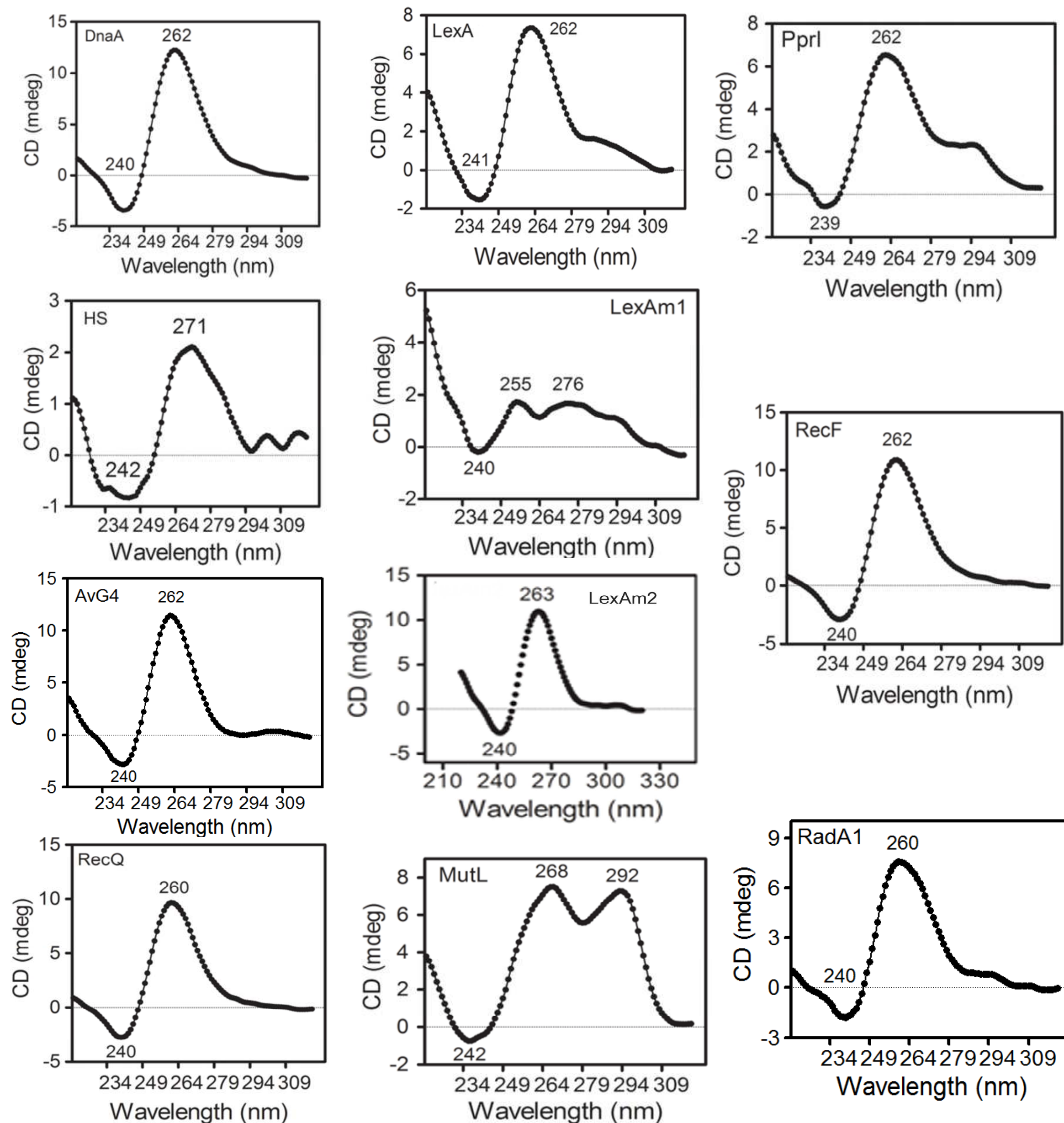

Fig. S1. Circular dichroism analysis of G-motifs present upstream to DNA repair genes in the genome of *D. radiodurans*. The G-motifs upstream to *lexA* (LexA), *pprI* (PprI), *recQ* (RecQ), *recF* (RecF), *mutL* (MutL), *dnaA* (DnaA) and *radA* (RadA) were synthesized and CD spectra were recorded as described in materials and methods. The spectral characteristics of these sequences were compared with a known G4 DNA forming sequence AvG4 (5'GGGTGGGTTGGGTGGG3') and a hairpin forming sequence (5' TCCTGCATCTTCAGGC 3') (HS) as a negative control as described earlier (Cahoon and Seifert 2009). The G4 motif upstream to *lexA* was mutagenized at 6G → A (LexAm1) and 5A → T (LexAm2) and the effects of the change in single nucleotide on CD spectral characteristics were recorded.