Characterization of DRA0282 from *Deinococcus radiodurans* for its role in bacterial resistance to DNA damage

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DRA0282, a hypothetical protein, was found in a pool of nucleotide-binding proteins in *Deinococcus radiodurans* cells recovering from gamma radiation stress. This pool exhibited an unusual inhibition of nuclease activity by ATP. The N terminus of DRA0282 showed similarity to human Ku80 homologues, while the C terminus showed no similarities to known proteins. The recombinant protein required Mn^{2+} for its interaction with DNA and protected dsDNA from exonuclease III degradation. The binding of the protein to supercoiled DNA with a K_d of ~ 2.93 nM was nearly 20-fold stronger than its binding to ssDNA and nearly 67-fold stronger than its binding to linear dsDNA. *Escherichia coli* cells expressing DRA0282 showed a RecA-dependent enhancement of UV and gamma radiation tolerance. The $\Delta drA0282$ mutant of *D. radiodurans* showed a dose-dependent response to gamma radiation. At 14 kGy, the $\Delta drA0282$ mutant showed nearly 10-fold less survival, while at this dose both $pprA::cat\Delta drA0282$ and $pprA::cat$ mutants were nearly 100-fold more sensitive than the wild-type. These results suggested that DRA0282 is a DNA-binding protein with a preference for superhelical DNA, and that it plays a role in bacterial resistance to DNA damage through a pathway in which PprA perhaps plays a dominant role in *D. radiodurans*.

INTRODUCTION

Deinococcus radiodurans R1 shows extraordinary tolerance to several abiotic stresses, including ionizing and non-ionizing radiation (Makarova *et al.*, 2001; Cox & Battista 2005; Blasius *et al.*, 2008). This bacterium possesses an efficient DNA double-strand break (DSB) repair mechanism (Minton, 1994; Daly & Minton, 1996) and strong oxidative stress tolerance mechanisms (Markillie *et al.*, 1999; Daly *et al.*, 2004, 2007). DSB repair in this organism displays RecA-dependent biphasic kinetics (Daly *et al.*, 1994; Slade *et al.*, 2009). Phase I involves extended synthesis-dependent strand annealing (ESDSA) processes (Zahradka *et al.*, 2006), followed by the second phase of the maturation of individual chromosomes, which presumably involves homologous recombination (Daly & Minton, 1996). The *D. radiodurans* R1 genome (White *et al.*, 1999) shows the absence of *recBC* and *sbcB/sbcA* genes, while the *recD2* protein (Montague *et al.*, 2009) and components of the complete RecF pathway are present (Blasius *et al.*, 2008). Expression of *sbcB* (Misra *et al.*, 2006) and *recBC* (Khairnar *et al.*, 2008) genes of *Escherichia coli* in

D. radiodurans makes this bacterium sensitive to gamma radiation by delaying its DSB repair, further supporting the contention that the RecF recombination pathway contributes to DSB repair and radiation resistance in this bacterium. The role of the RecFOR pathway in DSB repair through an ESDSA mechanism has been suggested in *D. radiodurans* (Bentchikou *et al.*, 2010). However, the significance of DNA protection in radiation resistance and DSB repair during the early phase of post-irradiation recovery (PIR) has also been shown. Two proteins, namely DdrA (Harris *et al.*, 2004) and PprA (Narumi *et al.*, 2004), have been characterized for their roles in DNA protection and radiation resistance in *D. radiodurans*. Also, recently, the involvement of reversible protein phosphorylation in the regulation of nucleolytic degradation during PIR has been suggested (Kamble *et al.*, 2010).

Here, we report the characterization of DRA0282 from *D. radiodurans* for its role in DNA protection from exonuclease degradation *in vitro* and in radiation resistance *in vivo*. The recombinant protein showed stronger binding to superhelical DNA and protected dsDNA from exonuclease III (ExoIII) degradation *in vitro*. The $\Delta drA0282$ deletion mutant ($\Delta drA0282$) of *D. radiodurans* showed a dose-dependent effect, and these cells displayed nearly 10-fold less resistance as compared with the wild-type at a 14 kGy dose of gamma radiation. Deletion of *drA0282* in a *pprA*-minus background ($pprA::cat \Delta drA0282$), however,

Abbreviations: DSB, double-strand break; ExoIII, exonuclease III; FTICR-MS, Fourier transform ion cyclotron resonance-MS; PIR, post-irradiation recovery.

Five supplementary figures and two supplementary tables are available with the online version of this paper.

showed no additive effect to the *pprA* mutant phenotype, although a double mutant showed nearly a 10-fold higher sensitivity than the $\Delta drA0282$ mutant. *E. coli* cells expressing DRA0282 showed a RecA-dependent enhancement of UV (254 nm) and gamma radiation resistance. Although SOS-deficient but recombination-proficient *E. coli* cells expressing DRA0282 showed an increase in DNA damage tolerance, the increase was significantly less than that of wild-type cells expressing the same protein. This indicated that both recombination and SOS mechanisms are important for DRA0282 function *in vivo*. Thus, we characterized DRA0282 as a DNA-binding protein with a preference for superhelical DNA, and demonstrated that it contributes to bacterial resistance to DNA damage by mechanism(s) that require RecA, at least in *E. coli*.

METHODS

Bacterial strains and materials. *D. radiodurans* ATCC13929 was a generous gift from Dr M. Schäfer, Germany (Schäfer *et al.*, 2000). The wild-type and its derivatives were grown aerobically in TGY (0.5% Bacto tryptone, 0.3% Bacto yeast extract, 0.1% glucose) with or without kanamycin (8 $\mu\text{g ml}^{-1}$). TGY agar plates were incubated at 32 °C for 48 h and colonies were counted. *E. coli* and its derivatives were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. Other recombinant techniques were as described in Sambrook & Russell (2001). Molecular biology grade chemicals were purchased from Sigma, Roche Biochemicals, New England Biolabs and Bangalore Genei.

DNA-binding protein fractionation. Exponentially growing cells were irradiated with 6 kGy of gamma radiation. Aliquots were collected at 1 h intervals and cell-free extract was prepared in lysis buffer (20 mM Tris/HCl, pH 7.6, 1 mM MgCl_2 , 1 mM DTT, 1 mM PMSF, 200 μg lysozyme ml^{-1}), as described previously (Kota & Misra, 2008). Soluble proteins were diluted with 10 volumes of buffer A (10 mM sodium phosphate, pH 7.0, 50 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM DTT, 1 mM PMSF) and passed through a heparin-Sepharose affinity column as described previously (Kamble *et al.*, 2010). Proteins were eluted with 500 mM NaCl in buffer A. The protein concentration was determined, and the heparin-purified fraction showing ATP inhibition of nucleolytic function was treated with alkaline phosphatase and refractionated on a MonoQ column by FPLC. Proteins were eluted with a salt gradient (50–500 mM NaCl) in 50 mM Tris/HCl buffer, pH 7.5, containing 10% (v/v) glycerol and 1 mM DTT. Fractions were assayed for nuclease activity in the presence and absence of 2 mM ATP. Fraction 12, which showed ATP inhibition of nucleolytic function, was analysed by Fourier transform ion cyclotron resonance-MS (FTICR-MS).

Functional domain analysis. Functional domain analysis of DRA0282 was carried out using standard online bioinformatics tools. In brief, a PSI-BLAST search was done with the Swiss-Prot database, with default parameters and the keyword 'DNA repair'. Initial iterations showed Ku80 and PRP19/PSO4 from yeast, and these were selected for further iterations. After five iterations, several Ku-like proteins were found. We selected these sequences, and multiple sequence alignment was carried out, including prokaryotic Ku proteins, in CLUSTAL_X. This procedure was repeated further to align DRA0282 with Ku proteins alone. DRA0282 again clustered with eukaryotic Ku. Next we built a neighbour-joining tree and bootstrapped it 1000 times. Secondary structure was inferred from PSIPRED, JNET and Professor at the Quick 2D server at the Max Planck Institute

for Developmental Biology. For 3D structure determination, we initially submitted the protein sequence to online servers such as I-TASSER and PHYRE, and the templates with closest matches were obtained from these servers. We subsequently built a 3D model in SWISS-MODEL, in the alignment mode, using 1JEY, the human Ku heterodimer structure, as the template (Arnold *et al.*, 2006), and residues 1–486 were modelled at minimum free energy stabilization.

Construction of a DR_A0282 expression plasmid and recombinant protein purification. Genomic DNA of *D. radiodurans* was prepared as described in Battista *et al.* (2001) and used for PCR amplification of *drA0282* coding sequences using a gene-specific forward primer 282F (5'-GAATTCATATGTTTCATGAAGAGCAAGG-3') and reverse primer 282R (5'-ACGGGATCCTAACTGACGGG-AGGTGA-3'), which incorporated restriction enzyme sites at the 5' ends of the primers. The 1.515 kb PCR product was ligated at *NdeI* and *BamHI* in pET28a+ to yield pET282, and at *NcoI* and *BamHI* sites in pTrc99 to yield pTrc282. The pTrc282 plasmid was used for studying the effect of DRA0282 expression on the radiation response of the wild-type (*E. coli* AB1157) and *recA* mutant (*E. coli* JC1553) of *E. coli* K-12 (Supplementary Table S1). The pET282 plasmid was transferred to *E. coli* BL21(DE3) pLysS, and cells were induced with 200 μM IPTG for purification of recombinant DRA0282 using metal affinity chromatography, as described previously (Misra *et al.*, 1998). DRA0282 purified under denaturing conditions was refolded by serial dilution of urea in buffer with an increasing concentration of DTT, as described previously (Khairnar & Misra, 2009). The refolded protein was dialysed in 10 mM Tris/HCl, pH 7.5, 15 mM KCl, 1 mM DTT and 50% (v/v) glycerol, and stored at –20 °C for further use.

Isolation of a deletion mutant of *D. radiodurans*. The *drA0282* deletion mutant was generated using protocols described previously (Khairnar *et al.*, 2008). In brief, the 1 kb upstream and downstream fragments of the coding sequences of *drA0282* were PCR-amplified using sequence-specific primers. The upstream fragment was amplified using primers 282upF (5'-ATGGGCCCCGCTTTGTACTCCAGAGCA-3') and 282upR (5'-CGGAATTCTTCATGAACACCAGGT-3'), and the downstream fragment was amplified using 282dnF (5'-ATGGATCCACCACCCGTCAGTAAGCT-3') and 282dnR (5'-CGTCTAGAAGCGCCCTGGTTGACCGT-3'). The products were cloned independently and sequentially in pNOKOUT (Khairnar *et al.*, 2008) to yield pNOK282. This plasmid was linearized with *ScaI* and transformed into *D. radiodurans* to generate the $\Delta drA0282$ mutant. PCR amplification was carried out using sequence-specific primers, and complete replacement of *drA0282* with the *nptII* gene was confirmed. These clones were considered as $\Delta drA0282$ mutants, and a single such clone was used for further studies.

To generate the double mutant of *pprA* and *drA0282*, linearized pNOK282 was transformed into the *pprA::cat* mutant of *D. radiodurans* (Narumi *et al.*, 2004). Recombinant cells were subcultured for many generations in TGY broth containing both chloramphenicol (5 $\mu\text{g ml}^{-1}$) and kanamycin (10 $\mu\text{g ml}^{-1}$). Genomic DNA was prepared, and the absence of *drA0282* in the *pprA* mutant background was ascertained by PCR amplification using gene-specific primers.

Cell survival studies. Cell survival studies were carried out as described previously (Rajpurohit *et al.*, 2008). In brief, mutant and wild-type *D. radiodurans* cells were grown in TGY broth to the late-exponential phase at 32 °C. Cells were suspended in sterile PBS and exposed to various doses of gamma radiation at a dose rate of 5.89 kGy h^{-1} (Gamma 5000, ^{60}Co , Board of Radiation and Isotopes Technology, Department of Atomic Energy, India). Appropriate dilutions were plated on TGY agar plates and incubated at 32 °C. *E. coli* cells expressing DRA0282 on pET282 and pTrc282 were treated with different doses of UV (254 nm) and gamma radiation as

described previously (Kota & Misra, 2006). In brief, exponentially growing *E. coli* cells induced with 200 μM IPTG were washed with and resuspended in PBS, and treated with various doses of gamma radiation at a dose rate of 356 Gy h^{-1} (Gammacell 220 irradiator, MDS Nordion) and UV at 254 nm. UV-treated cells were plated on LB agar plates containing kanamycin (25 $\mu\text{g ml}^{-1}$) and incubated in the dark. The plates were incubated at 37 °C overnight for *E. coli* and for 36 h at 32 °C for *D. radiodurans* before c.f.u. were counted.

DNA binding and ExoIII protection studies. The DNA-binding assay was carried out as described previously (Kamble *et al.*, 2010). In brief, 100 ng purified DRA0282 and 500 ng heparin-purified proteins were incubated with 200 ng PCR-amplified deinococcal DNA/super-helical form of pBluescript SK +/single-stranded linear DNA in buffer containing 10 mM Tris/HCl, pH 7.5, 5 mM MnCl_2 , 15 mM KCl and 2% (v/v) glycerol for 20 min at 37 °C. To determine the effect of ATP, the proteins were pre-incubated with 2 mM ATP for 20 min at 37 °C, followed by incubation with 200 ng DNA substrate for 30 min. The ExoIII protection assay was performed as described elsewhere (Wang *et al.*, 2005). In brief, 200 ng DNA substrate was pre-incubated with different concentrations of purified DRA0282 for 10 min in Exonuclease III Buffer (New England Biolabs). The reaction mixture was further incubated for 20 min in the presence of an increasing concentration of ExoIII. When required, the nucleoprotein complex was dissociated by heating at 65 °C in the presence of 95% formamide and 25 mM EDTA. Products were analysed on 1% agarose gels and DNA bands were visualized with ethidium bromide.

For determination of the affinity constant of DRA0282 for different DNA substrates, 200 ng DNA was incubated with an increasing concentration of recombinant DRA0282 (4 nM to 1.42 μM) for 10 min, and products were analysed on 0.8% agarose gels. Ethidium bromide-stained DNA fluorescence intensity was measured using ImageJ software (Abramoff *et al.*, 2004). The integrated intensity for bound and unbound fractions per unit area was measured separately, and the fraction bound to the protein was plotted as a function of the protein concentration using GraphPad Prism 5. The K_d for curve fitting of individual plots was determined by the software, working on the principle of the least-squares method and applying the formula $Y = B_{\text{max}} \times [X]/K_d + [X]$, where B_{max} is the maximum binding capacity, Y is the bound fraction and $[X]$ is the protein concentration.

RESULTS AND DISCUSSION

Identification of DRA0282 from a protein mixture exhibiting ATP-stimulated DNA protection

Heparin-binding proteins purified from cells undergoing PIR show kinetically the appearance of nucleolytic activity (Kamble *et al.*, 2010). The 1 h PIR proteins did not show any inherent nucleolytic activity, which, however, was expressed upon dephosphorylation with alkaline phosphatase. Alkaline phosphatase-treated 1 h PIR proteins were refractionated on a Mono-Q column (Fig. 1a). The fractions were checked for nuclease activity towards PCR-amplified DNA with and without ATP. It was found that the nucleolytic activity was fractionated (Fig. 1b), and only fraction 12 showed ATP inhibition of nucleolytic function (Fig. 1c). Fractions 6, 7 and 8 showed strong DNA-binding activity. Other fractions, such as 9, 10, 13–18 and 20, showed neither nucleolytic activity nor an effect of ATP on the quality and quantity of the DNA substrate as compared with the control. This suggested that the nucleolytic

function of fraction 12 was sensitive to ATP. This fraction was analysed by FTICR-MS, and matched a number of proteins represented by at least five or more peptides (Supplementary Table S2). These included some known proteins as well as a large number of uncharacterized ORFs, including DR_0129, DR_A0282, DR_1124, DR_1768, DR_2417 m and DR_2563. However, no proteins were annotated as nucleases or kinases in this fraction. Among these, the ORFs DR_0129, DR_1124, DR_1768, DR_2417 m and DR_2563 have also been found in a multiprotein DNA-processing complex identified independently in the same bacterium that also exhibits an uncharacterized ATP-sensitive exonucleolytic function (Kota & Misra, 2008). Fraction 12 also contained DRA0282, a hypothetical protein, with the largest number of FTICR-MS peptides (84), which was characterized here for its *in vitro* activity and *in vivo* role, if any, in radiation resistance.

DRA0282 shows structural similarities to Ku-type proteins

Multiple sequence alignments of DRA0282 with human Ku80 showed a close similarity to the β -barrel of the Ku

Fig. 1. Fractionation and nucleolytic activity assay of heparin-binding proteins from *D. radiodurans*. Heparin-binding proteins were isolated from cells allowed to recover for 1 h post-irradiation, and were fractionated on MonoQ columns by FPLC (a). Nearly 500 ng of proteins of each fraction was tested for nucleolytic activity with 100 ng dsDNA substrate in buffer containing 5 mM MnCl_2 , both in the absence (b) and the presence (c) of 2 mM ATP pre-incubated with the proteins for 20 min. (b, c) Asterisks mark fraction 12, which contained the hypothetical protein DRA0282.

core domain (Supplementary Fig. S1). Phylogenetic analysis showed DRA0282 clustering with eukaryotic Ku proteins (Supplementary Fig. S2). This was further verified by the alignment of DRA0282 with other Ku-like proteins from both prokaryotes and eukaryotes. Results showed that ~150 amino acid residues from the N terminus of DRA0282 were homologous to the N terminus of human and yeast Ku80. This forms the α/β domain, also known as the Rossman fold, which signifies the binding of nucleotides by this protein. Further, the 150 amino acid residues from 250–400 of DRA0282 showed the highest homology to the central domain known as the Ku core domain in Ku proteins, which is responsible for their binding to free DNA ends (Aravind & Koonin, 2001). The secondary structure predicted by PyMOL showed extensive β -sheets at regular intervals, interspersed by coils. Although the DRA0282 sequence showed the overall α/β domain structure of Ku80m, it lacked certain segments, such as $\alpha 2$ and $\beta 17$ (Supplementary Fig. S1 and Fig. 2). The overall similarities of this protein to Ku-like proteins prompted us to build a 3D model using the human Ku heterodimer as template (Arnold *et al.*, 2006). Regions with gaps had high root mean square deviation (RMSD), while regions with sequence overlap had a maximum RMSD of 4°, and the modal RMSD value was 0.8. Unlike human Ku, which is activated by ATP, DRA0282 was inhibited by it. This difference could be attributed to the absence of the $\alpha 2$ loop in DRA0282 and the resulting changes in its structure. The core domain of this protein resembled the Ku80 core domain. The $\alpha 7$ – $\beta 18$ loop, which is responsible for dsDNA binding in Ku80, showed a significant match with DRA0282, while $\beta 10$ – $\beta 13$ showed a poor structural alignment. The homology of DRA0282 to human Ku80 and its homologues was therefore through its N terminus,

while its C-terminal domain was different. It could be speculated that this protein has evolved through genomic rearrangement under the pressure of natural selection. The results from online servers were intriguing. PHYRE returned a receptor-type tyrosine protein phosphatase as the best match [Protein Data Bank (PDB) ID: 2V5Y], while I-TASSER returned a sialidase from *Micromonospora* spp. (PDB ID: 1EUT), and FUGUE returned measles virus haemagglutinin (PDB ID: 2ZB6). These proteins have in common extensive β -sheet structures. These results indicated the structural resemblance of DRA0282 to Ku80 protein in having DNA-binding domains.

DRA0282 is a DNA-binding protein with an ability to protect dsDNA in solution

The purified recombinant DRA0282 protein (Supplementary Fig. S3) was incubated with ssDNA and linear dsDNA in the presence of different metal ions. The protein showed strong binding to both dsDNA and ssDNA in the presence of Mn^{2+} , while this activity was not observed in the presence of Ca^{2+} and Mg^{2+} (Fig. 3a). DRA0282 showed stronger binding to ssDNA than to dsDNA in the presence of Mn^{2+} (Fig. 3b). It did not bind to dsDNA in the presence of Zn^{2+} , while its binding to ssDNA in the presence of Zn^{2+} was minor compared with its binding in the presence of Mn^{2+} . Recombinant DRA0282 showed strong binding to superhelical plasmid DNA, without affecting the topology or size of the plasmid DNA. This was confirmed by the complete recovery of the substrate in its original form upon heating with 95 % formamide and 25 mM EDTA at 65 °C (Fig. 3b), which breaks the nucleoprotein interaction *in vitro* (O'Donovan *et al.*, 1994). This indicated that DRA0282 was a Mn^{2+} -dependent

Fig. 2. Structural similarities of DRA0282 and human Ku80. The 3D models of DRA0282 and human Ku80 were compared to find similarities in the dsDNA-binding motifs. The N-terminal Von Willebrand domain is coloured blue in DRA0282 and red in human Ku80, while the Ku core domain is coloured green in both molecules.

Fig. 3. Characterization of the DNA-binding activity of DRA0282. (a) One hundred nanograms of purified recombinant DRA0282 was incubated with 200 ng M13mp18 ssDNA and 200 ng dsDNA in buffer supplemented with 5 mM ZnCl_2 , MnCl_2 , MgCl_2 or CaCl_2 , and products were analysed on 1% agarose. (b) DRA0282 (P) was incubated with both linear (PCR) and superhelical (plasmid) dsDNA (S), with BSA (BS) as a control. One set of reaction mixtures was treated with 95% formamide, 25 mM EDTA (F), while another set was kept as a control (N), and both sets were analysed on 1% agarose gels. (c) Autoradiogram showing the 200 bp dsDNA labelled with ^{32}P at the 5' end (lane S) incubated with either 100 ng BSA (lane 1) or 100 ng DRA0282 (lanes 2–9). For the competition assay, the protein was pre-incubated separately with 50 ng *Bam*HI- (lane 3), *Eco*RV- (lane 4) or *Pst*I-digested dsDNA (lane 5), M13mp18 ssDNA (lane 6) or supercoiled plasmid DNA (lane 7), before the addition of radiolabelled probe. DNA-binding activity (lane 2) was also checked in the absence (lane 8) and presence (lane 9) of 5 mM Mn^{2+} + 2 mM ATP. Products were separated by 6% native PAGE and dried, and the autoradiogram was developed. (d) The effect of 2 mM ATP, AMP, GTP or GMP on the ssDNA-binding activity of DRA0282 was monitored on an agarose gel.

DNA-binding protein. The effects of the types of DNA ends and ssDNA on dsDNA binding were monitored by a competition assay. A 200 bp ^{32}P -labelled dsDNA probe was used to study the binding preferences of DRA0282. dsDNA with different types of ends generated by *Bam*HI, *Eco*RV or *Pst*I did not disrupt the binding of DRA0282 to the probe, which, however, was readily disrupted by ssDNA (Fig. 3c). These activities were Mn^{2+} -dependent and sensitive to ATP (Fig. 3c). Further, the purified protein showed the highest binding constant (K_d) of 2.93 nM for superhelical plasmid, followed by 60.22 nM for linear ssDNA and 196.9 nM for linear dsDNA (Fig. 4).

Since this protein contains a Ku80-type DNA-binding pocket and nucleotide interaction domain, the effect of nucleoside phosphates on DNA binding and the protection of DNA ends from ExoIII degradation were examined. The DNA–DRA0282 interaction was inhibited by ATP and GTP, while AMP and GMP did not show any measurable effect on this activity (Fig. 3d). These results suggested that DRA0282 was a DNA-binding protein with a relatively higher affinity for superhelical DNA and linear ssDNA as

compared with linear dsDNA. The inhibition of the DNA–protein interaction by nucleoside triphosphates and not by monophosphates suggested the involvement of terminal phosphates rather than the adenyl group in the inhibition of the DNA-binding activity of this protein. Similarly, recombinant DRA0282 protected linear dsDNA from ExoIII degradation, whereby as much as 50 U nuclease could not degrade the dsDNA substrate completely after pre-incubation with DRA0282 (Fig. 5a). An increasing concentration of DRA0282 afforded an increase in protection of DNA when challenged with 10 U ExoIII, which was able to degrade the dsDNA substrate completely in the absence of DRA0282 (Fig. 5b). Since DNA binding by DRA0282 was inhibited by ATP, the effect of ATP on the dsDNA protection of DRA0282 was examined. In the presence of ATP, DRA0282 failed to protect DNA from ExoIII degradation, and eventually more than 90% of the DNA was degraded, in spite of the presence of 72 nM DRA0282 (Fig. 5c), which had shown complete DNA protection in an earlier assay (Fig. 5b). Since ATP alone showed no effect on ExoIII activity (Fig. 5c, lane 6), and the

Fig. 4. Determination of the DNA-binding constant of DRA0282. Two hundred nanograms of linear dsDNA (●), superhelical DNA (▲) and linear ssDNA (■) substrates were incubated with increasing concentrations of recombinant DRA0282 for 10 min at 37 °C. Products were analysed on a 1% agarose gel. The DNA–protein bound fraction was plotted against protein concentration, and affinity constants of the protein for different DNA substrates were computed using GraphPad Prism 5 graphics, as described in Methods.

protection of dsDNA from the action of ExoIII was not seen in the presence of BSA as a control (Fig. 5c, lane 7), it is possible that the binding of ATP to DRA0282 prevents its binding to DNA, eventually leading to DNA degradation by ExoIII. These results suggest that DRA0282 is able to protect dsDNA from nucleolytic degradation by direct binding to the DNA substrate. Its higher affinity for superhelical DNA indicated a strong possibility of this protein interacting directly with the bacterial genome. The possibility that DRA0282 has a role in the DNA damage response of bacteria was further investigated by expressing this protein in *E. coli* and by generating a knockout of this gene in *D. radiodurans*.

DRA0282 enhances the UVC and gamma radiation tolerance of *E. coli*

The effect of DRA0282 on DNA damage tolerance was monitored in wild-type and mutant strains of *E. coli* (Supplementary Table S1). Wild-type cells expressing DRA0282 showed a nearly 10-fold higher tolerance to gamma radiation at a dose of 200 Gy (Fig. 6a), and a nearly three-log increase in UVC tolerance at a dose of 1.2 kJ m⁻², as compared with untransformed cells (Fig. 6c). Interestingly, the *recA* mutant (JC1553) expressing DRA0282 did not show any increase in gamma radiation (Fig. 6b) and UVC tolerance (Fig. 6d). This indicated that DRA0282 functions in *E. coli* through RecA. Since RecA is involved in both homologous recombination and SOS response mechanisms in *E. coli*, the contribution of RecA in supporting DRA0282 function in

Fig. 5. DRA0282 protection of dsDNA from ExoIII degradation. The ~150 nM 1 kb PCR-amplified dsDNA was pre-incubated with ~18 nM DRA0282 for 5 min before increasing concentrations of ExoIII were added (a). Similarly, the DNA substrate was pre-incubated with increasing concentrations (18–72 nM) DRA0282 in the presence of 2 mM MnCl₂ (b) and 2 mM ATP along with 5 mM Mn²⁺ (c) before 10 U ExoIII was added. Reaction mixtures containing both BSA (BS) and ExoIII, and ExoIII alone, were used as controls. All reactions were carried out at 37 °C for 20 min, and products were analysed on a 1% agarose gel.

E. coli via these pathways was examined. DRA0282 was expressed in *E. coli* strain DM49 (*E. coli* K-12, *recA*⁺ *lexA3*) (Mount *et al.*, 1972), and the effect of both UVC and gamma radiation was monitored. These cells showed an enhanced tolerance to both UV and gamma radiation (Fig. 7), albeit slightly lower than that observed in the wild-type background. The DM49 strain has a wild-type copy of RecA but a point mutation in the RecA co-protease cleavage site of LexA (*lexA3*), making it SOS-deficient but still recombination-proficient. These results suggested that DRA0282 plays an important role in the DNA damage tolerance of *E. coli* and works through mechanisms that involve RecA.

DRA0282 deletion does not alter the *pprA* mutant phenotype in *D. radiodurans*

The Δ *dra0282* mutant showed a dose-dependent response to gamma radiation-induced DNA damage. It showed nearly a 10-fold reduction with respect to wild-type tolerance at 14 kGy gamma radiation (Fig. 8), while no difference in the gamma radiation response was noted in either a *pprA* single or a *pprA::cat* Δ *dra0282* double mutant. Both these mutants showed a nearly two-log

Fig. 6. Effect of DRA0282 overexpression on the DNA damage response of *E. coli*. *E. coli* AB1157 (wild-type; WT) and JC1553 (*recA*) cells expressing DRA0282 (■) and corresponding controls (●) were treated with different doses of gamma and UV (254 nm) radiation. c.f.u. were monitored after overnight incubation at 37 °C. Cell survival of 100 % corresponds to 5.423×10^7 and 3.124×10^7 cells ml⁻¹ of the wild-type and *recA* mutant, respectively.

decrease with respect to the wild-type tolerance of gamma radiation (Fig. 8). This suggests that although both these proteins contribute to the radiation resistance of this bacterium, PprA plays the dominant role. The levels of expression of the *dra0282* gene were increased nearly 20-fold within 1 h of post-irradiation growth (Supplementary Fig. S4), further supporting the possible involvement of DRA0282 in the radiation response of *D. radiodurans*.

Previously it has been reported that gamma-irradiated *D. radiodurans* R1 cells show rapid DNA degradation, which is arrested within 90 min of PIR (Khairnar *et al.*, 2008; Slade *et al.*, 2009). During this period the cells show a higher rate of DNA synthesis, leading to a net increase in DNA content and DSB repair. This period coincides with the expression of DNA-protecting proteins such as PprA (Narumi *et al.*, 2004) and DdrA (Harris *et al.*, 2004), and many uncharacterized ORFs, including DR_A0282 (Liu *et al.*, 2003; Tanaka *et al.*, 2004; Supplementary Fig. S4). The protection of linear dsDNA from ExoIII activity by PprA, DdrA and now the DRA0282 protein (Fig. 5), which is also found to have a role in radiation resistance of *D. radiodurans* (Fig. 8), suggested that DNA protection from nucleolytic degradation plays an important role in the

radiation resistance of this bacterium. The fact that DRA0282 enhanced the UVC and gamma radiation resistance of wild-type *E. coli* and a *lexA3* mutant, although to a lesser extent than the wild-type, while not doing so in a *recA* mutant, suggests a role for this protein in recombination-dependent mechanism(s) that contribute to DNA repair in *E. coli*. On the other hand, the molecular mechanisms that support the role of this protein in the radiation resistance of *D. radiodurans* are not yet clear. However, DRA0282 showed structural homology to Ku homologues, and the different roles of Ku-type proteins, including non-homologous end-joining (NHEJ) and DSB repair, have been shown in higher organisms (Downs & Jackson, 2004). For example, human hPso4, a protein having similarity to DRA0282 (Supplementary Fig. S5), has been shown to bring Metnase, a human DSB repair protein with SET and transposase/nuclease domains (Lee *et al.*, 2005), to the sites of DSB repair (Zhang *et al.*, 2005; Lu & Legerski, 2007; Beck *et al.*, 2008). hPso4 is known to bind dsDNA non-specifically, and has pleiotropic functions in DNA recombination and error-prone repair. The hPso4 protein is induced 15- to 30-fold in cells exposed to gamma radiation and chemical mutagens, but not UV radiation (Mahajan & Mitchell, 2003). These findings indicate the

Fig. 7. Effect of DRA0282 on DNA damage tolerance of SOS-deficient and recombination-proficient *E. coli*. *E. coli* DM49 (*lexA3* mutant) cells expressing DRA0282 (■) or harbouring the expression vector (●) were treated with different doses of gamma (a) and UV (254 nm) (b) radiation, and cell survival was monitored at 37 °C. Cell survival of 100 % corresponds to 7.21×10^7 cells ml^{-1} .

possible role of Ku homologues in DSB repair by both NHEJ and recombination functions. Although the existence of NHEJ-type activity has not been demonstrated in *D. radiodurans*, its genome encodes several other hypothetical proteins which could constitute an NHEJ pathway, and therefore the occurrence of such a mechanism cannot be ruled out in this bacterium. The existence of an NHEJ-type mechanism has been reported in other bacteria, including *Bacillus subtilis* (Weller *et al.*, 2002) and *Mycobacterium* (Gong *et al.*, 2005; Pitcher *et al.*, 2007). Recently, an alternative mechanism of DNA ends joining has been demonstrated in *E. coli* (Chayot *et al.*, 2010). Although further studies will be required to understand the molecular mechanism underlying the roles of DRA0282 in the radioresistance of *D. radiodurans*, this study reports the characterization of the hypothetical protein DRA0282 as a DNA-binding protein with a higher affinity for superhelical DNA and with a role in dsDNA protection, as well as its

Fig. 8. Effect of *drA0282* deletion on the gamma radiation tolerance of *D. radiodurans*. Cells of *D. radiodurans* R1 (wild-type; ●) and its $\Delta drA0282$ single mutant (■), *pprA::cat*Δ*drA0282* double mutant (▼) and *pprA::cat* single mutant (▲) were treated with different doses of gamma radiation, and cell survival was monitored. Cell survival of 100 % corresponds to 1.45×10^7 , 4.12×10^6 , 2.54×10^7 and 1.72×10^6 cells ml^{-1} for the wild-type, $\Delta drA0282$, *pprA::cat* and *pprA::cat*Δ*drA0282* strains, respectively.

contribution to bacterial resistance to DNA damage by a mechanism that requires a functional RecA, at least in *E. coli*.

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