DNA polymerase X from *Deinococcus radiodurans* implicated in bacterial tolerance to DNA damage is characterized as a short patch base excision repair polymerase

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The *Deinococcus radiodurans* R1 genome encodes an X-family DNA repair polymerase homologous to eukaryotic DNA polymerase β . The recombinant deinococcal polymerase X (PolX) purified from transgenic *Escherichia coli* showed deoxynucleotidyltransferase activity. Unlike the Klenow fragment of *E. coli*, this enzyme showed short patch DNA synthesis activity on heteropolymeric DNA substrate. The recombinant enzyme showed 5'-deoxyribose phosphate (5'-dRP) lyase activity and base excision repair function *in vitro*, with the help of externally supplied glycosylase and AP endonuclease functions. A *polX* disruption mutant of *D. radiodurans* expressing 5'-dRP lyase and a truncated polymerase domain was comparatively less sensitive to γ -radiation than a *polX* deletion mutant. Both mutants showed higher sensitivity to hydrogen peroxide. Excision repair mutants of *E. coli* expressing this polymerase showed functional complementation of UV sensitivity. These results suggest the involvement of deinococcal polymerase X in DNA-damage tolerance of *D. radiodurans*, possibly by contributing to DNA double-strand break repair and base excision repair.

INTRODUCTION

Cells exposed to DNA-damaging agents produce different types of structural changes depending upon the nature of the DNA damage. DNA strand breaks produced upon radiation exposure are mainly repaired by a recombination process (Shinohara & Ogawa, 1995). Alkylating agents cause abnormal base methylation, which sometimes leads to formation of AP (apurinic/apyrimidinic) sites on DNA. Methyl groups added onto nucleotide bases are corrected by either respective methyltransferases (Moore *et al.*, 1994) or DNA glycosylases, or both. *Escherichia coli* contains class I alkyl glycosylase encoded by *tagA*, and class II glycosylase encoded by *alkA*. The AlkA enzyme shows a broad range of substrate specificity and can remove a wide range of alkylated bases while TagA glycosylase is highly specific to 3-methyladenine (3-MeAde) and 3-methylguanine (3-MeGua) modifications (Bjelland *et al.*, 1993, 1994; O'Brien & Ellenberger, 2004). Both these enzymes show differential inhibition by excised methylated bases (Tudek

et al., 1998). Bulky adducts formed by UV action create a block in the replication fork, which is removed by the combined action of multienzyme nucleotide excision machinery followed by the DNA resynthesis step (de Laat *et al.*, 1999). DNA damage produced by oxidizing agents such as hydroxyl and peroxide radicals (Friedberg *et al.*, 1995) causes only slight distortion of DNA molecules and such defects are corrected by the base excision repair (BER) mechanism. The 5'-deoxyribose phosphatase (5'-dRPase) activity in BER DNA polymerases along with DNA glycosylases and AP endonucleases plays an important role in correcting such DNA lesions (Seeberg *et al.*, 1995; Parikh *et al.*, 1999). Non-removal of methylated bases from DNA also induces an SOS response leading to a higher mutation rate (Grzesiuk *et al.*, 2001).

Deinococcus radiodurans R1 is characterized by its extraordinary tolerance to radiation, desiccation and other DNA-damaging agents (Battista, 2000; Makarova *et al.*, 2001; Blasius *et al.* 2008). A DNA double-strand break produced by these abiotic agents is repaired in two phases. Phase I is RecA independent and involves the extended synthesis-dependent strand-annealing (ESDSA) process (Zahradka *et al.*, 2006); phase II follows RecA-dependent slow crossover events of homologous recombination (Daly & Minton, 1996). It is reported that DNA synthesis in γ -irradiated cells occurs at a much faster rate than in

Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; dRP, deoxyribose phosphate; MMC, mitomycin C; NER, nucleotide excision repair; PHP, polymerase and histidinol phosphatase; PolX, polymerase X; Pol β , polymerase β ; T/P, template/primer; UDG, uracil DNA glycosylase.

Two supplementary tables and four supplementary figures are available with the online version of this paper.

unirradiated controls, indicating the higher activity of DNA polymerase during post-irradiation recovery (Zahradka *et al.*, 2006). Apart from replicative DNA polymerases, the *D. radiodurans* genome was annotated for the X-family DNA-repair polymerase (PolX) encoded by DR0467, which is homologous to eukaryotic DNA polymerase β (Pol β) (Aravind & Koonin, 1999). Pol β plays a crucial role in BER and UV lesion bypass DNA synthesis in mammals (Kubota *et al.*, 1996; Servant *et al.*, 2002; Dianova *et al.*, 2004). *D. radiodurans* is endowed with a strong oxidative stress-tolerance mechanism, which contributes to the extraordinary radiation tolerance of this bacterium (Markillie *et al.*, 1999). *D. radiodurans* has the complete nucleotide excision repair (NER) system (Evans & Moseley, 1983; Agostini *et al.*, 1996; Narumi *et al.*, 1997), similar to *E. coli*, and a poorly understood mechanism of BER. The absence of a *lexA* mutation effect on the expression of *recA* and absence of *umuDC* in *D. radiodurans* suggested the absence of the SOS response in this bacterium (Narumi *et al.*, 2001; Bonacossa de Almeida *et al.*, 2002; Sheng *et al.*, 2004). However, the role of LexA2 in radioresistance by over expression of *pprA* indicated the possibility of LexA2 functioning through a different mechanism in *D. radiodurans* (Satoh *et al.*, 2006). Since *D. radiodurans* contains PolX, a homologue of Pol β , along with other components of a BER system, the role of this enzyme in radiation and oxidative stress-induced DNA-damage repair merited investigation.

Here we report the role of PolX in the bacterial response to DNA damage and characterization of the recombinant enzyme for its short patch DNA synthesis and BER activity *in vitro*. *D. radiodurans* lacking a complete PolX molecule responded differently to γ -radiation damage compared to cells expressing the N-terminal domain of polymerase. Although these cells showed a relatively smaller effect on mitomycin C and UV radiation responses, PolX showed functional complementation of *E. coli* mutants defective for excision repair functions. These results suggest the functional role of this protein in oxidative stress tolerance in *D. radiodurans* and indirectly indicate a less significant role of the polymerase and histidinol phosphatase (PHP) hydrolase domain in polymerase activity *in vivo*. In addition, the short-length DNA synthesis, 5'-deoxyribose phosphate (5'-dRP) lyase activity and the repair of the damaged base in the presence of exogenously added glycosylase and AP endonuclease *in vitro* might characterize this enzyme as a short patch BER enzyme, and may imply the possible mechanism by which this enzyme could be beneficial in bacterial tolerance to DNA damage.

METHODS

Bacterial strains, plasmids and growth conditions. *Deinococcus radiodurans* strain R1 was a generous gift from Dr M. Schafer (Schafer *et al.*, 2000) and *E. coli* mutants were obtained from Professor Elzbieta Grzesiuk (Grzesiuk *et al.*, 2001). See Supplementary Table S1, available with the online version of this paper, for details of all strains used. *D. radiodurans* R1 was grown at 32 °C in TGY broth

(0.5 % Bacto Tryptone, 0.1 % glucose, 0.3 % Bacto Yeast Extract). *E. coli* and its derivatives were grown at 37 °C in LB medium supplemented with kanamycin (25 $\mu\text{g ml}^{-1}$) for pET28+ (Novagen) and its derivatives, and ampicillin (100 $\mu\text{g ml}^{-1}$) for pRadgro (Misra *et al.*, 2006) and its derivatives. All the molecular biology grade chemicals, including restriction enzymes and DNA-modifying enzymes, were purchased from Sigma, Roche Biochemicals, New England Biolabs and Bangalore Genei.

Construction of recombinant plasmid for *polX* expression in *E. coli*. Genomic DNA was prepared from *D. radiodurans* R1 as described earlier (Battista *et al.*, 2001). Standard recombinant DNA techniques including plasmid DNA isolation were as described by Sambrook & Russell (2001). The coding region of *polX* (White *et al.*, 1999) was PCR amplified from the total genomic DNA of *Deinococcus* using gene-specific primers (polXF and polXR) as detailed in Supplementary Table S2. The restriction endonuclease sites were added at the 5' end of the respective primers. PCR was carried out in 50 μl containing 50 ng DNA template, 400 nM of each primer, 100 μM deoxynucleotide triphosphate, using a GC-rich amplification system (Roche Molecular Biochemicals) for 5 min at 94 °C, 2 min at 50 °C and 2 min at 72 °C followed by 25 cycles of 2 min at 94 °C, 2 min at 50 °C and 2 min at 72 °C. The PCR product was sequenced to confirm gene identity and absence of mutation in the coding region of the gene. The 1.7 kb PCR product was cloned at *EcoRI* and *HindIII* sites in pET28a+ and at *ApaI* and *XbaI* sites in pRadgro (Misra *et al.*, 2006). The recombinant plasmids containing the *polX* gene under IPTG-inducible T7 and constitutively expressing *groESL* promoters were named as pETpolX and pGropolX, respectively.

Generation of *polX* mutants of *D. radiodurans*. The pPolX::cat plasmid was constructed by inserting the *cat* cassette, expressing in *Deinococcus*, at the *SacII* site in pETpolX. In brief, the *cat* gene was PCR amplified from pRAD1 (Meima *et al.*, 2001) using CatF and CatR primers and ligated at the blunt-ended *SacII* site in pETpolX to yield pPolX::cat (Supplementary Fig. S1). Recombinant plasmid was linearized with *BspMI* and transferred into *D. radiodurans*. The transformants were grown for several generations in TGY supplemented with chloramphenicol (Cm; 5 $\mu\text{g ml}^{-1}$), and the complete replacement of a wild-type copy of 1.7 kb *polX* with 2.7 kb gene (*polX::cat*) was confirmed by PCR amplification. Cells lacking the wild-type allele of *polX* were named as *polXnok1*.

The *polX* deletion mutant was generated using protocols similar to that described earlier (Khairnar *et al.*, 2008). In brief, the sequences 1 kb upstream and 1 kb downstream of *polX* (DR0467) were PCR amplified from the *D. radiodurans* genome using NPK1 and NPK2 for downstream and NPK3 and NPK4 for upstream, respectively. These products were cloned sequentially at *ApaI*–*EcoRI* followed by *BamHI*–*XbaI* sites, respectively. The resultant plasmid pNOKpolX (Supplementary Fig. S2) was linearized with *ScaI* and transformed into *D. radiodurans*. These cells were grown for several generations in the presence of kanamycin (8 $\mu\text{g ml}^{-1}$) until the complete replacement of *polX* with *nptII* was achieved, which was confirmed by PCR amplification. The mutant showing the homozygous replacement of *polX* with *nptII* was named as *polXnok2*.

Cell-survival studies. *E. coli* cells expressing PolX were grown to late exponential phase and treated with different doses of DNA-damaging agents as described earlier (Khairnar *et al.* 2007; Kota & Misra, 2006). For γ -radiation treatment, the late-exponential-phase *E. coli* cells were treated with different doses of γ -radiation on ice at a dose rate of 7 Gy min $^{-1}$ (cobalt-60, Gamma cell 220). For UV irradiation, the cells were plated on respective agar plates at different dilutions, air-dried and then exposed to different doses of UV radiation at 254 nm, at a dose rate of 0.295 J s $^{-1}$ m $^{-2}$, in the dark. Hydrogen peroxide treatment was applied to *E. coli* using a modified protocol as

described earlier (Arrage *et al.*, 1993). In brief, *E. coli* cells were treated with different concentrations of hydrogen peroxide (0–5 mM) for 30 min with vigorous aeration in the dark. Mitomycin C (MMC) treatment was given as described earlier (Keller *et al.*, 2001). Treated cells were plated in triplicate and c.f.u. were scored after 20 h incubation at 37 °C. The effect of these agents on the cell survival of *D. radiodurans* and its derivatives was studied as described earlier (Misra *et al.*, 2006).

Purification of recombinant PolX and immunoblotting.

Transgenic *E. coli* BL21(DE3) pLysS cells harbouring pETpolX were induced with 100 µM IPTG and the cells expressing recombinant protein were used for protein purification by nickel-affinity chromatography using the manufacturer's protocols (Qiagen). In brief, the clear supernatant was mixed with 500 µl 50 % Ni-NTA agarose slurry, and hexahistidine-tagged recombinant protein was eluted from the matrix with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions were analysed on SDS-PAGE and the protein sample was dialysed in buffer containing 1 mM DTT. Western blotting using antibodies against PolX and His₆ was carried out as described earlier (Sambrook & Russell, 2001). Protein concentration was determined using Bradford's dye-binding method.

Nucleotide incorporation and polymerase activity characterization.

The cross-linking of template/primer (T/P) with enzyme and deoxynucleotidyltransferase activity assay of DNA polymerase was monitored on a UV cross-linked T/P–enzyme complex as described earlier (Misra *et al.*, 1998). In brief, the 19mer oligonucleotide (NPK5) was mixed with 27mer complementary template (NPK6) in a reaction mixture containing 20 mM Tris/HCl, pH 7.5, 1 mM MgCl₂ and 1 mM EDTA. The mixture was heated at 95 °C for 5 min and allowed to slowly cool to room temperature. The 50 nM T/P was mixed with 500 nM enzyme in a reaction mixture containing 50 mM Tris/HCl, pH 7.5, 1 mM DTT, 2 mM MgCl₂ and 5 % (v/v) glycerol in a final volume of 50 µl and cross-linked with UV light (320 nm) at 300 MJ cm⁻². The enzyme–T/P complex was assayed for its deoxynucleotidyltransferase activity and the incorporation of [α -³²P]dCTP at the 3' end of the primer was monitored by 8 % SDS-PAGE followed by autoradiography.

The primer extension activity of DNA polymerase was measured as described earlier (Misra *et al.* 1998). In brief, the 50 nM enzyme was incubated with T/P prepared by annealing the 33mer template (NPK7) with 17mer (NPK8) primer labelled at the 5' end in a reaction mixture containing 50 mM Tris/HCl, pH 7.5, 100 µM of each dNTP and 2 mM DTT. The reaction was initiated with 5 mM MgCl₂, and 100-fold excess molar concentration of unlabelled T/P was added. MgCl₂ was substituted with 2 mM MnCl₂ in reactions monitoring the effect of manganese ions on enzyme activity. The reaction mixture was incubated for 30 min at 37 °C and products were analysed on a 16 % urea-PAGE gel. The signals were detected by autoradiography.

5'-dRP lyase and BER activity assay. Preparation of the deoxyribose phosphate (dRP) lyase substrate and 5'-dRP lyase activity assay of recombinant enzyme were carried out essentially as described by Prasad *et al.* (2003). In brief, a 34mer oligonucleotide (NPK9) having dU at the 16th position was labelled with ³²P at the 3' end with terminal deoxynucleotidyltransferase and annealed with 34mer complementary oligonucleotide (NPK10). The dsDNA substrate was treated with uracil DNA glycosylase (UDG) in a standard reaction buffer (New England Biolabs). The 100 nM UDG-treated substrate was incubated with AP endonuclease (APE1, New England Biolabs) and subsequently with 10 nM recombinant PolX at 37 °C for 30 min in a reaction mixture containing 50 mM HEPES, pH 7.5, 20 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA and 2 mM DTT. The reaction was terminated with DNA loading dye (95 % formamide,

25 mM EDTA, 0.05 % bromophenol blue and 0.05 % xylene cyanol); products were analysed on 8 M urea-10 % PAGE gel and signals were detected by autoradiography.

The BER activity of PolX was assayed using a modification of the protocol described by Kubota *et al.* (1996). In brief, a 30mer oligonucleotide (NPK11) with internal modification at the 15th position was labelled at the 3' position with ³²P using terminal deoxynucleotidyltransferase and annealed with the complementary oligonucleotide (NPK12) having a normal cytosine complementary to the modified base. The reaction was carried out with 5 units Fpg DNA glycosylase (New England Biolabs) and 1 unit AP endonuclease I (New England Biolabs) in different combinations with 50 nM polymerase and 66 cohesive units of ligase, *in vitro*. The reaction was carried out in 20 µl reaction mixture containing dsDNA oligonucleotide labelled at the 5' end of the modified oligonucleotide only and 100 µM dNTPs as required, under standard assay conditions as described earlier (Kubota *et al.*, 1996).

RESULTS

The *polX* disruption mutant expresses truncated polymerase polypeptide

Two different types of *polX* mutants, named *polXnok1* and *polXnok2*, were generated in *D. radiodurans*. In mutant *polXnok1*, the *polX* gene was interrupted by the insertion of a *cat* cassette (Fig. 1). PCR amplification of *D. radiodurans* clones selected on chloramphenicol as selection marker showed a ~850 bp fragment with *cat*-specific primers (Fig. 1a) and a ~2.7 kb DNA fragment that includes *polX* along with *cat*, in the *polX* disruption mutant (Fig. 1b). These cells did not amplify the native *polX* gene (1.7 kb) and showed expression of a C-terminal truncated polypeptide of 24 kDa (Fig. 1d). The *cat* insertion mutant was expected to express the complete 5'-dRP lyase domain and the majority of the polymerase domain before the reading frame is interrupted at amino acid position 184 in the *polX* gene (Supplementary Fig. S3). However, the 24 kDa chimeric protein comprises a total of 220 amino acids, including 184 N-terminal amino acids of PolX and 36 amino acids of the translation readthrough in the *cat* cassette sequence before the stop codon (Fig. 1d). Mutant *polXnok2* showed the complete replacement of the *polX* gene with the *nptII* cassette (Fig. 1c). The homozygous *polXnok2* mutant cells showed a complete absence of any protein cross-reacting with PolX antibodies, whereas *polXnok1* cells expressed a 24 kDa protein cross-reacting with antibodies and wild-type cells expressed a polypeptide similar to purified proteins and cross-reacting with PolX antibodies (Fig. 1d). This confirmed that both mutants were homozygous for *polX* mutation and showed different expression of the polymerase domain. These cells were subsequently checked for their DNA-damage responses.

The *polX* mutants show different responses to oxidative stress and γ -radiation tolerance

The two *polX* mutants were tested for their survival in response to γ -radiation, MMC and hydrogen peroxide

Fig. 1. Generation of PolX mutant derivatives of *D. radiodurans*. (a, b) The complete replacement of the native *polX* gene with a disrupted copy of *polX* (*polX::cat*) in the *polXnok1* (*nok1*) mutant was ascertained by PCR amplification of the *cat* (a) and *polX* (b) genes from the genomic DNA from wild-type (WT), mutant cells (Mutant) and pPolX::cat (Con) plasmid. (c) Similarly, PCR amplification of the *polX* gene was checked in genomic DNA of putative *polXnok2* (*nok2*) clones (1, 2, 3) and the *D. radiodurans* (D) genome. The sizes of the PCR products, as indicated with arrows, were obtained by comparison with the size markers (M). Clone no. 1 was taken for subsequent studies. (d) Total proteins from wild-type (WT), *polXnok1* (*nok1*), *polXnok2* (*nok2*) mutant and purified PolX (Con) were separated on SDS-PAGE and blotted with antibodies against PolX.

treatment. The *polXnok1* cells showed a nearly sixfold decrease in survival relative to wild-type cells after γ -irradiation at 10 kGy (Fig. 2a), and an almost 1.5 log cycle decrease in tolerance of hydrogen peroxide at 20 mM (Fig. 2b). Interestingly, the *polXnok2* mutant was more sensitive to γ -radiation than the *polXnok1* mutant, showing an almost 2 log cycle decrease in γ -radiation survival at 10 kGy. The *polXnok2* cells showed a similar response to hydrogen peroxide to that of *polXnok1* cells. The slightly higher rate of *polXnok2* cell survival than *polXnok1* in response to hydrogen peroxide might suggest the

possibility of an error-prone repair by truncated polymerase enzyme (PHP minus polymerase domain) in *polXnok1* cells. The two mutants differed in their survival after treatment with MMC (Fig. 2d) and UVC (Fig. 2c): unlike *polXnok1*, the *polXnok2* cells showed sensitivity to higher doses of UV radiation and to longer exposure with MMC ($20 \mu\text{g ml}^{-1}$) as compared to wild-type. The differential effect of *polX* mutation on γ -radiation response and relatively higher sensitivity to hydrogen peroxide suggest that PolX has a greater role in oxidative stress tolerance of *D. radiodurans*. The higher sensitivity of *polXnok2* cells to γ -radiation than *polXnok1* cells indicated the enigmatic role of the PHP domain in PolX activity regulation.

Deinococcal PolX shows typical short patch DNA repair characteristics

The *polX* gene (DR0467) of *D. radiodurans* was cloned in pET28a+ to yield pETpolX and recombinant protein was expressed under the T7 inducible promoter in *E. coli* BL21 (Supplementary Fig. S4). Recombinant PolX was purified from transgenic *E. coli* to near homogeneity (Fig. 3a) and was checked for its nucleotide incorporation specificity and primer extension activity. The enzyme-T/P complex was cross-linked by UV irradiation and incubated with dNTP mixture containing [α - ^{32}P]dCTP as one of the deoxynucleotides. Since the enzyme was cross-linked with DNA, its intermolecular dynamics would be restricted and the incorporation of [α - ^{32}P]dCTP, a complementary base to G in the template, would be possible only if the enzyme has bound DNA in polymerase mode. The results showed that PolX binds T/P in polymerase mode and could add [α - ^{32}P]dCTP at the primer terminus (Fig. 3b) in the enzyme-T/P cross-linked complex. SDS-PAGE analysis followed by autoradiography showed a major band of about 84 kDa (enzyme-T/P) along with two smaller-sized products of approximately 27 kDa and 40 kDa. The nature of the smaller-sized products is not clear. However, the possibility of recombinant PolX undergoing proteolytic cleavage into the polymerase domain cannot be ruled out. Antibodies generated against the complete PolX molecule reacted with an additional smaller-sized protein of *D. radiodurans* (Fig. 1d). *E. coli* DNA polymerase I undergoes *in vivo* cleavage into different smaller-sized products exhibiting deoxynucleotidyltransferase activity in gel (Yoshida & Cavalieri, 1971). Our results confirmed earlier reports that the recombinant PolX of *D. radiodurans* is an active enzyme, which binds the double-stranded DNA substrate in its polymerase mode. The primer extension ability of PolX was tested using 5'-radiolabelled primer in T/P and incubation with unlabelled dNTPs. PolX showed addition of only two nucleotides in a single binding event at the primer end while *E. coli*'s Klenow fragment showed full-length primer extension (Fig. 3c). It was previously shown that a putative *D. radiodurans* *polX* gene encoded an active DNA polymerase enzyme with hairpin nuclease activity (Lecointe *et al.*, 2004; Blasius *et al.*, 2006). While

Fig. 2. DNA-damage response of *polX* mutants of *D. radiodurans*. The wild-type (■), *polXnok1* (●) and *polXnok2* (▲) mutant cells were treated with γ -radiation (a), hydrogen peroxide (b), UV irradiation (254 nm) (c) and different periods with MMC (20 $\mu\text{g ml}^{-1}$) (d), and cell survival was monitored as described in Methods.

confirming the polymerase activity of the enzyme, the short patch DNA synthesis nature of this polymerase was also evident from our study.

Deinococcal PolX shows 5'-dRP lyase and BER activity

The short patch DNA polymerase function of PolX was correlated with its role in BER. The 5'-dRP lyase and BER activity of this polymerase was assayed with internally modified double-stranded oligonucleotides using standard protocols. For 5'-dRP lyase activity, the 3'-labelled dU-DNA substrate (dU-S) was treated with UDG and the substrate with the apurinic site was incubated with AP endonuclease with and without recombinant deinococcal PolX. The results showed the release of an oligonucleotide close to 19 nt in length (dRP-S) upon AP endonuclease treatment (Fig. 4). The incubation of PolX along with AP endonuclease resulted in the conversion of the majority of the dRP-S into an 18mer product. Since dRP-S was generated from AP endonuclease action on UDG-treated substrate, it would have a dRP residue from the apurinic site located at its 5' end. The generation of an 18mer product from dRP-S, which could have resulted from the

removal of the dRP residue, suggests the presence of 5'-dRP lyase activity in this enzyme.

For BER activity, the 8-oxoG incorporated oligonucleotide annealed with normal complementary oligonucleotide (Fig. 5a) was incubated with recombinant PolX in different combinations and permutations of recombinant DNA glycosylase, AP endonuclease and DNA ligase. The results showed the addition of a single nucleotide by this polymerase at the lesion produced by combined action of glycosylase and AP endonuclease (Fig. 5). Incubation of modified dsDNA substrate with glycosylase, AP endonuclease and PolX, separately (Fig. 5b, lanes 2–4), did not show the cleavage of labelled nucleotide at the modified base position, i.e. 15. However, incubation of DNA substrate with glycosylase, AP endonuclease and PolX together, without DNA ligase, cleaved the dsDNA substrate at the 15th position and cleavage products of 16 nt were seen (Fig. 5b, lanes 5–9). In samples incubated with PolX, the release of a product of 15 nt, possibly due to 5'-dRP lyase activity of PolX, was also seen. Incubation of these enzymes with DNA ligase gave a full-length product in the presence of dGTP (Fig. 5b, lane 11). Incubation with dTTP (lane 10) and dCTP (lane 13) did not show recovery of full-length DNA product. However, low activity was also

Fig. 3. Activity characterization of recombinant PolX. The purified deinococcal DNA PolX (a) as shown in fraction no. 4 was taken for further enzyme activity assay. (b) The T/P primer was UV cross-linked with polymerase enzyme and incubated either with dCTP, a correct nucleotide, or with dATP for incorporation at the primer terminus. (c) The primer extension assay was carried out either with PolX (P) or with Klenow fragment of *E. coli* (K) in the presence of Mg^{2+}/Mn^{2+} as described in Methods and products were separated by 12% urea-PAGE gel followed by autoradiography.

observed in the presence of dATP alone (Fig. 5b, lane 12), indicating the possibility of this enzyme lacking stringent substrate specificity *in vitro*. The regeneration of full-length oligonucleotides from the glycosylase and AP endonuclease cleaved fragments could be possible only when 5'-dRP lyase activity is enzymically replaced with normal dGTP/dATP. This

Fig. 4. The 5'-dRP lyase activity of deinococcal PolX. The recombinant PolX was assayed for 5'-dRP lyase activity using 3'-labelled [^{32}P]dU-substrate (dU-S, 1) as described in Methods. The UDG-treated dU template (2) was incubated with AP endonuclease (APE) (3) to yield 5'-dRP substrate (dRP-S). The AP-treated dU-S was incubated with PolX (4) and the reaction mixture containing product (P) of the PolX reaction was analysed by 10% urea-PAGE and radiolabelled products detected by autoradiography.

further supports the presence of 5'-dRP lyase activity for this enzyme (Fig. 4) and suggests the role of recombinant PolX in the repair of modified bases *in vivo* by combined action with other BER enzymes such as DNA glycosylase and uncharacterized AP endonuclease.

Deinococcal PolX complements AlkA glycosylase and UvrA deficiencies in *E. coli*

To study the phenotypic effect of PolX in *E. coli* AB1157 and its mutant derivatives, the *polX* coding sequence along with six histidine codons was PCR amplified from pETpolX and subcloned in pRadgro, under the P_{groESL} promoter, to yield pGropolX (Fig. 6a). *E. coli* mutants defective in BER and NER (Supplementary Table S1) were transformed with pGropolX. Since pGropolX is expected to express the recombinant protein with an N-terminal hexahistidine tag, the expression of His₆-PolX in various mutant backgrounds was ascertained by immunoblotting using hexahistidine antibodies and also with anti-PolX serum. Both anti-His antibodies (Fig. 6b) and anti-PolX serum cross-reacted with a similar protein band from *E. coli* cells harbouring pGropolX, while the cells harbouring the expression vector did not show cross-reactivity of any other proteins with both antibodies. Anti-PolX serum cross-reacted with only one protein band from pGropolX-expressing clones (data not shown). This indicated the expression of recombinant PolX in *E. coli* under the *groESL* promoter of *D. radiodurans*. The functioning of the deinococcal *groESL* promoter in *E. coli* was demonstrated earlier (Kota & Misra, 2006).

Fig. 5. BER activity of DNA PolX. (a) The double-stranded oligonucleotide substrate was prepared with the ^{32}P -labelled modified oligonucleotide having 8-oxo-guanidine (*) at the 15th position and annealed with complementary oligonucleotides. (b) The labelled substrate (1) was mixed with Fpg glycosylase (2), AP endonuclease (3) and polymerase (4) separately, and together without DNA ligase (5–9) and with DNA ligase (10–13) in the presence of dTTP (5, 10), dCTP (6, 13), dGTP (8, 11), dATP (7, 12) and all four dNTPs (9) under standard excision repair assay conditions as described in Methods. The products were analysed by 16% urea-PAGE followed by autoradiography.

The effect of transgenic PolX on the DNA-damage response of these *E. coli* mutants was checked. The results showed a 2 log cycle improvement in UVC tolerance of the *alkA* (Fig. 7a) and *uvrA* (Fig. 7b) mutants, although this did not equal the basal level of wild-type tolerance. The *alkA* and *uvrA* mutants did not show significant improvement in their resistance to hydrogen peroxide, MMC and γ -radiation exposure (data not shown). Surprisingly, the *tagA* mutant expressing PolX showed contrasting responses to UV and MMC effects (Figs 7 and 8). On the one hand, these cells showed higher sensitivity to UVC radiation, which was suppressed by the additional mutation in the *alkA* gene (Fig. 7a). On the other hand, they showed nearly fourfold improvement in MMC tolerance as compared to *tagA* mutant control at a concentration of $1\ \mu\text{g ml}^{-1}$ (Fig. 8). AB1157 expressing PolX showed improved tolerance to MMC as compared to the control (Fig. 8). MMC is a bifunctional agent that causes alkylation of bases and cross-linking on DNA, which can be repaired by both excision repair and double-strand break repair mechanisms. The improved MMC tolerance of wild-type cells expressing PolX suggested the role of this polymerase in these DNA repair processes.

Fig. 6. Cloning of *polX* in *E. coli* expression vector and confirmation of recombinant protein expression by immunoblotting. *polX* along with six histidine codons was cloned in pRadgro (a), and transformed into *E. coli tagA* (Tg), *alkA* (Ak), *tagAalkA* (TgAk), *uvrA* (Uv) mutants and the parental strain AB1157 (AB). The expression of recombinant protein (PolX) in transgenic cells (C) and its absence in vector controls (V) was confirmed by immunoblotting with anti-histidine serum (b).

DISCUSSION

This paper reports (i) the *in vivo* role of an X-family DNA polymerase in oxidative stress and γ -radiation tolerance of *D. radiodurans* and (ii) the short patch DNA synthesis and BER activity of recombinant PolX, *in vitro*. The different γ -radiation responses of the *polX* mutant lacking the complete polymerase molecule and the mutant lacking only the PHP domain suggest a role for the N-terminal polymerase in γ -radiation tolerance and DNA repair. Hydrogen peroxide affected both categories of *polX* mutants more than wild-type cells, supporting the role of this polymerase in oxidative stress tolerance. Earlier studies have reported a similar effect of *polX* deletion mutation on the γ -radiation response of *D. radiodurans* (Lecointe *et al.*, 2004).

PolX shows amino acid sequence similarities with eukaryotic Pol β (Aravind & Koonin, 1999), which has been shown to be a BER and DNA lesion bypass repair enzyme (Kubota *et al.*, 1996; Servant *et al.*, 2002; Dianova *et al.*, 2004). Unlike replicative DNA polymerases such as DNA Pol α , DNA Pol γ and DNA PolIII, which can polymerize long-stretch DNA (Holmes *et al.*, 1990; Lahue *et al.*, 1989), the DNA repair Pol β catalyses the short patch BER function by incorporation of one or two nucleotides on damaged sites similar to very short patch repair in bacteria

Fig. 7. Effect of PolX expression on the UV response of excision repair mutants. (a) *E. coli* mutants *tagA* (■, □), *alkA* (●, ○), *tagAalkA* (▲, △) and (b) *uvrA* (▲, △) and parental strain AB1157 (■, □) transformed with pGropolX (open symbols) were treated with UV radiation (254 nm) and the effect on cell survival was compared with untransformed controls (filled symbols) as described in Methods.

Fig. 8. Effect of MMC on cell survival of *E. coli* expressing PolX. *E. coli* *tagA* and *alkA* mutants and the parental strain AB1157 expressing PolX (*tagA* PolX, *alkA* PolX and 1157 PolX, respectively) were treated with MMC at 1 µg ml⁻¹ (light grey bars) or 2 µg ml⁻¹ (dark grey bars) for 30 min and cell survival was compared with untreated controls (white bars).

(Wiebauer & Jiricny, 1990; Lieb, 1987). Our results from primer extension activity assay (Fig. 3c), 5'-dRP lyase assay (Fig. 4) and BER assay (Fig. 5) strongly support the possibility of PolX acting as a short patch DNA repair polymerase similar to eukaryotic Pol β , and it may not necessarily be a processive and replicative enzyme. However, the role of a processive DNA polymerase A of *D. radiodurans* R1 in lesion bypass activity has also been reported recently (Heinz & Marx, 2007). The recently published 3D structure of PolX solved at 2.46 Å resolution indicated an unusual stretched-out conformation instead of the usual closed right-hand conformation as normally observed with other DNA polymerases (Leulliot *et al.*, 2009). The functional complementation of PolX to the UVC response of *uvrA* and *alkA* mutants and wild-type *E. coli* cells (Fig. 7) suggests the contribution of this polymerase in UV repair of *E. coli*. Since the *polX* deletion mutant of *D. radiodurans* showed marginal sensitivity to UVC at higher doses and greater improvement in UVC response of *E. coli*, the role of this polymerase in UV tolerance seems to require the otherwise functional SOS response in the host cell. The higher sensitivity of the *tagA* mutant expressing PolX was suppressed by *alkA* mutation in *alkAtagA* double mutants. Similarly, the *alkAtagA* double mutant showed higher tolerance to UVC as compared to single mutations (Fig. 7a). It has been suggested that the accumulation of 3-MeAde and 7-MeGua on the genome of *alkAtagA* double mutants of *E. coli*, respectively, induces the SOS response (Grzesiuk *et al.*, 2001). Therefore, the possibility of PolX contributing to excision/SOS repair of UV-modified bases in *E. coli* could be speculated. Functional complementation of eukaryotic Y-family DNA polymerase in DNA repair of *E. coli* lacking non-replicative Y-family polymerases such as UmuDC and DinB has been demonstrated (Yamada *et al.*, 2006).

Also, it is noteworthy that *polX* mutants differing in the expression of the N-terminal domain of PolX showed different responses to γ -radiation and MMC. This indicates, although indirectly, the lesser importance of the PHP domain in the N-terminal domain of this DNA polymerase. Recently, the crystal structure of this polymerase was resolved at 2.46 Å, which also clearly suggested that the nuclease active site of the PHP domain is obstructed by the polymerase domain (Leulliot *et al.*, 2009). On the contrary, this enzyme showed the short patch DNA synthesis and BER function in conjunction with other BER proteins *in vitro*, as observed in this study, and others have also reported the endonuclease activity of this enzyme (Lecointe *et al.*, 2004), *in vitro*. These findings invite speculation about a novel mechanism of PHP and polymerase domain interactions during catalytic reactions both *in vitro* and *in vivo* and would be worth investigating independently. An orthologue of the PolX reported from *Bacillus subtilis* shows the identical arrangement of PHP and polymerase domains (Baños *et al.*, 2008). The action of the *B. subtilis* enzyme on DNA gaps with a downstream 5'-phosphate group, a prerequisite reaction for BER, has been

demonstrated. Thus the 5'-dRP lyase activity observed with the *D. radiodurans* PolX (Fig. 4) and earlier findings on the *B. subtilis* enzyme suggest that polymerases having an N-terminal polymerase domain similar to Pol β and a PHP domain similar to the *B. subtilis* enzyme would come under the category of BER polymerase. Taken together, the results presented here support the close functional similarities of PolX with Pol β in short patch BER *in vitro*, and the involvement of this enzyme in bacterial oxidative stress tolerance and UV resistance, possibly by a short patch excision repair mechanism.

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