Characterization of an ATP-regulated DNA-processing enzyme and thermotolerant phosphoesterase in the radioresistant bacterium *Deinococcus radiodurans*

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A multiprotein DNA-processing complex identified from *Deinococcus radiodurans* exhibits uncharacterized ATP-sensitive nuclease functions. DR0505 was one of the 24 polypeptides identified from the complex. It contains two 5′ nucleotidase motifs, one is at the C-terminal end of the N-terminal CPDD (calcineurin phosphodiesterase domain), with the second at the C-terminal end of the protein. Recombinant DR0505 showed both phosphomonoesterase and phosphodiesterase activities with chromogenic substrates, showing higher affinity for bis-(p-nitrophenyl) phosphate than for p-nitrophenyl phosphate. The enzyme exhibited pH optima ranging from 8.0 to 9.0 and metal-ion-dependent thermotolerance of esterase functions. Both mono- and di-esterase activities were stable at temperatures up to 50°C in the presence of Mg²⁺, whereas monooesterase activity was observed at temperatures up to 80°C in the presence of Mn²⁺ and up to 50°C with Ca²⁺. The purified enzyme showed 5′ nucleotidase activity on a wide range of natural mononucleotides including cyclic mononucleotides and 8-oxo-GMP. DR0505 showed a nearly 7-fold higher activity on ADP than AMP, but this activity was inhibited with ATP. Interestingly, DR0505 also showed single-stranded endonuclease and 3′ → 5′ exonuclease activities on both single-stranded and double-stranded DNA-substrates. Unlike for the exonuclease activity, the single-stranded endonuclease activities observed on stem-loop substrates and at the single strand–double-strand junction in forked-hairpin substrates were not inhibited with ATP. These results suggested that DR0505 is an ATP-regulated DNA-processing enzyme and a thermotolerant esterase *in vitro*. We therefore suggest possible roles of this enzyme in nucleotide recycling and DNA processing, which is required for efficient double-strand break repair and the high radiation tolerance observed in *D. radiodurans*.

Key words: 5′-nucleotidase, *Deinococcus radiodurans*, DNA-end-processing, DR0505, dual-function enzyme, thermotolerant esterase.

INTRODUCTION

The nucleoside analogues used in medicine as drugs, are phosphorylated by nucleotide kinases before they undergo metabolic degradation by the combined action of deaminases and nucleotidase family proteins [1]. Two types of nucleotidases, 5′ nucleotidases (EC 3.1.3.5) and 3′ nucleotidases (EC 3.1.3.6), have been reported from different organisms. The 5′ nucleotidases are the main enzymes involved in regulating intracellular deoxynucleotide pools [2–4]. Several 5′ nucleotidases, differing in their subcellular localization, protein sequence and substrate affinities, have been identified from both mammalian and bacterial systems. The roles of these enzymes have been greatly emphasized in nucleoside drug metabolism in eukaryotes and in purine metabolism in bacteria [5]. The pathogenic bacteria use this process as a defence mechanism to escape from the human immune system [6]. Bacteria exposed to abiotic stresses, such as radiation, desiccation and oxidants, suffer a wide range of base changes in their genome. The proficient excision repair mechanisms involve the excision of damaged bases followed by re-synthesis of new DNA strands. The excised nucleotides are released into the cellular pool of nucleotides and recycled by indigenous mechanisms. Recycling of chemically altered nucleotides is required to avoid their re-incorporation during DNA synthesis and thus prevent mutagenesis. The spontaneous mutation rates differ in different bacteria conferring different levels of abiotic stress tolerance [7,8]. Mutation rates are significantly lower in bacteria exhibiting extraordinary tolerance to oxidative stress produced by ionizing radiation, desiccation and chemical oxidants.

*Deinococcus radiodurans* strain R1 has an extraordinary tolerance to various abiotic stresses, including radiation, desiccation and chemical oxidants [9,10]. Its extreme phenotype is attributed to an efficient DNA DSB (double-strand break) repair [11,12] and strong oxidative stress tolerance mechanisms. This bacterium contains all the components of base excision repair, like a DNA repair polymerase [13], AP (apurinic/apyrimidinic)-endonuclease-type activity [14] and various types of DNA glycosylases [15]. In addition, the bacterium also encodes enzymes responsible for both the pathways of nucleotide excision repair [16,17] and mismatch repair [18,19]. Through these excision repair pathways both damaged and normal nucleotides are released into the cellular pool of nucleotides. Similarly, *D. radiodurans*, exposed to high doses of γ-radiation, showed a burst of *in vivo* DNA degradation [20,21] before DNA synthesis is resumed. These processes would eventually generate a large pool of natural, as well as damaged, deoxynucleotides in the cell. Therefore it has been suggested that there are mechanisms that help to recycle these mononucleotides and ‘clean’ the radiation-damaged, potentially mutagenic, nucleotide species from dividing cells in *D. radiodurans* [10]. The genome of this bacterium encodes a large number of NUDIX (for nucleoside

Abbreviations used: AP, apurinic/apyrimidinic; BNPP, bis-(p-nitrophenyl) phosphate; ds, double-stranded; DIG, digoxigenin; DSB, double-strand break; DTT, dithiothreitol; Ni-NTA, Ni²⁺-nitritotriacetaete; NUDIX, nucleoside diphosphate linked to some other moiety < ORF, open reading frame; PDE, phosphodiesterase; PME, phosphomonoesterase; PNPP, p-nitrophenyl phosphate; ss, single-stranded.

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diphosphate linked to some other moiety x family enzymes and uncharacterized nucleotide metabolic enzymes and hence the roles of these proteins in recycling and detoxification of mononucleotides are worth investigating.

In the present study we functionally characterized DR0505, a 5′ nucleosidase family protein, from D. radiodurans. The recombinant DR0505 showed both PME (phosphomonoesterase) and PDE (phosphodiesterase) activities and DNA-processing activities in vitro. The PME activity of the enzyme was resistant to increasing temperature, up to 50°C with Mg2+ and 80°C with Mn2+, with PDE function resistant up to 50°C with both of these metal ions. The enzyme dephosphorylated normal nucleotides, 8-oxo-GMP and 2′,3′-cyclic nucleotides at different rates. The enzyme showed ATP-sensitive 3′ → 5′ exonuclease activities on both ss (single-stranded) DNA and ds (double-stranded) DNA substrates; however, the ssDNA endonuclease activities on stem-loop and forked-hairpin substrates were not inhibited with ATP. These results suggest that DR0505 is a bifunctional enzyme exhibiting temperature-resistant esterase functions and ATP-regulated DNA-processing activities that are possibly required for recycling of nucleotides and DSB repair during the post-irradiation recovery of D. radiodurans.

MATERIALS AND METHODS

Bacterial strains and materials

The D. radiodurans strain ATCC13939 was a gift from Dr M. Schaefer (Division of Epigenetics, Deutsches Krebsforschungszentrum, Heidelberg, Germany) [22]. Wild-type bacteria and their derivatives were grown aerobically in TGY (0.5% Bacto tryptone, 0.3% Bacto yeast extract and 0.1% glucose) broth or on agar plates as required at 32°C. Escherichia coli expression vector pET28a+ and its derivatives were maintained in E. coli strain HB101 in the presence of 25 μg/ml kanamycin. Other recombinant techniques used were as described in [23]. All the molecular biology grade chemicals including restriction enzymes and DNA-modifying enzymes were purchased from Sigma–Aldrich, Roche Biochemicals, New England Biolabs or Bangalore Genei.

Construction of DR0505 expression plasmid

Genomic DNA of D. radiodurans R1 was prepared as described previously [24]. The 1.7 kbp coding sequence of DR0505 was PCR-amplified from the genomic DNA of D. radiodurans using the gene-specific forward primer (5′-GGAAATTCATGAAAGAAAACCTGTGCT-3′) and reverse primer (5′-CAGGATCTTACCACTTGAGCTATGGAACAAAGAAAACCTGTGCT-3′). The PCR product was cloned between the Ndel and EcoRI sites in pET28a+. Restriction analysis and nucleotide sequencing ascertained the identity and correctness of the gene cloned in expression vector. The recombinant plasmid pET0505 was transformed into E. coli BL21 (DE3) pLysS for expression of recombinant proteins.

Expression and purification of recombinant protein

The recombinant DR0505 was purified from transgenic E. coli BL21 (DE3) pLysS, harbouring pET0505, by Ni-NTA (Ni2+-nitrilotriacetate) affinity chromatography as chromographed previously [25]. In brief, the IPTG (isopropyl β-D-thiogalactopyranoside)-induced cell pellet was dissolved in lysis buffer containing 20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA and 1 mM PMSF, and was incubated with 200 μg/ml lysozyme for 30 min. The mixture was treated with 0.25% Triton X-100 for 30 min at 37°C and sonicated using a 4 μm tip at 40% duty cycle by giving 10 s pulses with intermittent cooling on ice for 2 min. The majority of the recombinant protein was separated from clear supernatant by centrifugation at 12000 g for 30 min. The pellet was dissolved in buffer B (10 mM Tris/HCl, pH 8.0, 100 mM NaH2PO4, and 8 M urea). The mixture was incubated at room temperature (25°C) under shaking for 1 h and centrifuged at 12000 g for 20 min. The Ni-NTA matrix (Qiagen) was directly added to this supernatant and the protein was allowed to bind to the resin under gentle shaking conditions for 2 h. The resin, with protein bound, was passed through a 3 kDa cut-off filter column and washed thoroughly with buffer C (buffer B at pH 6.3). The proteins were eluted with buffer D (buffer B at pH 5.9) and subsequently with buffer E (buffer B at pH 4.5) and analysed via SDS/PAGE. The fractions eluted with buffer E, containing nearly 99% pure protein, were pooled and dialysed sequentially in a buffer containing 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM DTT (dithiothreitol) and 1 mM EDTA with decreasing concentrations of urea (from 8 M to zero). Finally the pooled fractions were dialysed in a buffer containing 20 mM Tris/HCl, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM PMSF and 50% (v/v) glycerol. The protein was stored in small aliquots at −20°C for subsequent use. The MS analysis of purified protein and peptide mass fingerprints generated from MALDI (matrix-assisted laser-desorption ionization) MS confirmed both the purity and identity of the recombinant protein as a DR0505.

Measurements of enzyme activity

The PME activity was measured with the disodium salt of PNPP (p-nitrophenyl phosphate), using a standard method as described previously [26]. In brief, the activity assay was carried out at 37°C in a 0.5 ml reaction volume containing 500 ng of enzyme in 50 mM Tris/HCl, pH 8.0, 5 mM of MnCl2, MgCl2, CaCl2, and 10 mM PNPP. After an 20 min incubation the reaction was stopped with 6 M NaOH and the concentration of p-nitrophenol was determined from the absorbance at 410 nm against substrate blank. Enzyme activity was expressed as nmol or μmol of p-nitrophenol formed/min per mg of protein. Similarly, the total PDE activity was assayed with 500 ng of purified protein in a 0.5 ml reaction volume containing 500 ng of enzyme in 50 mM Tris/HCl, pH 8.0, 5 mM of MnCl2, MgCl2, CaCl2, and 1 mM PNPP [bis-(p-nitrophenyl) phosphate] and incubated at 37°C for 20 min. The reaction was stopped with 3 M NaOH and release of p-nitrophenol was measured at 410 nm as described previously [27]. Subsequently, all enzyme assays were carried out in the presence of 5 mM MnCl2, unless mentioned.

The pH optima of enzyme were determined as described previously [28]. In brief, the activities at different pH were assayed in different buffers: 50 mM sodium citrate buffer for the pH range from 3 to 6; 50 mM Tris/HCl for the pH range from 7 to 9; and 100 mM carbonate/bicarbonate for the pH range from pH 10 to 11. For temperature optima determination, the esterase activities were measured at different temperatures and at pH 8.0 in the presence of 5 mM MnCl2, 5 mM MgCl2 or 5 mM CaCl2. Similarly, to determine the ATP effect and the various DNA-processing activities, 500 ng of protein was pre-incubated with different concentrations of ATP in a 0.5 ml reaction volume containing 50 mM Tris/HCl, pH 8.0, and 5 mM MnCl2, for 20 min at room temperature, followed by an esterase assay with 10 mM PNPP and different types of DNA substrates.
The nucleotidase activity on natural nucleotide substrates was assayed as described previously [28]. In brief, a 0.1 ml reaction mixture, containing 50 mM Tris/HCl, pH 8.0, 5 mM MnCl₂, 0.2 mM 8-oxo-dGMP (or 0.5 mM for other nucleotides) and 0.2 μg of protein sample, was incubated at 37°C for 20 min. The reaction was stopped by the addition of a 1/4 volume of Malachite Green reagent. The mixture was incubated further for 20 min and then levels of P₈ were measured at 630 nm. Similarly, the PDE activity with 2',3'-cyclic nucleotide monophosphates was measured essentially as described in [29]. The reaction was performed in 80 μl reaction volumes containing 50 mM Tris/HCl, pH 8.0, 5 mM MnCl₂, 0.5 mM 2',3'-cAMP or 2',3'-cGMP and 0.2 μg of protein and incubated for 40 min at 37°C. The reaction was stopped by the addition of Malachite Green reagent and the amount of Pi released was measured after a 20 min incubation at 630 nm. For the Kₘ and Vₘₐₓ determination, the enzyme assay was performed with 500 ng of purified protein with different concentrations of substrates (1–10 μM for PNPP; 0.1–10 μM for BNPP; 10–200 nM for AMP; and 2–100 nM for cAMP) and the P₈ released was measured with Malachite Green as described above.

**DNA-binding and nuclease activity assay**

The DNA-binding assay was performed in 50 μl reaction mixture volumes containing 50 mM Tris/HCl, pH 8.0, 5 mM MnCl₂, 75 mM NaCl, 0.1 mM DTT, 200 ng of dsDNA substrates and 200 ng of protein at 37°C for 5 min. Products were analysed on a 0.8% agarose gel. The nuclease activity was checked on dsDNA and circular ssDNA (M13mp18) substrates. The 200-bp PCR-amplified linear dsDNA was labelled at the 3' ends with DIG (digoxigenin)–dUTP using Klenow enzyme and DIG-labelling kit protocols (Roche Biochemicals). An approx. 50 ng equivalent of DIG–dUTP linear dsDNA and 500 ng of unlabelled circular M13mp18 ssDNA were incubated with 500 ng of purified protein in a reaction mixture containing 50 mM Tris/HCl, pH 8.0, 75 mM NaCl, 0.1 mM DTT and 5 mM MnCl₂ (or other bivalent metal ions) at 37°C for a defined time period. Products from the DIG–dUTP-labelled dsDNA substrate were separated by native PAGE (5% gels), transferred on to nylon membranes (Hybond; GE Healthcare), blotted with an anti-DIG–AP antibody (Roche Molecular Biochemicals) and chemiluminescent signals were detected as described in the manufacturer’s protocol. For the temperature effect on DNA-binding activity and the effect of ATP on nuclease activity, 200 ng of protein was pre-incubated in reaction buffer containing 50 mM Tris/HCl, pH 8.0, 5 mM MnCl₂, 75 mM NaCl and 0.1 mM DTT for 20 min at different temperatures and at different ATP concentrations respectively. Treated samples were mixed with DNA substrates and incubated at 37°C for 5 min, in the case of the DNA-binding assay, and for different time periods for the nuclease activity assay. The mixture was heated at 65°C with 95% formamide containing 25 mM EDTA to dissociate the nucleoprotein complex as described previously [30]. Products from unlabelled linear dsDNA, circular ssDNA and superhelical plasmid DNA (pBluescript SK+) were analysed on agarose gels and detected by ethidium bromide staining.

**DNA-end-labelling and processing activity assay**

The DNA-processing activity of the protein was assayed on DNA substrates with different topologies and structures of DNA ends. These substrates were labelled at either 3' or 5' ends using standard protocols as described in [23]. In brief, the substrates were labelled at the 3' end with [α-32P]dCTP using Klenow enzyme and at the 5' end with [γ-32P]ATP using polynucleotide kinase. The unincorporated nucleotides were removed using G-25 spin columns (GE Healthcare).

DNA-end-processing activities were assayed as described previously [31,32]. In brief, 160 nM enzyme was mixed with ~10 nM each of the labelled homopolymer of dA₄₀ (40× deoxyadenosine phosphate substrate), HP₂ (hairpin-forked substrate, 5'-AAAAAGACCTGGCACGTAGGACAGCAGCTGCTGTCCTACGTGCCAGGTCAAAAAA-3') [33] or HP78 (stem-loop substrate, 5'-GTTTCTATTCAGCCCCCTTGACGTAATCCAGCCCGGGGCTGATTACGTCAAAGGCTGAATAGAAAC-3') [34]. The reaction mixture was incubated at 37°C for 1 h with HP78, for 2 h with HP2 and for various times with the dA₄₀ substrate. The ss products of HP2, HP78 and dA₄₀ were separated by urea/PAGE (8 M urea/12% acrylamide gels). Radiolabelled products were detected by autoradiography.

**Statistics**

All the experiments were repeated at least three times. Results presented without an S.D. are from a representative experiment.

**RESULTS AND DISCUSSION**

A multiprotein DNA-processing complex isolated from *D. radiodurans* R1 has been shown previously to be an ATP-sensitive exonuclease and to have an ATP-stimulated DNA-end-joining activity, along with bivalent ion and ATP-independent topoisomerase functions [35], DR0690 (topoisomerase 1B type), DRB0100 (an ATP-type DNA ligase) and a few uncharacterized ORFs (open reading frames) including DR0505 (a putative 5' nucleotidase family enzyme) were among the 24 polypeptides identified from the multiprotein complex. Hence a further study on the uncharacterized ORFs was performed to identify the ATP-sensitive nuclelease functions of the complex.

**DR0505 showed structural similarities with 5' nucleotidase enzyme of *Thermus* species**

All of the uncharacterized proteins detected in the complex were checked for the presence of putative functional domains in *silico*. Among them, DR0505 was found to have three distinct functional signatures: (i) a leader peptide in the first 27 amino acids at the N-terminus; (ii) a calcineurin-type PDE domain from amino acids 32–297; and (iii) two 5' nucleotidase domains, one from 225–297 and a second from 363–557 towards the C-terminal end of the polypeptide (Supplementary Figure S1 at http://www.BiochemJ.org/bj/431/bj4310149add.htm). Structure prediction using I-TASSER (iterative threading assembly refinement) tools indicated that this protein was likely to form a structure resembling the 5' nucleotidase family protein from *Thermus* species at the most stable energy minimization state (TM score of 0.63 ± 0.13 and root mean square deviation values of 9.2 ± 4.6 Å). (results not shown). This indicated the possibility that DR0505 conferred both nuclease and esterase activities and hence this protein was characterized subsequently.

**Recombinant DR0505 was characterized as a dual esterase**

The recombinant DR0505 protein was purified to near homogeneity with >99% purity (Supplementary Figure S2
at temperatures (50°C for PME) remained nearly the same at higher temperatures (50°C for PME) and 50°C for PDE activity. The activity increased at temperatures up to 80°C in the presence of Mn2+ ions (Figure 2B). The K_m and V_max for both PME and PDE activities were determined at 80°C and 50°C. The K_m and V_max were 3.652 ± 1.137 μM and 20.46 ± 3.215 μmol/min per mg of protein for PME activity, and 0.739 ± 0.213 μM and 69.86 ± 7.512 μmol/min per mg of protein for PDE activity respectively. Although the K_m values measured at higher temperatures (50°C for PDE and 80°C for PME) remained nearly similar to that measured at 37°C, the V_max was nearly 2-fold higher on both the substrates in presence of Mn2+ ions at higher temperatures. This suggested that DR0505 was a mesophilic thermotolerant esterase. Although, the thermotolerant nature of DR0505 in D. radiodurans, a mesophilic bacterium, may indicate a vestigial property of an enzyme that evolved to operate under primordial conditions, it might provide a higher stability in order to perform functions under conditions of high γ-irradiation, when the cellular temperature would increase. Other thermotolerant enzymes isolated from mesophilic bacteria have been reported with similar temperature characteristics, i.e. a wide

**Figure 1 Phosphoesterase activity characterization of DR0505 protein in vitro**

(A) The phosphoesterase activities of purified recombinant DR0505 was assayed on chromogenic substrates PNPP and BNPP in the presence of 5 mM of each MnCl2 (1), CaCl2 (2) or MgCl2 (3).

(B) The PME (with PNPP) and PDE (with BNPP) activities of DR0505 was assayed at different pH in the presence of 5 mM MnCl2. (C) The effect of ATP on PDE activity of DR0505 was measured at 0.5 mM (2), 1.0 mM (3) and 2.0 mM (4) ATP and compared with the control (1) without ATP. All the experiments were repeated at least three times in triplicate and the results are means ± S.D.

The K_m and V_max for PME and PDE activities were measured using double-reciprocal plots (Supplementary Figure S3 at http://www.BiochemJ.org/bj/431/bj4310149add.htm). The enzyme showed a bivalent ion preference for its pH optimum in alkaline conditions (pH 9) at 37°C. Although the ATP inhibition of esterase function was found to be a unique feature of this enzyme, the functional significance of this regulation in the radioresistance of D. radiodurans is unclear. Recently, we showed that in D. radiodurans ATP levels increased within 1 h post-irradiation [36], with the levels subsequently dropping with a concurrent increase in DNA synthesis and reassembly of the shattered genome. The level of expression of DR0505 gene is also highest at 1 h post-irradiation [37], although they remained high for 4 h. These results indicate that although the level of DR0505 was high, ATP might serve to regulate the enzyme’s activity during early in the initial post-irradiation response in D. radiodurans.

**DR0505 protein can be characterized as a thermotolerant mesophilic enzyme**

While determining the temperature optimum for PME activity using PNPP, we observed that DR0505 showed a continuous increase in its activity at temperatures up to 45°C. Hence, both the esterase activities of DR0505 were measured as a function of temperature at pH 8.0. Interestingly, the enzyme showed a bivalent ion preference in temperature tolerance. The PME activity of the enzyme was significant at 37°C, and the activity increases at temperatures up to 80°C at pH 8.0 in the presence of Mn2+ ions (Figure 1C). These results indicated that the DR0505 was a better PDE than PME enzyme in the presence of both Mn2+ and Mg2+ ions (Figure 1C); DR0505 did not show an ADP-related effect on its esterase activity (results not shown). A differential response of nucleotidases to ATP and ADP has been reported from mammalian systems with the distinctions between the ADP and ATP effect on nucleotidase activity primarily depending upon the cellular location of the enzyme in multicellular organisms [5].

The K_m and V_max for PME and PDE activities (Figure 1B) at 37°C. ATP inhibited the esterase function of this enzyme in the presence of both Mg2+ and Mn2+ ions (Figure 1C); DR0505 did not show an ADP-related effect on its esterase activity (results not shown). A differential response of nucleotidases to ATP and ADP has been reported from mammalian systems with the distinctions between the ADP and ATP effect on nucleotidase activity primarily depending upon the cellular location of the enzyme in multicellular organisms [5].

The K_m and V_max for PME and PDE activities were measured using double-reciprocal plots (Supplementary Figure S3 at http://www.BiochemJ.org/bj/431/bj4310149add.htm). The enzyme had a K_m and V_max of 2.52 ± 0.245 μM and 5.558 ± 0.845 μmol/min per mg of protein with PNPP, and 0.423 ± 0.053 μM and 35.037 ± 2.873 μmol/min per mg of protein with BNPP respectively at pH 8.0 and 37°C. These results indicated that the DR0505 was a better PDE than PME with its pH optimum in alkaline conditions (pH 9) at 37°C. Although the ATP inhibition of esterase function was found to be a unique feature of this enzyme, the functional significance of this regulation in the radioresistance of D. radiodurans is unclear. Recently, we showed that in D. radiodurans ATP levels increased within 1 h post-irradiation [36], with the levels subsequently dropping with a concurrent increase in DNA synthesis and reassembly of the shattered genome. The level of expression of DR0505 gene is also highest at 1 h post-irradiation [37], although they remained high for 4 h. These results indicate that although the level of DR0505 was high, ATP might serve to regulate the enzyme’s activity during early in the initial post-irradiation response in D. radiodurans.
Deinococcus thermotolerant esterase with DNA-processing functions

DR0505 was characterized as 5’ nucleotidase in vitro

D. radiodurans has a very high tolerance to γ-irradiation and oxidative stress, which induce different types of DNA base damage that are repaired by efficient excision/incision repair pathways [10,40]. As a result, the levels of both damaged and undamaged nucleotides are bound to increase in the cell. These nucleotides are subsequently recycled in fresh nucleotide biosynthesis and/or are exported by NUDIX family enzymes [41]. This helps the cells by avoiding re-incorporation of chemically altered (mutagenic) bases in DNA during replication. How this process operates in Deinococcus is not understood; however, the mutation rate following irradiation is extremely low in this bacterium [11] and the possibility of an efficient system for recycling of non-toxic nucleotides and detoxification of mutagenic nucleotides cannot be ruled out. Nucleotidases are known for their involvement in nucleotide recycling in living cells. Therefore the 5’ nucleotidase activity of DR0505 was assessed with different nucleotides including 8-oxo-GMP, a biomarker of oxidative damage of DNA and a potent mutagenic agent. The enzyme showed differential activity on different mononucleotides and cyclic-nucleotides (Table 1). The enzyme dephosphorylated dCMP and GdMP ~1.5–2.0-fold faster than CMP and GMP respectively. This activity on cAMP, cGMP and 8-oxo-GMP substrates was ~1.5-fold higher than AMP and GMP respectively. The enzyme showed a nearly 7-fold faster ADP degradation than AMP degradation, indicating the possible activation of this enzyme by ADP (an ADP-mediated activation of nucleotidases has been reported in mammalian systems [5]).

As the level of cAMP changes in response to γ-irradiation and it has been shown to be an important regulator of stress-response enzymes, the affinity of DR0505 toward cAMP was checked and compared with AMP. The $K_\text{m}$ and $V_{\text{max}}$ of this enzyme were determined for AMP and cAMP. For AMP, the $K_\text{m}$ and $V_{\text{max}}$ were 56.948 ± 12.345 nM and 70.375 ± 9.143 nmol/min per mg of protein, for cAMP, these parameters were 7.875 ± 0.0156 nM, and $V_{\text{max}}$ and 497.742 ± 72.021 nmol/min per mg of protein respectively. This indicated the higher affinity of DR0505 enzyme toward cAMP. Recently, we demonstrated the kinetic change in the levels of ATP and cAMP during the post-irradiation response of D. radiodurans [36] and argued that the higher cAMP levels post-irradiation is the net effect of low cyclic PDE and higher adenylate cyclase activities. The cyclic PDE activity starts increasing after 2 h post-irradiation and has reached a maximum in 4 h post-irradiation. The transcriptome analysis also shows γ-irradiation-induced expression of DR0505 1 h post-irradiation and onwards [37]. DR0505, which gets induced during this period, was incidentally found to have cyclic PDE activity indicating the possible role of this enzyme in maintaining cellular levels of cAMP during the post-irradiation response. The identification and characterization of another calcineurin-like phosphoesterase [42] and structure-function studies of a phosphotriesterase homologue [43] have been reported from Deinococcus. These findings indicate that esterases have a significant role in this bacterium and suggest the possible role of DR0505 in recycling of both natural and chemically altered nucleotides in vivo.

DR0505 was characterized as an ATP-sensitive exonuclease

In order to study the significance of the PDE activity of DR0505 in DNA metabolism, the DNA-binding activity of recombinant DR0505 protein was first checked with both circular and linear dsDNA substrates. The enzyme showed DNA-binding activity with both linear dsDNA (Figure 3A) and superhelical plasmid DNA (Figure 3B) at 37°C. However, the pre-incubation of purified DR0505 at higher temperature abolished its interaction with DNA at 37°C (Figure 3). These results suggest that DR0505 is a non-specific DNA-binding protein. Unlike the esterase activities, the DNA–protein interaction function of this protein was destroyed when at higher temperatures.

On longer incubations, the enzyme showed metal ion preference for nucleolytic degradation of both linear dsDNA and circular ssDNA substrates. Both of these activities were inhibited by ATP.
in vitro (Figures 4 and 5). The enzyme showed nuclease activity on circular M13mp18 ssDNA in the presence of both Mg\(^{2+}\) and Mn\(^{2+}\) ions but these activities were higher in the presence of Mn\(^{2+}\) as compared with Mg\(^{2+}\) (Figures 4A and 4B). As the endonucleolytic cleavage of circular DNA is a prerequisite of exonuclease activity, the degradation of the circular ssDNA clearly indicated the presence of endonuclease activity in DR0505. A time course assay of DR0505 on circular ssDNA showed the progressive degradation of ssDNA in the absence of ATP, which was completely inhibited in presence of ATP (Figure 4C).

The nuclease activity on linear dsDNA labelled at 3' termini was highest in the presence of Mn\(^{2+}\) and the majority of the 3' label was rapidly hydrolysed in less than 40 min incubation at 37°C (Figure 5A). This activity was significantly lower in the presence of other bivalent metal ions, such as Mg\(^{2+}\), Ca\(^{2+}\), Zn\(^{2+}\) and Co\(^{2+}\). ATP inhibition was observed with both Mn\(^{2+}\)- (Figure 5) and Mg\(^{2+}\)- (results not shown) dependent nuclease activities. Incubation of the enzyme with 3'-labelled dsDNA for various times at 37°C showed a rapid removal of the 3' label on dsDNA and more than 60% of the total 3' labels were removed in a 10 min incubation (Figures 5B and 5C). These results indicated that DR0505 is an ATP-sensitive Mn\(^{2+}\)-dependent nuclease. Absence of endonucleolytic products on dsDNA substrate and faster removal of label from 3'-labelled dsDNA indicated that DR0505 was possibly a 3' → 5' dsDNA exonuclease. Although the distinction of exo- from endo-nuclease activity could not be made at this stage, the enzyme showed time-dependent hydrolysis of circular ssDNA, which is the characteristic of an exonuclease degradation of DNA. ATP treatment of DR0505 quickly inhibited both ssDNA and dsDNA exonuclease activities. After a longer incubation of ATP-treated DR0505 with circular ssDNA there were detectable levels of smaller size DNA products as obtained from density scanning of agarose gels (Figure 4C and results not shown) suggesting a low level of ssDNA exonuclease activity. This result might argue that DR0505 was a non-specific nuclease, and ATP inhibited its exonuclease activities on both ssDNA and dsDNA in vitro. Further distinction of both the endonuclease and exonuclease activities were next obtained with different structured DNA substrates.

**Figure 3** Effect of temperature on DNA-binding activity of DR0505

The 200 bp (A) linear dsDNA and (B) superhelical (CCC) plasmid DNA pBluescript SK+ substrates were incubated at 37°C for 5 min with recombinant DR0505 pretreated at different temperatures for 20 min. Products were analysed on 0.8 % agarose gels and DNA bands were visualized with ethidium bromide. Products were compared with EcoRI-linearized plasmid DNA (linear) to rule out the possibility that superhelical dsDNA is converted into linear dsDNA by DR0505.

**Figure 4** Nucleolytic activity assay of DR0505 on circular ssDNA substrates in presence of different bivalent ions

(A) M13mp18 circular ssDNA substrate was incubated at 37°C with (2–5) or without (1 and 6) recombinant DR0505 for 40 min, in the presence of 5 mM MnCl\(_2\) (1, 2 and 3) or 5 mM MgCl\(_2\) (4, 5 and 6), and with (2 and 5) or without (3 and 4) 2 mM ATP. The mixture was heated at 65°C in presence of 95% formamide and 25 mM EDTA for 15 min as detailed in the Materials and methods section. Products were analysed on 0.8 % agarose gels. (B) The fluorescence intensity of DNA substrates (light bars) and products (dark bars) in (A) were obtained by density scanning of the intact DNA band and the smear in the running track respectively. (C) M13mp18 circular ssDNA substrate was incubated at 37°C with DR0505 protein for 10, 20, 30 and 40 min in the absence and presence of 2 mM ATP. The products were analysed on 0.8 % agarose gel and compared with DNA without protein.

**Figure 5** DR0505 showed ssDNA endonuclease and ATP-sensitive 3' → 5' exonuclease

DR0505 showed ATP-mediated regulation of ssDNA/dsDNA exonuclease activity and circular ssDNA endonuclease. It did not show dsDNA endonuclease activity (Figure 5). Since M13mp18 ssDNA forms numerous islands of dsDNA resulting in the formation of ssDNA–dsDNA (ss–ds) junctions, the possibility of DR0505 cutting at the ss–ds DNA junction has not been ruled out. For this, the DNA processing activity of DR0505 was checked on synthetic oligonucleotides forming a linear ss homopolymer (dA40), stem-loop (HP78) or forked-hairpin (HP2) substrates with homopolymeric forks and heteropolymeric dsDNA stems. These 5'32P-labelled DNA substrates were incubated with protein and products were analysed by urea/PAGE. Results indicated a successive although slow degradation of dA40 incubated for...
different time periods, and both sizes and amount of substrate (dA40) decreased concurrently with the increase in levels of intermediate products and 5′ terminal nucleotide ([32P]dAMP) (Figure 6A). This activity was completely inhibited in the presence of ATP. The enzyme showed successive degradation of 5′-labelled HP78 in the absence of ATP, producing intermediate products and terminal mononucleotide ([32P]dGMP). This activity was attenuated in the presence of ATP and three prominent features of this enzyme. ATP inhibition of nuclease activity has not been shown for bacterial nucleases to date. In contrast, the stimulation of exonuclease activity with ATP (exonuclease V) and a Mn2+-dependent Mre11/Rad50-type function of the SbcCD complex have been shown in both E. coli [31,45] and in D. radiodurans [32]. Purified DR0505 showed differential regulation of its exonuclease and endonuclease activities by ATP (compare Figures 4, 5 and 6) in vitro, indicating the significance of ATP regulation in DR0505 functions during the post-irradiation response in D. radiodurans. These functions might have a direct relevance in recycling of nucleotide pools and DNA processing to order to achieve efficient DSB repair in D. radiodurans. As both the ssDNA endonuclease and the hairpin endonuclease activities of this protein were insensitive to ATP, the functional significance of these activities, irrespective of ATP status, during the post-irradiation response can be speculated upon. The radiation-induced synthesis of DR0505 [37] and increased ATP levels [36] coincides with the early-phase of the post-irradiation response in D. radiodurans. This might mean that attenuation of the exonuclease activity of DR0505, which could lead to the degradation of genetic materials, is required due to the higher ATP levels at 1 h post-irradiation. Subsequently, from 1 h onwards when ATP is low, the enzyme contributes to DNA end-processing for efficient recombination repair. Since, γ-irradiation induces different types of DNA breaks and their processing is required for efficient reassembly, it is therefore possible that the ss–ds DNA junction endonuclease activity of this protein is active during the post-irradiation response. It is noteworthy that a deletion mutant of dr0505 did not show any significant change in tolerance to γ-irradiation compared with the wild-type strain (results not shown) suggesting possible functional redundancy for this protein in vivo and that DR0505 seems to be one of the members of a protein family doing similar or related functions in this bacterium. In previous studies, the phosphoesterases [42,43] and DNA-end-processing activity of an X family DNA polymerase [46] and SbcCD proteins [32,47] were reported in D. radiodurans. Thus DR0505, characterized in the present study, represents a novel protein conferring two important functions, the thermotolerant esterase and endo- and exo-nuclease activities in vitro, reponses which are differentially regulated by ATP. Further studies are required to understand the nature of micro-environment responsible for the differential temperature responses in the esterase and nuclease activities in same protein.
molecule and the exact role of this protein in DNA metabolism and radiation tolerance of *D. radiodurans*.

**AUTHOR CONTRIBUTION**

Swathi Kota identified DR0505 from the multiprotein complex, proposed possible functions for this protein, conducted experiments, analysed the results, and discussed and wrote paper. Vijay Kumar obtained and analysed results. Hari Misra, conceived the idea, planned experiments, wrote the paper and served as the principal investigator for the project.

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Deinococcus thermotolerant esterase with DNA-processing functions


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SUPPLEMENTARY ONLINE DATA

Characterization of an ATP-regulated DNA-processing enzyme and thermotolerant phosphoesterase in the radioresistant bacterium *Deinococcus radiodurans*

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**Figure S1** Schematic representation of different functional domains in DR0505 polypeptide

DR0505 contains a leader peptide (LP) followed by a well-defined calcineurin phosphodiesterase domain (CPDD) at the N-terminus and 5′ nucleotidase domain at the C-terminus of the protein.

**Figure S2** Expression and purification of recombinant DR0505 protein from *E. coli*

The dr0505 coding sequences were PCR amplified and cloned at the NdeI and EcoRI sites in pET28a+ to yield pET505. Transgenic *E. coli* harbouring pET505 was grown to exponential phase and induced with IPTG (isopropyl β-D-thiogalactopyranoside). (A) Total proteins from uninduced (1) and induced (2) cells were analysed via SDS/PAGE. (B) The recombinant protein was purified (P) from transgenic *E. coli* expressing DR0505 protein on pET505 and size was ascertained. Lane M shows standard size protein markers.

**Figure S3** Lineweaver–Burk plots of enzyme activity as a function of substrate concentrations

Enzyme activity of DR0505 was determined at different concentrations of PNP and BNPP in the presence of 5 mM MnCl₂ as described in the Materials and methods section in the main paper. Reciprocals of both enzyme activity (1/V) and substrate concentration (1/[S]) were plotted for determination of *Kₘ* and *Vₘₐₓ* values. All the experiments were repeated three times and the results are means ± S.D.