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DNA Repair 3 (2004) 163–169
DNA REPAIR
www.elsevier.com/locate/dnarepair

Multiple uracil-DNA glycosylase activities in *Deinococcus radiodurans*

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Accepted 23 October 2003

Abstract

The extremely radiation resistant bacterium, *Deinococcus radiodurans*, contains a spectrum of genes that encode for multiple activities that repair DNA damage. We have cloned and expressed the product of three predicted uracil-DNA glycosylases to determine their biochemical function. DR0689 is a homologue of the *Escherichia coli* uracil-DNA glycosylase, the product of the *ung* gene; this activity is able to remove uracil from a U:G and U:A base pair in double-stranded DNA and uracil from single-stranded DNA and is inhibited by the Ugi peptide. DR1751 is a member of the class 4 family of uracil-DNA glycosylases such as those found in the thermophiles *Thermotoga maritima* and *Archaeoglobus fulgidus*. DR1751 is also able to remove uracil from a U:G and U:A base pair; however, it is considerably more active on single-stranded DNA. Unlike its thermophilic relatives, the enzyme is not heat stable. Another putative enzyme, DR0022, did not demonstrate any appreciable uracil-DNA glycosylase activity. DR0689 appears to be the major activity in the organism based on inhibition studies with *D. radiodurans* crude cell extracts utilizing the Ugi peptide. The implications for *D. radiodurans* having multiple uracil-DNA glycosylase activities and other possible roles for these enzymes are discussed.

Keywords: Uracil-DNA glycosylase; *Deinococcus radiodurans*; *Escherichia coli*

1. Introduction

Uracil-DNA glycosylase is a widespread enzyme found in eukaryotes and prokaryotes, and is a crucial enzyme for maintaining genomic integrity and preventing mutations [1–3]. This enzyme removes uracil that is present in DNA resulting from deamination of cytosine or the misincorporation of dUMP in place of dTMP [4–6]. Recently, uracil-DNA glycosylase has also been found to be involved in B cell hypermutation in conjunction with activities such as the activation-induced cytosine deaminase (AID) [7,8].

Four major families of uracil-DNA glycosylases are known to exist in nature [9,10]: family 1, the enzymes having homology to the activities found in *Escherichia coli*, humans, and DNA-containing viruses; family 2, the MUG/TDG enzymes; family 3, the SMUG enzymes found in humans, *Drosophila*, and *Xenopus*; and family 4, the prokaryotic enzymes such as those found in the thermophilic organisms *Thermotoga maritima* and *Archaeoglobus fulgidus*, as well as in other prokaryotes. In addition, a putative fifth family (UDGX) has been identified for certain bacterial species such as *Campylobacter jejuni* and *Neisseria meningitidis* [10]. A sixth putative family member has only been seen in *Deinococcus radiodurans* (DRUDG) [10]. Our discovery of the class 4 uracil-DNA glycosylases began with the characterization of the activity in *T. maritima* [11,12]; we also characterized the activity in the archaeon *A. fulgidus* [13]. Both of these enzymes are able to remove uracil from both double- and single-stranded DNA, and can function at high temperatures. We have also found that these two enzymes contain an iron–sulfur cluster which has also been found in other family 4 members [14]; this motif is not seen in any of the other uracil-DNA glycosylase family member proteins.

Most prokaryotes either have a family 1 uracil-DNA glycosylase or a family 4 uracil glycosylase, but occasionally,

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doi:10.1016/j.dnarep.2003.10.011

some species have both, such as *Helicobacter pylori* and *Mycobacterium tuberculosis* [10]. In addition, the family 2 enzymes are present in several prokaryotes. The Mug protein has been shown to specifically remove uracil from U:G base pairs, and has also been shown to remove ethenopurine and ethenopyrimidine adducts from DNA [15–17]. *D. radiodurans* has four putative uracil-DNA glycosylases [10,18]. Comparative genomic [19] and whole genome transcriptome analyses [20] support the view that recovery of *D. radiodurans* from irradiation is likely determined by a complex network, within which DNA protection, DNA repair, and metabolic functions play critical roles.

In this study, we aimed to determine which of the predicted multiple uracil-DNA glycosylases were active in *D. radiodurans*. We have expressed and characterized a family 1 enzyme (DR0689) that is homologous to the *E. coli* Ung protein, as well as a family 4 analog (DR1751). Both of these proteins were active. In addition, we expressed a novel putative DRUDG activity (DR0022). The protein DR0022 did not display any measurable uracil-DNA glycosylase activity. The significance of these findings is discussed.

2. Materials and methods

2.1. Bacterial strains and DNA

The *E. coli* strain DH5α was used for all cloning experiments and for plasmid amplifications. The *ung* deficient strain BW310(DE3) was prepared as described previously [11,13]. In addition, BW310(DE3) was transfected with the plasmid pLys (Novagen), obtained from the strain BL21(DE3)pLysS, yielding the strain BW310(DE3)pLysS. Genomic DNA was isolated from *D. radiodurans* strain R1 [18].

2.2. Cloning of the D. radiodurans DR0689, DR1751, and DR0022 genes

PCR was carried out using *D. radiodurans* R1 genomic DNA as a template and the following oligonucleotides: for DR0689, 5′ GGGGAAGCTAGCATGACCGACCAACCCGAC 3′, and 5′ GGCCGGAAGCTTTCATTCCTCCGTCACCGT 3′; for DR1751, 5′ GGGGAACATATGACCGGCTCCCTTCCCCCG 3′, and 5′ GGCCGGAAGCTTTCATTCCAATTCACTGAA 3′; for DR0022, 5′ GGGGAAGCTAGCATGCCGCACCACGTCCCG 3′, and 5′ GGCCCGAAGCTTTCAGGAAGACCAACTGGG 3′. All of the oligonucleotides contained *Nhe*I and *Hind*III restriction sites at the 5′ and 3′ ends, respectively. All of the PCR amplified DNA sequences were cloned into pET28a and were transformed into *E. coli* BW310(DE3) cells; pET28a-DR1751 was transformed into BW310(DE3)pLysS. The DNA sequence of the inserts was confirmed by DNA sequencing.

2.3. Protein purification

BW310(DE3)pET28a-DR0689 and BW310(DE3)pET 28a-DR0022 were inoculated into LB medium containing 34 μg/ml kanamycin (LB-kan) and grown overnight at 37 °C. BW310(DE3)pLysS pET28a-DR1751 was inoculated into LB-kan containing 34 μg/ml chloramphenicol. The saturated cultures were diluted 1:100 in 150 ml LB-kan medium and grown with shaking at 37 °C until OD_{600} reached 0.9, then IPTG was added to a final concentration of 1 mM and the cell cultures were incubated for an additional 3 h at 30 °C. Cells were pelleted by centrifugation at 3000 × *g* for 5 min at 4 °C and then resuspended in either 5 ml ice cold binding buffer containing 5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9 (DR0689 and DR0022) or 4 ml ice cold binding buffer containing 10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0 (DR1751). Cells were lysed by sonication with 5 × 10 s bursts. The sonicates were clarified by centrifugation at 12, 000 × *g* at 4 °C for 30 min.

For DR00689 and DR0022, the supernatants were added to a 1.2 ml His•Bind Resin Ni^{2+} column (Novagen), pre-equilibrated with five column volumes of binding buffer. Unbound proteins were eluted with 12 column volumes of binding buffer. The histidine-tagged proteins were eluted from the columns with six column volumes of buffer containing 60, 100, 250, and 500 mM imidazole in 500 mM NaCl, 20 mM Tris–HCl, pH 7.9. For DR1751, crude cell extract was applied to 1.2 ml of Ni-NTA His•Bind Resin (Novagen) at a flow rate of 0.5 ml/min. The resin was washed with 2 × 5 ml wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole). Protein was eluted with 2 ml elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole, fraction I).

DR0689 protein was eluted in 100 mM imidazole buffer and DR0022 was eluted in 250 mM imidazole buffer (fraction I). DR0022, which demonstrated no detectable uracil-DNA glycosylase activity, was not purified further. DR0689 protein was subsequently purified by gel filtration chromatography; an aliquot of fraction I was loaded onto a Superdex 75 FPLC column (Pharmacia), equilibrated with a buffer containing 50 mM Tris–HCl, pH 7.8, 0.5 mM Na_2EDTA, 100 mM NaCl and 5% glycerol. The DR0689 protein eluted from the column with a corresponding molecular weight of ∼28–30 kDa. Fractions were checked for uracil-DNA glycosylase activity and pooled. An equal volume of glycerol was added for storage. The DR0689 protein was stored in aliquots at −20 °C without any significant loss of activity for up to 6 months.

DR1751 (fraction I), was loaded onto a NAP-10 gel filtration column and eluted with 1.5 ml of buffer A (50 mM Hepes-KOH, pH 7.8, 0.1 mM Na_2EDTA, 1 mM DTT, 5% glycerol). The elutant was then loaded onto a MonoQ HR 5/5 column (Pharmacia) and was eluted with a 20 ml linear gradient from buffer A to buffer A containing 1 M NaCl. Active fractions were pooled, mixed with an equal vol-

ume of glycerol, aliquoted and stored at −20 °C. The purity of the DR0689, DR1751, and DR0022 proteins was analyzed on a 12% SDS-PAGE gel, stained with Coomassie Blue.

2.4. DNA substrates

Double-stranded DNA containing [^{3}H] labeled uracil was prepared by nick translation of calf thymus DNA as described previously [11,12]. Oligonucleotide substrates containing U/G, U/A, and T/G base pairs were prepared as described previously [13] but in place of 5′ end labeling with [^{32}P], the uracil-containing oligonucleotides were synthesized (Invitrogen) with a 5′-hexachloro-fluorescein phosphoramidite (HEX) group [21].

2.5. Reactions with single- or double-stranded DNA containing [^{3}H] labeled uracil

Single stranded DNA substrate containing [^{3}H] uracil was made by denaturing the double-stranded substrate at 95 °C for 10 min followed by immediate chilling on ice. Reactions (100 μl) contained 0.75 pmol double- or single-stranded DNA substrate containing [^{3}H] labeled uracil (375 Bq), 50 mM MOPS-KOH, pH 7.8, 0.1 mM Na_2EDTA, 1 mM DTT, 100 μg/ml BSA and 2–4 ng protein (DR0689, DR1751, DR0022) were incubated at 37 °C for 30 min. Reactions were stopped by the addition of 110 μl 10% trichloroacetic acid and 11 μl of calf thymus DNA (2.5 mg/ml). The samples were then centrifuged at $10,000 \times g$ for 5 min. Radioactivity contained in the supernatant was determined by liquid scintillation counting.

2.6. Reactions with HEX-labeled oligonucleotides

Reactions with HEX-labeled single- or double-stranded oligonucleotides (16 μl) contained 50 mM Hepes-KOH, pH 7.8, 1 mM DDT, 5 mM Na_2EDTA, 100 μg/ml BSA, 1 pmol of labeled DNA and 0.6 μg of *D. radioduarans* extract or 0.1 pmol of purified protein. The resulting abasic sites were subsequently cleaved by addition of 8 μl of 300 mM NaOH in 95% deionized formamide (without dye markers) and were incubated at 99 °C for 10 min. The reaction products were resolved on denaturing 20% polyacrylamide (PAGE) gels containing 6 M urea. The products were visualized using a Molecular Imager FX System (Bio-Rad).

3. Results

3.1. Properties of the family 1 uracil-DNA glycosylase DR0689

Following the complete sequencing of the *D. radiodurans* genome [18], a homologue of the family 1 class of uracil-DNA glycosylases was identified (DR0689). This gene encodes a 247 amino acid protein with a deduced molecular weight of 27,745 Da and a predicted pI of 6.97. This gene was expressed in *E. coli* BW310, a strain deficient in the Ung protein [13]. The expression product was purified as a His–tag fusion protein as shown in Fig. 1.

As expected for a family 1 uracil-DNA glycosylase, the protein was active on removing uracil from both double-stranded and single-stranded DNA. The K_m for release of uracil from this substrate was determined from Lineweaver Burk analysis using initial reaction rates and was found to be 0.70 μM, over a substrate range of 0.2–1.6 μM. The enzyme was capable of removing uracil from U/G and U/A base pairs and from single-stranded DNA (Fig. 2); the enzyme was unable to remove thymine from a T/G base pair.

In order to determine the contribution of DR0689 to repair of uracil residues in *D. radiodurans*, bacterial extracts were prepared and were incubated with Ugi, a peptide inhibitor of the class 1 family of uracil-DNA glycosylases. As shown in Fig. 2, the Ugi inhibitor effectively inactivated the purified DR0689 protein; Ugi also reduced activity in *D. radiodurans* crude cell extracts, as shown in Fig. 3. Using a double stranded DNA substrate containing [^{3}H] labeled uracil opposite adenine, Ugi reduced the overall enzyme activity in the extracts greater than 95% (data not shown). These findings suggest that DR0689 is a major uracil-DNA glycosylase in *D. radiodurans*.

3.2. Properties of the family 4 uracil-DNA glycosylase DR1751

Family 4 uracil-DNA glycosylases are found exclusively in prokaryotes, and are found in several organisms that grow at extreme temperatures [9,10]. The family 4 analogue in *D. radiodurans* is a 237 amino acid protein with a deduced molecular weight of 25,649 Da and a predicted pI of 8.34. The DR1751 gene was expressed in *E. coli* BW310 and the expression product was purified as a His–tag fusion protein as shown in Fig. 4. The molecular weight of the purified protein (MonoQ fraction) was confirmed by MALDI–TOF mass spectrometry to 30,165 Da, the higher molecular weight resulting from the presence of the N-terminal histidine tag. In addition, the activity co-elutes on gel filtration chromatography with proteins in the molecular weight range of ∼28–30 kDa (data not shown).

As with DR0689, the protein was active on removing uracil from both double-stranded and single-stranded DNA. The K_m for the release of uracil from the double-stranded DNA substrate containing [^{3}H] labeled uracil was determined from Lineweaver Burk analysis using initial reaction rates and was found to be 2.5 nM, over a substrate range of 2–20 nM. The enzyme was capable of removing uracil opposite both G and A (Fig. 5); it was ineffective in removing thymine from a T/G base pair. Unlike the enzymes previously isolated from *T. maritima* and *A. fulgidus*, DR1751

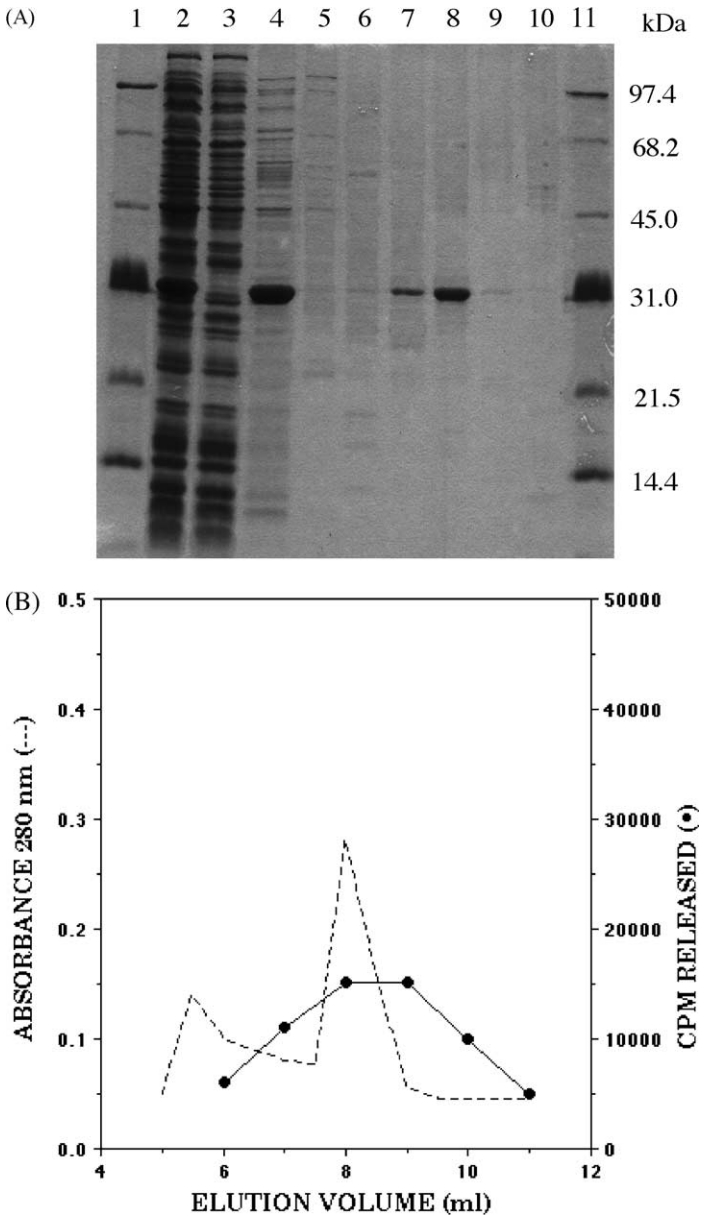

Fig. 1. (A) Purification of DR0689. The purity of the enzyme was evaluated on a 12% SDS-PAGE gel which was stained with Coomassie blue. Lanes 1 and 11, molecular weight markers; lane 2, crude cell lysate (10 μg); lane 3, unbound proteins eluted from the His•Bind column (5 μg); lane 4, bound proteins eluted from the His•Bind column (2 μg); lanes 5–10, fractions 12, 14, 16, 18, 20, and 22, respectively, eluted from the Superdex 75 column. The sizes of the molecular weight markers (lyoszyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b) are given in the margin in kDa. (B) Co-elution of uracil-DNA glycosylase activity and protein following elution on a Superdex 75 gel filtration column. DR0689 protein eluted from the column with a corresponding molecular weight of ∼28–30 kDa. There is a ∼0.3 ml delay between UV detection and fraction collection.

was inactivated by heating (data not shown). No detectable AP endonuclease activity was observed (less than 1% conversion of supercoiled pBR322 plasmid DNA containing one AP site/molecule to open circular DNA).

3.3. Properties of a putative uracil-DNA glycosylase DR0022

DR0022 was identified as a putative uracil-DNA glycosylase with homology to the family 4 class of enzymes [10]. The protein was expressed in *E. coli* and found to have no appreciable activity using the DNA substrate containing [^{3}H] labeled uracil. In addition, it showed no activity on an oligonucleotide substrate containing a U/G base pair (Fig. 6). The protein was also expressed in cell free system using a reticulocyte in vitro transcription–translation system (Novagen). Using this system, no activity was detected for DR0022, whereas uracil-DNA glycosylase activity was evident in parallel reactions expressing DR0689 and DR1751. We conclude that DR0022 does not function as a uracil-DNA

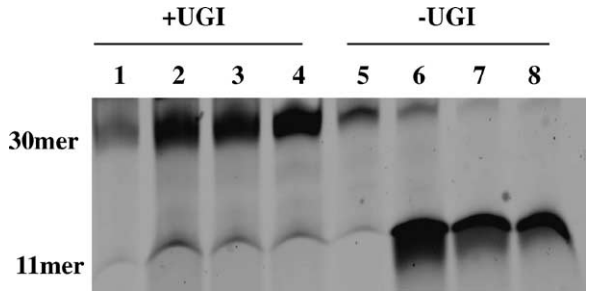

Fig. 2. DR0689 removes uracil from single-stranded DNA and from double-stranded oligonucleotides containing either a U/G or U/A base pair. The 30 mer double- or single-stranded HEX-labeled oligonucleotides (1 pmol each) were incubated in a 16 μl reaction mixture containing 50 mM Hepes-KOH, pH 7.8, 1 mM DDT, 5 mM Na_2EDTA, 100 μg/ml BSA, 0.1 pmol DR0689, for 20 min at 37 °C. The reactions were stopped by the addition of 20 μl of 0.1 M NaOH and the samples were heated at 90 °C for 30 min to cleave the phosphodiester bonds at the abasic sites. The samples were resolved on a 20% PAGE gel containing 6 M urea. Lane 1, (T/G) 30 mer; lane 2, (U/A) 30 mer; lane 3, (U/G) 30 mer; lane 4, single-stranded 30 mer; lane 5, (T/G) 30 mer; lane 6, (U/A) 30 mer; lane 7, (U/G) 30 mer; lane 8, single-stranded 30 mer. Reactions in lanes 1–4 also contained 3 units (0.5 pmol) of Ugi inhibitor.

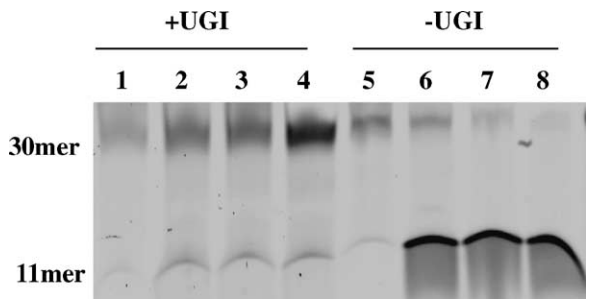

Fig. 3. Ugi inhibits uracil-DNA glycosylase activity in *D. radiodurans* crude cell extracts. The 30 mer double- or single-stranded HEX-labeled oligonucleotides (1 pmol each) were incubated in a 16 μl reaction mixture containing 50 mM Hepes-KOH, pH 7.8, 1 mM DDT, 5 mM Na_2EDTA, 100 μg/ml BSA, 300 ng extract for 30 min at 37 °C. The reactions were stopped by the addition of 20 μl of 0.1 M NaOH and the samples were heated at 90 °C for 30 min to cleave the phosphodiester bonds at the abasic sites. The samples were resolved on a 20% polyacrylamide gel containing 6 M urea. Lane 1, (T/G) 30 mer; lane 2, (U/A) 30 mer; lane 3, (U/G) 30 mer; lane 4, single-stranded 30 mer; lane 5, (T/G) 30 mer; lane 6, (U/A) 30 mer; lane 7, (U/G) 30 mer; lane 8, single-stranded 30 mer. Reactions in lanes 1–4 also contained 3 units (0.5 pmol) of Ugi inhibitor.

glycosylase under the reaction conditions favorable for the class 1 and 4 family of enzymes.

4. Discussion

We have examined the activity of multiple uracil-DNA glycosylases that are present in *D. radiodurans*. A surprising result was that the majority of the activity found in cell extracts seems to result from expression of the DR0689 protein, the class 1 uracil-DNA glycosylase with homology to the major activity found in *E. coli*. Both the purified enzyme and the overall uracil-DNA glycosylase activity were substantially diminished when reactions included the Ugi peptide inhibitor. DR1751 activity was not affected by the addition of Ugi (data not shown); we have demonstrated previously that other class 4 enzymes from *T. maritima* and *A.*

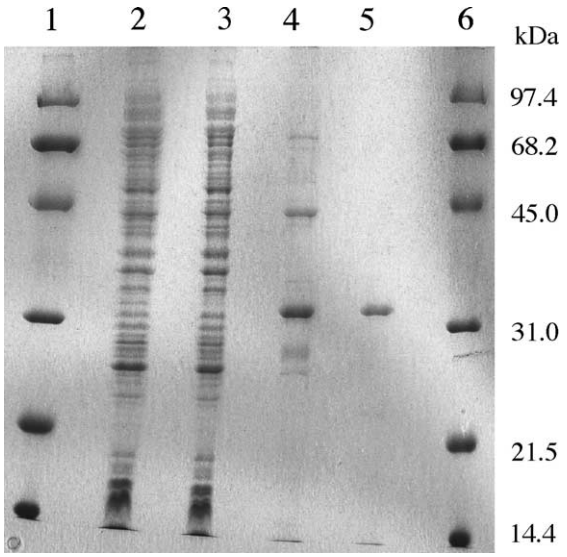

Fig. 4. Purification of DR1751. The purity of the enzyme was evaluated on a 12% SDS-PAGE gel which was stained with Coomassie blue. Lanes 1 and 6, molecular weight markers; lane 2, crude cell lysate (1 μg); lane 3, unbound proteins eluted from the Ni-NTA column (7 μg); lane 4, bound proteins eluted from the Ni-NTA column (0.6 μg); lane 5, MonoQ fraction (0.9 μg). The sizes of the molecular weight markers are given in the margin in kDa.

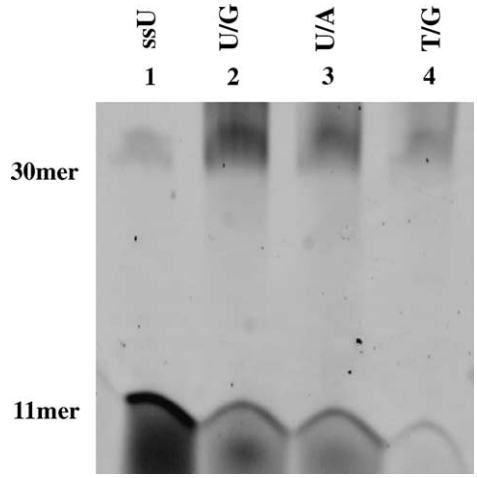

Fig. 5. DR1751 removes uracil from single-stranded DNA and from double-stranded oligonucleotides containing either a U/G or U/A base pair. The 30 mer double- or single-stranded HEX-labeled oligonucleotides (1 pmol each) were incubated in a 16 μl reaction mixture containing 50 mM Hepes-KOH, pH 7.8, 1 mM DDT, 5 mM Na_2EDTA, 100 μg/ml BSA, 0.1 pmol enzyme for 30 min at 37 °C. The reactions were stopped by the addition of 20 μl of 0.1 M NaOH and the samples were heated at 90 °C for 30 min to cleave the phosphodiester bonds at the abasic sites. The samples were resolved on a 20% polyacrylamide gel containing 6 M urea. Lane 1, single-stranded 30 mer; lane 2, (U/G) 30 mer; lane 3, (U/A) 30 mer; lane 4, (T/G) 30 mer.

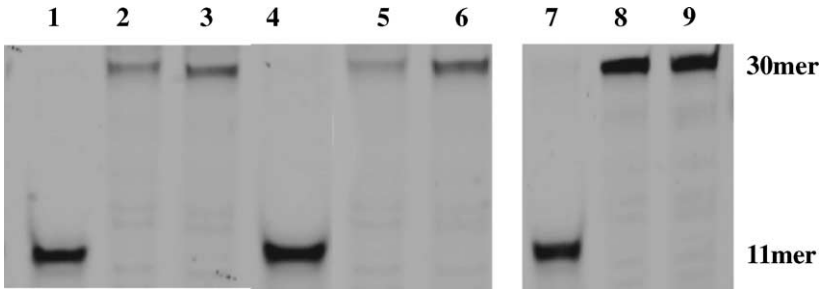

Fig. 6. DR0022 is unable to remove uracil from single-stranded DNA and from double-stranded oligonucleotides containing either a U/G or U/A base pair. The 30 mer double- or single-stranded HEX-labeled oligonucleotides (1 pmol each) were incubated in a 16 μl reaction mixture containing 50 mM Hepes-KOH, pH 7.8, 1 mM DDT, 5 mM Na_2EDTA, 100 μg/ml BSA, 0.1 pmol enzyme for 30 min at 37 °C. The reactions were stopped by the addition of 20 μl of 0.1 M NaOH and the samples were heated at 90 °C for 30 min to cleave the phosphodiester bonds at the abasic sites. The samples were resolved on a 20% polyacrylamide gel containing 6 M urea. Lane 1, (U/G) 30 mer treated with DR0689; lane 2, (U/G) 30 mer treated with DR0022; lane 3, (U/G) 30 mer, no enzyme; lane 4, (U/A) 30 mer treated with DR0689; lane 5, (U/A) 30 mer treated with DR0022; lane 6, (U/A) 30 mer, no enzyme; lane 7, single-stranded 30 mer treated with DR0689; lane 8, single-stranded 30 mer treated with DR0022; lane 9, single-stranded 30 mer, no enzyme.

fulgidus are also not subject to inhibition by Ugi [11,13].

In a study examining genome wide predicted uracil-DNA glycosylase activities [10] DR0022 was predicated to be a member of a novel uracil-DNA glycosylase family. In our hands, the expressed protein did not demonstrate any appreciable UDG activity either following purification from *E. coli* or by expression using an in vitro transcription–translation system. The function of this protein awaits further characterization. In the recently reported transcriptome dynamics of *D. radiodurans* following high dose irradiation and recovery [20], DR0022 had an induction profile similar to *recA* and remained induced throughout recovery; this suggests it has a role in DNA repair. In contrast, DR0689 and DR1751 did not show induction following irradiation, and it is possible that these glycosylases are constitutively expressed in *D. radiodurans*, as are many of its stress response genes including superoxide dismutase A (DR1270) and catalases (DR1998, DRA0259) [22].

In addition to the three enzymes characterized in this study, *D. radiodurans* has a gene (DR0715) that encodes an analogue of the *E. coli* mismatch specific uracil-DNA glycosylase or Mug protein [15–17]. We have performed preliminary studies with this enzyme; it was found to poorly remove uracil from an oligonucleotide with a U/G base pair. However, the enzyme did show some activity on an oligonucleotide substrate containing an ethenocytosine residue. The *E. coli* protein has shown robust activity removing etheno adducts from DNA, and this activity would be expected in the *D. radiodurans* analogue [15,16]. Overall, we do not believe that this activity contributes substantially to the UDG activity found in *D. radiodurans* extracts.

Why does *D. radiodurans* have multiple genes for activities to remove uracil from DNA? A higher level of hydrolytic deamination of cytosine may be expected in an organism such as *D. radiodurans* that can survive exposure to high-level acute irradiation (17 kGy) [23], grow under chronic irradiation (60 Gy/h) [24], as well as resist cycles of desiccation/hydration [25]. Another answer may lie in a more specialized role of these repair enzymes. For example in archaea, the family B DNA polymerase will stall at sites of uracil in DNA during replication, and may recruit a uracil-DNA glycosylase to remove this lesion [26,27]. This mechanism seems to occur in place of the multiple bypass polymerases found in higher organisms. Whether such protein–protein interactions occur for DR1751 and DNA polymerases in *D. radiodurans* is being further investigated.

Acknowledgements

W.A.F. was supported by a grant, CA52025, from the National Cancer Institute and a Howard Hughes Medical Institute—Research Resources Program for Medical Schools Pilot Research Project Award. M.J.D was supported by the Department of Energy (Office of Biological and Environmental Research, Office of Science) grants DE-FG02−01ER63220 from the Genomes to Life Program and DE-FG02-97ER62492 from the Natural and Accelerated Bioremediation Research Program.

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