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Characterization *in vitro* and *in vivo* of the DNA helicase encoded by *Deinococcus radiodurans* locus DR1572

Zheng Cao, Douglas A. Julin*

Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742, United States

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ABSTRACT

Deinococcus radiodurans survives extremely high doses of ionizing and ultraviolet radiation and treatment with various DNA-damaging chemicals. As an effort to identify and characterize proteins that function in DNA repair in this organism, we have studied the protein encoded by locus DR1572. This gene is predicted to encode a Superfamily I DNA helicase, except that genome sequencing indicated that it has a one-base frameshift and would not encode a complete helicase. We have cloned the gene from two different *D. radiodurans* strains and find that the frameshift mutation is not present. The corrected gene encodes a 755 residue protein that is similar to the *Bacillus subtilis* YvgS protein and to helicase IV of *Escherichia coli*. The purified protein (helicase IV_{Dr}) has ATP hydrolysis and DNA helicase activity. A truncated protein that lacks 214 residues from the N-terminus, which precede the conserved helicase domain, has greater ATPase activity than the full-length protein but has no detectable helicase activity. Disruption of locus DR1572 in the *D. radiodurans* chromosome causes greater sensitivity to hydrogen peroxide and methylmethanesulfonate compared to wild-type cells, but no change in resistance to gamma and ultraviolet radiation and to mitomycin C. The results indicate that locus DR1572 encodes a complete protein that contributes to DNA metabolism in *D. radiodurans*.

1. Introduction

The bacterium *Deinococcus radiodurans* has been the object of much recent study because of its extraordinary ability to survive high levels of radiation, oxidizing chemicals, desiccation, and other agents that can damage DNA and other cellular components [1–4]. In particular, the organism can withstand high doses of ionizing radiation that cause extensive damage including double-strand breaks (dsb) to DNA, as well as damage to protein and lipids.

Several factors are believed to contribute to the ability of *D. radiodurans* to survive high levels of DNA damage from ionizing radiation. The organism has multiple copies of its chromosomes at all stages of growth, which may facilitate double-strand DNA break repair via homologous recombination [5,6]. Its nucleoid takes on a condensed structure which may maintain broken DNA ends in proximity for rapid repair [7–9]. Finally, high intracellular manganese and low iron concentrations in *D. radiodurans* are proposed to protect against the damaging effects of reactive oxygen species produced in irradiated aqueous solutions, during desiccation, or during treatment with oxidants such as hydrogen peroxide [10–12]. Manganese ion is proposed to act specifically as an antioxidant that prevents damage to proteins, which retain their activity and are able to repair the damaged DNA [13,14].

Double-strand break repair in most bacteria is initiated by the RecBCD or AddAB enzyme and requires the RecA protein [15–17]. However, the dsb-repair mechanism in *D. radiodurans* must be different from that in other bacteria since the organism lacks homologues of both RecBC and AddAB [18–20]. The severe radiation sensitivity of *recA* [21–23] and *polA* mutant *D. radiodurans* strains [24] indicate that the RecA protein and DNA polymerase I are involved in repair of radiation-induced double-strand breaks [25]. However, the identities of other enzymes with activities likely to be important in the repair process, including helicases and nucleases, are largely unknown.

As an effort to identify and characterize enzymes that might be involved in DNA repair and other DNA transactions in *D. radiodurans*, we have studied the properties of the enzyme encoded by *D. radiodurans* locus DR1572. This protein has amino acid sequence similarity to several DNA helicases found in other bacteria, including PcrA, UvrD and rep [26]. The nearest *B. subtilis* homologue of the *D. radiodurans* protein has been proposed to be a homologue of the *Escherichia coli* helicase IV [27] (see Section 3). For this reason, we refer to the protein encoded by locus DR1572 as helicase IV_{Dr}, and the gene as *helD*. We have expressed helicase IV_{Dr} in *E. coli*. The purified protein has ATP hydrolysis and DNA unwinding activity. We also disrupted the *helD* gene in the *D. radiodurans* chromosome and find that the mutant is somewhat more sensitive than the

* Corresponding author. Tel.: +1 301 405 1821; fax: +1 301 314 9121.
E-mail address: djulin@umd.edu (D.A. Julin).

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wild-type to treatment with hydrogen peroxide and with methylmethanesulfonate. However, the mutant is as resistant as wild-type to gamma and UV irradiation, and to treatment with mitomycin C. The results show that locus DR1572 encodes a protein that contributes to the resistance of *D. radiodurans* to some DNA-damaging agents.

2. Materials and methods

2.1. Materials

Wild-type *D. radiodurans* strain R1 was purchased from the ATCC, Manassas, VA. Wild-type *D. radiodurans* strain BAA-816 was a gift from Dr. Michael Daly, Uniformed Services University of the Health Sciences, Bethesda, MD. The plasmid pTNK101 was a gift from Dr. John Battista, Louisiana State University. *E. coli* strain AB1157 (=WA234 [28]) was a gift from Dr. Wilfried Wackernagel, University Oldenburg, Germany.

D. radiodurans was grown in TGY medium (0.5% tryptone, 0.3% yeast extract, and 0.1% glucose). *E. coli* was grown in LB medium (1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride). Antibiotic concentrations used for *D. radiodurans* were: chloramphenicol, 3 μg/ml; kanamycin, 8 μg/ml; and streptomycin, 5 μg/ml. Concentrations used for *E. coli* were: chloramphenicol, 15 μg/ml; kanamycin, 30 μg/ml; and ampicillin, 100 μg/ml.

The sequences of all oligodeoxyribonucleotides used for PCR are given in Supplementary Table 1.

2.2. helD gene cloning and sequencing

D. radiodurans genomic DNA was isolated as described [29]. PCR reaction mixtures (50 μl) contained 200 ng genomic DNA or 1 ng plasmid DNA template, 0.6 μM primers, 200 μM of each dNTP, glycerol (15% v/v), and 2.5 units *Pfu* DNA polymerase (Stratagene Corp.). A 2267 bp fragment encompassing the entire putative *helD* open reading frame (bp # 1,591,892 to 1,594,158 of *D. radiodurans* chromosome 1, Genbank Acc. # AE000513) was amplified from *D. radiodurans* strain BAA-816. A 1625 bp fragment encoding a truncated version of the protein (bp # 1,592,534 to 1,594,158) was amplified from both strains BAA-816 and R1. The amplified DNA products were ligated into the pCR-blunt vector (Invitrogen Corp). The complete sequences of both the 1.6 and 2.3 kb *helD* gene inserts were determined at the DNA sequencing facility in the Center for Biosystems Research, University of Maryland (see Section 3). The predicted amino acid sequences of the proteins encoded by these DNA fragments are given in Supplementary Fig. 1.

2.3. Helicase IV_{Dr} protein expression and purification

The two forms of the *helD* gene cloned from strain BAA-816 (2.3 and 1.6 kb, see above) were transferred to pET15-b (Novagen Corp.) using *Nde*I and *BamH*I sites introduced by PCR, to make pET-helIV_{Dr}-83 (2.3 kb insert) and pET-helIV_{Dr}-59 (1.6 kb insert). These plasmids encode respectively the 83 and 59 kDa forms of helicase IV_{Dr} (see Section 3). A single colony of *E. coli* strain Rosetta2(DE3) (Novagen Corp.) containing pET-helIV_{Dr}-83 was used to inoculate 10 ml of LB medium containing ampicillin (200 μg/ml) and grown overnight at 37 °C. The overnight culture was diluted 1:100 into ZYP-5052 medium (1% tryptone, 0.5% yeast extract, 1 mM $MgSO_4$, 0.33% $(NH_4)_2SO_4$, 0.68% KH_2PO_4, 0.71% Na_2HPO_4, 0.5% glycerol, 0.05% dextrose, and 0.2% lactose) with ampicillin (100 μg/ml). The culture was grown at room temperature (~23 °C) with vigorous shaking for 20 h for autoinduction of helicase IV_{Dr}-83 expression. The cells were harvested by centrifugation at 6000 rpm and stored at −80 °C.

The plasmid pET-helIV_{Dr}-59 was transformed into BL21(DE3)pLysS cells (Novagen Corp.) to express the helicase IV_{Dr}-59 protein. The transformed cells were grown at 37 °C with vigorous shaking to OD_{600} = 0.4–0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to 0.5 mM, and the cells were incubated at 30 °C for 3 h. The cells were harvested by centrifugation and stored at −80 °C.

The frozen cell pellets from each culture (~8–10 g cells) were thawed on ice and suspended in 50 ml of native binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8) containing 20 mM imidazole. Phenylmethanesulfonylfluoride (1 mM) and protease inhibitor cocktail for polyhistidine-tagged proteins (Sigma–Aldrich, 50 μl/g cells) were added to the suspension. The cells were lysed by sonication and the cell extract was applied to a 3 ml Ni^{2+}-NTA column (ProBond resin, Invitrogen Corp.). The column was washed with 30 ml of native wash buffer (20 mM sodium phosphate, pH 7.8, 500 mM NaCl) containing 60 mM imidazole, and the helicase IV_{Dr} protein was eluted in a 60-ml gradient of 60–500 mM imidazole in native wash buffer.

The Ni^{2+}-NTA column fractions containing helicase IV_{Dr}-83 protein were dialyzed at 4 °C against Buffer A (20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) containing 50 mM NaCl. Protein that precipitated after dialysis was removed by centrifugation and the supernatant was loaded onto a 5 ml single-stranded DNA (ssDNA) cellulose column (Sigma) in Buffer A containing 50 mM NaCl. The column was washed with 50 ml of Buffer A containing 50 mM NaCl, and then with 20 ml of 300 mM NaCl and 20 ml of 600 mM NaCl in Buffer A. The fractions containing helicase IV_{Dr}-83 were collected and concentrated by ultrafiltration (Amicon).

The Ni^{2+}-NTA column fractions containing helicase IV_{Dr}-59 protein were dialyzed at 4 °C against buffer A with no added salt, applied to a ssDNA column in buffer A (no salt), and the column washed with the same buffer. The helicase IV_{Dr}-59 protein was eluted mainly in the wash fractions, after the major contaminating proteins remaining from the Ni^{2+}-NTA column. The fractions containing helicase IV_{Dr}-59 were dialyzed against Buffer A containing 10% (v/v) glycerol and concentrated by ultrafiltration (Amicon).

The protein concentrations were determined from the absorbance at 280 nm, using ϵ_{280} = 73,465 M^{-1} cm^{-1} for His-tagged helicase IV_{Dr}-83 and ϵ_{280} = 49,390 M^{-1} cm^{-1} for His-tagged helicase IV_{Dr}-59 (calculated using the program ProtParam at ca.expasy.org/tools/protparam.html). The yield of helicase IV_{Dr}-83 was low (~0.3 mg from 9 g cells (1 l culture)), due to the precipitation after the Ni^{2+} column (see above). The yield of helicase IV_{Dr}-59 was higher (~10 mg from 7.5 g cells), as there was no precipitation during dialysis.

The His-tag peptides on helicase IV_{Dr}-83 and helicase IV_{Dr}-59 were removed using a Thrombin Cleavage Capture kit (Novagen Corp.). The protein (0.1 mg) was treated with thrombin (0.44 units) at 4 °C overnight. Removal of the His-tag was verified by analysis on 10% SDS-PAGE and by western blotting using anti-His-tag monoclonal antibody (Novagen Corp.) (data not shown).

2.4. Enzyme activity assays

2.4.1. ATP hydrolysis

ATP hydrolysis was measured using [γ-^{32}P]ATP and analysis by thin layer chromatography on polyethyleneimine-cellulose plates (J.T. Baker), as described [30]. The standard reaction mixture (20 μl total volume) contained 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, DNA co-substrate, 1 mM DTT, 5% (v/v) glycerol, 0.1 mg/ml BSA, 250 μM [γ-^{32}P] ATP and was incubated at 30 °C.

Table 1
Oligonucleotides used for helicase substrates.

Name	Length (nt)	Sequence (5′-3′)	Helicase substrate structures
Oligo-1	32	GCCGTAGTATGCACATCGACATCCATCGACAT	1 + 2: 20 bp, 12 nt 3′-tail
Oligo-2	20	GTCGATGTGCATACTACGGC	2 + 3: 20 bp, blunt ends
Oligo-3	20	GCCGTAGTATGCACATCGAC	3 + 4: 20 bp, 12 nt 5′-tail
Oligo-4	32	TACAGCTACCTAGTCGATGTGCATACTACGGC	1 + 4: 20 bp, forked end

2.4.2. DNA unwinding

Four oligodeoxyribonucleotides (see Table 1) were annealed in pairs to make a 20-base pair double-stranded DNA molecule with blunt ends (oligos 2 and 3), a 12 nt single-stranded 5′-overhang (oligos 3 and 4), a 12 nt single-stranded 3′-overhang (oligos 1 and 2), or a forked end of 12 nt non-complementary single-stranded DNA (oligos 1 and 4). Oligonucleotides were 5′-^{32}P-end-labeled and annealed in 50 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, as described [31]. DNA unwinding reaction mixtures (20 μl) contained 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, 5% (v/v) glycerol and 1 nM of duplex substrate. Reactions were incubated at 30 °C and samples were quenched and analyzed on non-denaturing polyacrylamide gels as described [31].

2.4.3. Chromosomal helD gene disruption

The *helD* gene was disrupted by replacing the DNA corresponding to the 1.6 kb fragment described above with a chloramphenicol-resistance gene (*cat* gene). Three overlapping PCR fragments were generated: a 968 bp chromosomal DNA fragment located upstream of the 1.6 kb *helD* coding sequence, a 948 bp product located downstream of *helD*, and an 829 bp DNA fragment including the *cat* gene fused to the *kat* promoter from the plasmid pTNK101 [32]. The 5′ end of the amplified *cat* gene fragment overlapped the 3′ end of the *helD* upstream fragment and the 3′end of the *cat* fragment overlapped the 5′ end of the *helD* downstream fragment. These three PCR fragments were mixed in 1:1:1 ratio and a PCR reaction was done with the primers Fw2 and Rev3 (see Supplementary Table 1) to amplify the entire DNA fragment. The resulting 2705 bp product was used to transform *D. radiodurans* wild-type strain BAA-816 to resistance to chloramphenicol, as described [32–34]. The *cat* gene insertion into the *helD* gene was verified by PCR and southern blotting (see Supplementary Fig. 2).

2.4.4. DNA damage sensitivity

Cells for each assay were grown to mid-log phase ($OD_{600} \approx 0.5$, corresponding to $\sim 10^9$ CFU/ml) in TGY medium without antibiotics. Cells were treated as described below and then spread on TGY plates with no antibiotic and incubated at 30 °C for 2 days before colonies were counted.

UV irradiation. Cells were serially diluted in TGY and spread on TGY plates. After the culture soaked into the plates, the plates were opened and exposed to UV-B light from a 15 W germicidal lamp (FG15T8; Fisher) at 90 J/(m^2 min) (measured with a UVX Radiometer (UVP, Upland, CA)) for different time periods.

Gamma irradiation. Cells were irradiated with gamma rays from a ^{60}Co source (Neutron Products model 200324) at about 2.5 kGy/h in 5.0 ml portions on ice. Irradiated cells were serially diluted in triplicate in TGY medium and spread on TGY plates.

Mitomycin C (MMC). Cells were incubated in liquid TGY medium containing 10 μg/ml MMC with shaking at 30 °C. At selected time points aliquots from the culture were serially diluted with TGY medium and spread on TGY plates.

Hydrogen peroxide. Hydrogen peroxide was added in various concentrations to cells in TGY medium. The cells were incubated for 1 h at 30 °C without shaking and aliquots were diluted and spread on TGY plates.

Methyl methanesulfonate (MMS). Cells were incubated in liquid TGY medium containing 30 mM MMS with shaking at 30 °C. At selected time points cells from 0.5 ml of each culture were harvested by centrifugation and resuspended in 0.5 ml of fresh TGY. Resuspended cells were serially diluted with TGY medium and spread on TGY plates.

E. coli strain AB1157 was grown to $OD_{600} = 0.5$ in LB medium at 37 °C and treated with 30 mM MMS. The treated cells were spread on LB plates and colonies were counted after overnight incubation at 37 °C.

3. Results and discussion

3.1. helD gene cloning and sequencing

D. radiodurans locus DR1572 is predicted to encode a protein with amino acid sequence similarity to several Superfamily I helicases, including the PcrA, UvrD, and rep helicases. However, as with several other genes in the *D. radiodurans* R1 genome sequence, locus DR1572 appears to have a frameshift mutation, so that a reading frame shift would have to occur during translation to produce a complete protein similar to these other helicases. We cloned locus DR1572 from two wild-type *D. radiodurans* strains, including the strain used in the genome sequencing project (BAA-816). The DNA sequences of four different isolates of the gene cloned from strain BAA-816 and two isolates from strain R1 had an additional G after bp # 1,593,382 in *D. radiodurans* chromosome 1 (GenBank accession number AE000513), which corrected the apparent frameshift. The corrected sequence in the 2.3 kb gene clone (see Section 2) encodes a protein of 755 residues (83 kDa), beginning at a predicted TTG start codon (bp # 1,591,892 in chromosome 1). The DNA sequence and the corrected protein sequence are shown in Supplementary Fig. 1. Several other *D. radiodurans* genes with apparent frameshifts have been cloned and sequenced and the frameshift mutations were not found [35–37].

3.2. Protein sequence analysis

A BLASTp search of the GenBank database with the full-length 83 kDa helicase IV_{Dr} protein sequence as the query found similar protein sequences in several other bacteria, including *Bacilli*, *Clostridia*, *Streptomycetes*, *Lactobacilli*, and other genera. The alignment of a selection of these proteins is given in Supplementary Fig. 3. The highest scoring *E. coli* protein sequences were UvrD ($E = 0.013$) and helicase IV ($E = 0.17$).

The closest match to the *D. radiodurans* helicase IV_{Dr} protein in *B. subtilis* is the protein encoded by the *yvgS* gene. The YvgS protein has been proposed to be an orthologue of *E. coli* helicase IV, a superfamily I helicase encoded by the *helD* gene [27]. The sequence identity between *E. coli* helicase IV and *B. subtilis* YvgS is low, but they are proposed to be orthologues based on the spacing of the conserved helicase motifs found in all superfamily I helicases [27,38,39]. In particular, both helicase IV and YvgS have a long N-terminal region preceding the first conserved helicase motif (~211 aa in *E. coli* helicase IV) and a relatively short spacing between helicase motifs IV and V, compared to the UvrD, rep, and PcrA helicases found in other bacteria [27,38].

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                                   motif I
D. radiodurans   (228) AMRFPAGTPVIIQGAAGSGKTTIGFHR  (30)  YAARILPELGIGGVSVTTPEAWATALLG-LEK (211)
B. subtilis      (219) IIRNEKSKILIVQGAAGSGKTSAALQR  (28)  YVSSVLPELGEENMEQATFQEYIEHRLGRKFK (232)
E. coli          (203) AVVNGEHSLLVLAG-AGSGKTSVLVAR  ( 9)  ASPEQILLLAFGRKAAEEMDERIRERLHTEDI (160)
                                    * ******     *                *                 *

                      motif II                    motif III               motif IV
D. radiodurans   EPFDHVVLDEAQDYSP---LLYALLARAARPGHVTALGDLNQGMHG-  (16)  AQVLTLSRTYRSTRQITE (41)
B. subtilis      TKIKHLFIDEAQDYSP---FQMAYMRSIFPAASMTVLGDINQSIYAH  (16)  AEYVRLKRTYRSTRQIVE (40)
E. coli          SPWKHILVDEFQDISPQRAALLAALRKQNSQTTLFAVGDDWQAIYRF  (17)  GERCDLDTTYRFNSRIGE (41)
                     *   ** ** **      *              **  *                  *  ***    * *

                           motif V                             motif IV
D. radiodurans   GLVILPVSLAKG------LEFSAAIVTGANQTTYDESTEYERRLLYVAASRALHWLGLV (11)
B. subtilis      GVCVIPVYLAKG------IEFDAVLVYDASEEHYHT--EHDRRLLYTACTRAMHMLAVF (21)
E. coli          ADYVIIVGLQEGSDGFPAAARESIMEEALLPPVEDFPDAEERRLMYVALTRARHRVWAL (24)
                       * *  *                             *** * *  ** *
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Fig. 1. Amino acid sequence alignment of *D. radiodurans* helicase IV_{Dr}, *B. subtilis* YvgS, and *E. coli* helicase IV. The complete amino acid sequences of *D. radiodurans* helicase IV_{Dr} (corrected sequence, 755 residues, see Supplementary Fig. 1), *B. subtilis* YvgS (774 residues, Genbank acc. # CAB15350), and *E. coli* helicase IV (684 residues, Genbank acc. # P15038) were aligned using CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The six motifs conserved in Superfamily I helicases [39] that were found by CLUSTALW2 are highlighted. Residues in *E. coli* helicase IV that are considered to be motif Ia [38] are underlined. The number of residues omitted are indicated in parentheses (the complete alignment is shown in Supplementary Fig. 4).

A partial alignment of the *D. radiodurans* helicase IV_{Dr} protein with *B. subtilis* YvgS and *E. coli* helicase IV is shown in Fig. 1 (the complete alignment is given in Supplementary Fig. 4). The alignment program (CLUSTALW2) found six of the seven conserved helicase motifs in the three proteins. The exception is motif Ia, typically located just C-terminal to motif I [38]. The residues in *E. coli* helicase IV identified as motif Ia (Fig. 1; see [38]) do not align well with YvgS and helicase IV_{Dr}. There is a region in the helicase IV_{Dr} homologues found by BLAST that has several conserved residues and is just C-terminal to the highly conserved motif I (Supplementary Fig. 3), but these residues are not similar to *E. coli* motif Ia. Motif Ia is the most variable of the seven motifs in superfamily I helicases, with no specific conserved residues in the consensus sequence [38–40]. While the ambiguity about motif Ia makes it difficult to be certain of the relationship among these proteins, we nonetheless refer to the protein encoded by locus DR1572 as *D. radiodurans* helicase IV (helicase IV_{Dr}) and the gene as *helD*.

3.3. Expression and in vitro characterization of helicase IV_{Dr}

The corrected *helD* gene is predicted to encode a protein with 755 amino acid residues and molecular mass of 83 kDa, referred to below as helicase IV_{Dr}-83. The amino acid sequence analysis shows that the first conserved helicase motif is found at about residue # 240 (see Fig. 1 and Supplementary Fig. 4). While helicase IV has a long N-terminal sequence preceding helicase motif I, motif I is very close to the amino-terminus in other superfamily I helicases such as UvrD. For this reason, we also expressed a shorter form of the *D. radiodurans* protein, beginning from the ATG codon # 215. This truncated protein has 541 residues and predicted molecular mass of 59 kDa and it includes all of the conserved helicase motifs. This protein is referred to below as helicase IV_{Dr}-59. Both proteins were expressed with 20-residue N-terminal His-tags and purified as described in Section 2. The His-tags were removed from both proteins by thrombin digestion, which leaves a Gly-Ser-His tripeptide at the amino-terminus of the protein. No differences were seen between tagged and untagged proteins in any of the assays described below (data not shown).

Both proteins exhibited ATP hydrolysis activity (Fig. 2). The activity of the full-length helicase IV_{Dr}-83 protein was low and was stimulated only very slightly by ssDNA under these reaction conditions. We tested a number of variations in the reaction conditions

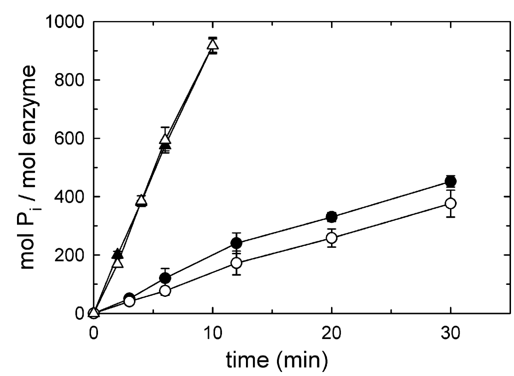

Fig. 2. ATP hydrolysis by the helicase IV_{Dr} proteins. ATP hydrolysis was measured as described in Section 2, with (filled symbols) 10 μM ssDNA (30 nt), or (open symbols) no DNA. Circles, reactions contained helicase IV_{Dr}-83 (0.12 μM). Triangles, reactions contained helicase IV_{Dr}-59 (0.09 μM).

(Table 2) and maximal activity was seen in the conditions used in Fig. 2. ATP hydrolysis catalyzed by helicase IV_{Dr}-59 was about 6-fold faster than that by helicase IV_{Dr}-83 under these reaction conditions (Fig. 2). However, ATP hydrolysis by helicase IV_{Dr}-59 was not

Table 2
ATP hydrolysis activity of helicase IV_{Dr}-83.

Reaction condition	Relative activity
Standard[a]	1.0[b]
10 mM $MgCl_2$	0.6
No DNA	0.3
ds circular DNA	0.8
ds linear DNA	0.4
23 °C	0.5
37 °C	1.1
+200 mM NaCl	0.4
+50 mM KCl	0.7
+1 mM $MnCl_2$	0.9
pH 6.5	0.7
pH 8.5	1.1

[a] Standard reaction conditions were 50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 10 μM ssDNA oligonucleotide (30 nt), 250 μM ATP, 0.53 μM His-tagged helicase IV_{Dr}-83, incubated at 30 °C for 15 or 30 min. Other conditions were as in Section 2.

[b] Activity was 8.5 μM ATP/(min μM helicase IV_{Dr}-83).

affected by the presence of ssDNA (a 30 nt oligonucleotide, 10 μM (Fig. 2)), nor by linear or circular dsDNA (1.35 μM bp for each; data not shown).

We attempted to detect DNA binding by both purified proteins using a gel-shift assay, with a 5′-^{32}P-labeled single-stranded oligodeoxyribonucleotide, as described [31]. We detected no binding by either protein in these assays (with 1 nM DNA and up to 120 nM helicase IV_{Dr}-83 or 720 nM helicase IV_{Dr}-59; data not shown). We also detected no DNA binding by helicase IV_{Dr}-83 in a nitrocellulose filter binding assay (data not shown). The fact that helicase IV_{Dr}-59 did not bind DNA is consistent with the fact that its ATPase activity was not stimulated by added ssDNA (Fig. 2) and that it did not bind well to the ssDNA-cellulose column during the purification procedure (see Section 2). Helicase IV_{Dr}-83 did bind to the ssDNA column (eluted at ~0.25 M NaCl), but its ATPase activity was stimulated only slightly by ssDNA (Fig. 2) and we were unable to detect DNA binding by helicase IV_{Dr}-83 in this assay.

3.4. DNA unwinding measurements

Helicase reactions were done with 20 bp dsDNA molecules with either blunt ends, a 3′- or 5′-ssDNA-extension, or with a 12 nt non-complementary ssDNA fork at one end of the duplex. Each DNA duplex was 5′-^{32}P-end-labeled at a blunt ds-end and samples were analyzed on non-denaturing gels. The results indicate that the helicase IV_{Dr}-83 protein has DNA helicase activity with the 5′-ss-tailed and forked substrates (Fig. 3). No unwinding was detected with either the 3′-ssDNA-tailed or the blunt-ended substrates, in experiments done under the same conditions as those in Fig. 3 (data not shown). The enzyme preparation also had a small amount of nuclease activity on ssDNA (Fig. 3, lane 14, and data not shown). The nuclease activity is a contaminating enzyme from *E. coli*, since the relative levels of helicase and nuclease activity were quite variable in different batches of helicase IV_{Dr}-83. These results show that helicase IV_{Dr}-83 is a helicase with 5′-3′-polarity on ssDNA. There has been no biochemical study of the *B. subtilis* YvgS protein, but interestingly the *E. coli* helicase IV unwinds DNA with 3′-5′-polarity [44].

Helicase IV_{Dr}-59 (tagged or untagged) had no detectable DNA unwinding activity (nor any nuclease activity) on any of the four helicase substrates tested (Fig. 4). The inability of helicase IV_{Dr}-59 to unwind DNA and the weak binding of this protein to DNA indicates that the amino terminal extension of helicase IV_{Dr}-83 that is missing in helicase IV_{Dr}-59 contributes to DNA unwinding. Thus helicase IV_{Dr}-59 has vigorous ATP hydrolysis activity (Fig. 2) but is unable to bind and unwind the DNA (Fig. 4). The presence of this N-terminal extension in helicase IV_{Dr}-83 appears to enable the ATP hydrolysis to power DNA unwinding. Similar behavior has been observed previously for other DNA helicases, where the motor domain that contains the helicase motifs has ATP hydrolysis activity, but additional domain(s) are required for DNA unwinding or to target the enzyme to particular DNA substrate structures [41–43].

3.5. In vivo effects of helD gene disruption

We disrupted the *helD* gene in order to test whether the protein has a role in cell growth or in resistance to DNA damage in *D. radiodurans*. The *helD* gene disruption, and lack of detectable wild-type *helD* sequence, were verified by PCR and southern blotting (Supplementary Fig. 2).

We first tested the growth of wild-type *D. radiodurans* and the *helD:cat* mutant under normal culture conditions (TGY medium, 30 °C). There was essentially no difference in the lag time, the rate of exponential growth, and the final optical density of the culture, between the mutant and the wild-type (Supplementary Fig. 5). The

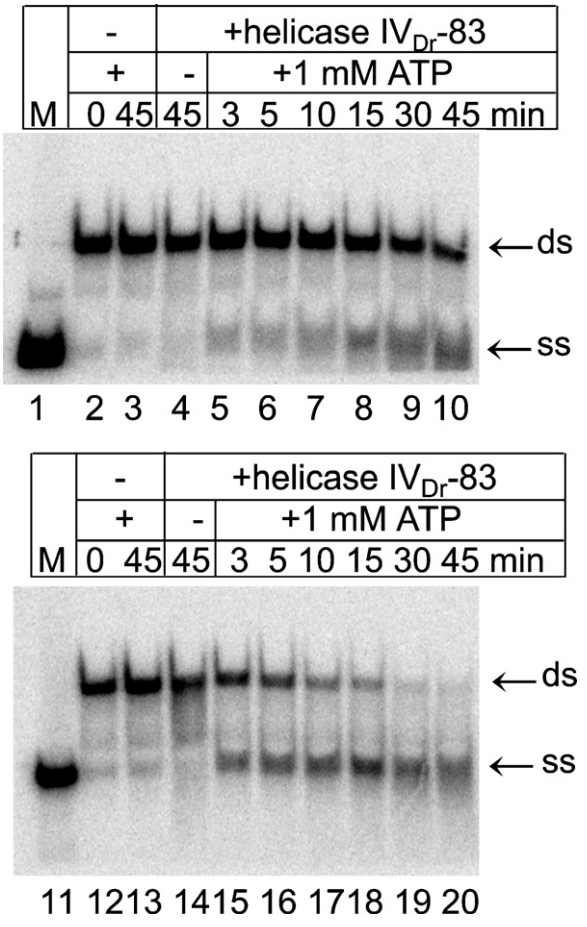

Fig. 3. DNA unwinding by helicase IV_{Dr}-83 DNA. Reaction mixtures were prepared as described in Section 2 and incubated at 30 °C. Samples were removed at the indicated times, quenched, and analyzed on non-denaturing polyacrylamide gels as described in Section 2. (Upper panel) Reactions contained the 5′-^{32}P-labeled 20 bp substrate with a 12 nt 5′-ssDNA end (oligos 3 + 4). Lane 1: ^{32}P-oligo-3 ssDNA marker. Lanes 2 and 3 contained no helicase IV_{Dr}-83. Lane 4–10 contained 0.12 μM helicase IV_{Dr}-83. Lane 4 had no ATP, while all other lanes had 1 mM ATP. (Lower panel) Reactions contained the 5′-^{32}P-labeled 20 bp substrate with a 12 nt ssDNA forked end (oligos 1 + 4). Lane 11: ^{32}P-oligo-1 ssDNA marker. Lanes 12 and 13 contained no helicase IV_{Dr}-83. Lanes 14–20 contained 0.12 μM helicase IV_{Dr}-83. Lane 14 had no ATP, while all other lanes contained 1 mM ATP.

helD:cat mutant is also as transformable as wild-type (data not shown), in experiments done as described [45].

We then tested the *helD:cat* mutant for its sensitivity to gamma and UV irradiation, and to the DNA alkylating and cross-linking agent mitomycin C. The mutant is as resistant as the wild-type to these three agents (Fig. 5). The *helD:cat* mutant is somewhat more sensitive than the wild-type to treatment with hydrogen peroxide or with MMS (Fig. 6). Hydrogen peroxide is an oxidant, and the extreme resistance of *D. radiodurans* to ionizing radiation is thought to be a manifestation of its resistance to oxidative conditions [4,13]. Thus it is not surprising that *D. radiodurans* is substantially more resistant to hydrogen peroxide than is *E. coli* [46]. However, MMS is a methylating agent which does not cause oxidative damage to DNA. We were interested in comparing directly the MMS-sensitivity of *D. radiodurans* to a wild-type (DNA repair proficient) *E. coli* strain. As observed for other DNA-damaging agents, *E. coli* strain AB1157 is significantly more sensitive to MMS than is the wild-type *D. radiodurans* strain (and the *helD:cat* mutant strain) (Fig. 6B).

The results in Fig. 6 indicate that helicase IV_{Dr} has a role in the ability of *D. radiodurans* to survive in the presence of some DNA damaging agents. Hydrogen peroxide is a precursor of hydroxyl radical, which can cause DNA base damage and ss- and ds-breaks. MMS

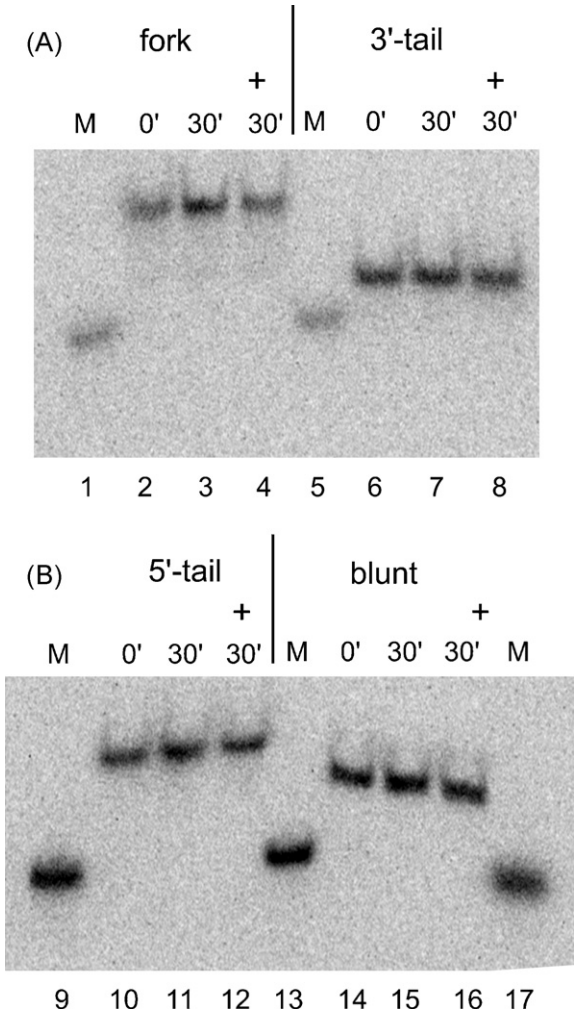

Fig. 4. DNA unwinding reactions with helicase IV_{Dr}-59. Reaction mixtures were prepared as described in Section 2, incubated at 30 °C, and analyzed as in Fig. 3. Only the lanes marked with '+' (lanes 4, 8, 12, 16) contained ATP (1 mM) and helicase IV_{Dr}-59 (0.8 μM). (A) Reactions contained 5′-^{32}P-labeled 20 bp substrate with (lanes 2–4) a 12 nt ssDNA forked end (oligos 1 + 4) or (lanes 6–8) a 12 nt 3′-ssDNA tail (oligos 1 + 2). Lanes 1 and 5: 5′-^{32}P-labeled oligo-1 marker. (B) Reactions contained a 5′-^{32}P-labeled 20 bp DNA duplex with (lanes 10–12) a 12 nt 5′-ssDNA tail (oligos 3 + 4), or (lanes 14–16) a 20 bp blunt-ended substrate (oligos 3 + 2). Lanes 9, 13, 17: 5′-^{32}P-labeled oligo-3 marker.

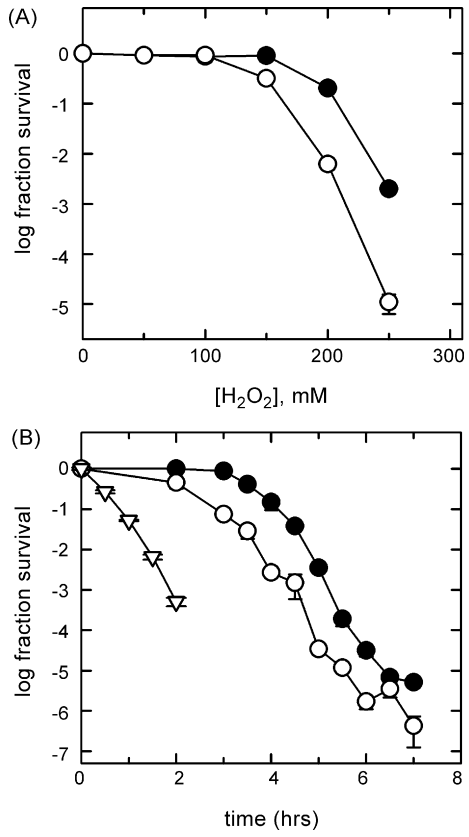

Fig. 6. Sensitivity of the *helD:cat* mutant to hydrogen peroxide (A) and methylmethanesulfonate (B). (A) Exponential phase cells were treated with the indicated amounts of hydrogen peroxide at 30 °C for 1 h, followed by serial dilution and plating on TGY plates. Filled circles: wild-type *D. radiodurans* (BAA-816). Open circles: *helD:cat* mutant. (B) Exponential phase cells were treated with 30 mM MMS, harvested, and resuspended in the same volume of fresh TGY, followed by serial dilution and plating on TGY plates. Closed circles: wild-type *D. radiodurans* (BAA-816). Open circles: *helD:cat* mutant. Open triangles: *E. coli* strain AB1157. Error bars in (A) and (B) are standard deviations of triplicate determinations.

methylates DNA bases at several positions, producing lesions that are generally repaired by direct repair or by base excision repair [47]. Methylated bases can also lead to dsb's by blocking replication fork progression [48]. Double-strand breaks require homologous recombination for their repair [16] and many *E. coli rec* mutants are sensitive to MMS [49]. Sensitivity of the *helD:cat* mutant to MMS could indicate that helicase IV_{Dr} acts in some aspect of homologous recombination or in processing stalled replication forks. However, the fact that the *helD:cat* mutant is not sensitive to IR shows that

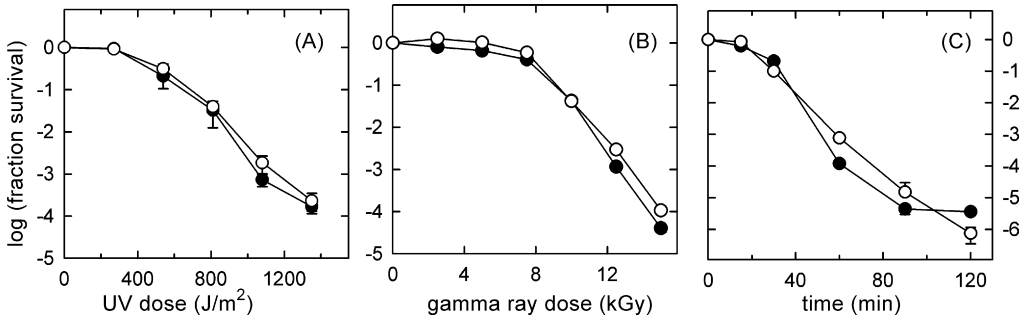

Fig. 5. Sensitivity of the *helD:cat* mutant to gamma and UV irradiation, and to mitomycin C. Wild-type *D. radiodurans* strain BAA-816 (filled circles) and *helD:cat* mutant (open circles) were grown to exponential phase and treated as follows. Error bars are standard deviations of triplicate samples. (A) After reaching OD_{600} = 0.5, cells were diluted and spread onto TGY plates and irradiated with a germicidal lamp at 90 J/(m^2 min). (B) Gamma irradiation was from a ^{60}Co source at 2.5 kGy/h. Cells were kept on ice during irradiation. (C) Cells were treated with 10 μg/ml MMC in TGY medium. Cells were harvested at the indicated times and resuspended with the same volume of fresh TGY, followed by serial dilution and plating on TGY plates.

helicase IV_{Dr} does not have an essential role in recombinational repair, which is thought to be the mechanism for dsb-repair in *D. radiodurans* [25].

The moderate phenotype of the *helD* mutation in *D. radiodurans* is similar to previous results with the *helD* and *yvgS* mutants in *E. coli* and *B. subtilis*, respectively. That work has indicated that the enzyme may act in the RecF pathway for DNA repair and homologous recombination [27,50,51]. The RecF pathway was first characterized in *E. coli* cells with mutations that inactivate RecBCD, exonuclease I (*sbcB*), and the SbcCD nuclease. The pathway requires the proteins encoded by several genes, including *recF*, *recO*, *recJ*, and others [15]. Thus, while a *B. subtilis yvgS* mutant in a wild-type background is slightly sensitive to MMS, a *yvgS recF* double mutant is less sensitive to MMS than the *recF* single mutant. The result suggests that YvgS produces DNA repair intermediates that require the RecF pathway for further processing and complete repair [27]. In the absence of YvgS, the damage from MMS is repaired by other pathways in *B. subtilis*. A *helD* mutation in *E. coli* has little or no effect on homologous recombination and resistance to UV or MMS in a wild-type genetic background. However, a *helD* mutation along with a mutation in *uvrD* or both *uvrD* and *recQ* substantially reduced the level of recombination in *recBC sbcB sbcC* cells, in which the recF pathway is operative [50,51]. The *helD* mutation had little effect on UV or MMS sensitivity in this genetic background [50,51].

The apparent role of helicase IV and YvgS in the RecF pathway is intriguing since *D. radiodurans* naturally lacks both RecBCD and exonuclease I (*sbcB*), and it has been suggested that the organism may use the RecF pathway to repair double-strand DNA breaks [52]. Indeed, mutation of one RecF pathway gene (*recO*) significantly reduced the radioresistance of *D. radiodurans* [53]. However, unlike in *E. coli* where inactivation of the SbcCD nuclease is necessary for RecF pathway function, *D. radiodurans* has *sbcCD* genes and, presumably, active SbcCD nuclease. Mutational inactivation of *sbcC* or *sbcD* renders *D. radiodurans* slightly more radiosensitive than the wild-type [54]. The MMS-sensitivity of the *D. radiodurans helD:cat* mutant, in a wild-type background, is also somewhat different from the phenotype of its putative *E. coli* and *B. subtilis* homologues. The role of helicase IV_{Dr} and the overall DNA repair pathways thus have properties that are unique to *D. radiodurans* as compared to these other, better-studied, bacteria.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2008.12.011.

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