The Stable, Functional Core of DdrA from *Deinococcus radiodurans* R1 Does Not Restore Radioleresistance In Vivo

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DdrA protein binds to and protects 3' DNA ends and is essential for preserving the genome integrity of *Deinococcus radiodurans* following treatment by gamma radiation in an environment lacking nutrients. Limited proteolysis was used to identify a stable and functional protein core, designated DdrA157, consisting of the first 157 residues of the protein. In vitro, the biochemical differences between wild-type and mutant proteins were modest. DdrA exhibits a strong bias in binding DNA with 3' extensions but not with 5' extensions. The mutant DdrA157 exhibited a greater affinity for 5' DNA ends but still bound to 3' ends more readily. However, when we replaced the wild-type *ddrA* gene with the mutant gene for *ddrA157*, the resulting *D. radiodurans* strain became almost as sensitive to gamma radiation as the *ddrA* knockout strain. These results suggest that while the stable protein core DdrA157 is functional for DNA binding and protection assays in vitro, the carboxyl terminus is required for important functions in vivo. The C terminus may therefore be required for protein or DNA interactions or possibly as a regulatory region for DNA binding or activities not yet identified.

The bacterium *Deinococcus radiodurans* is a polyextremophile with a noted ability to survive exposure to high doses of ionizing radiation (IR) (5). The capacity of *D. radiodurans* to survive DNA damage caused by IR has been attributed to its equally impressive ability to tolerate desiccation. Both IR and desiccation severely damage DNA (22, 29). In a cellular environment, both stresses would lead to damaged proteins, lipids, and nucleic acids, as well as an increased formation of reactive oxygen species.

*D. radiodurans* has a shoulder of IR resistance out to 5,000 Gy, where there is no measurable loss in viability (2). This level of IR reduces the genome of *D. radiodurans* to hundreds of DNA fragments as shown by pulsed-field gel electrophoresis (11). The amount of energy from 5,000 Gy of gamma radiation introduces thousands of DNA lesions, including hundreds of double-strand breaks (24). Therefore, the ability of *D. radiodurans* to survive IR treatment has been strongly linked to an enhanced capacity to repair DNA.

Survival after IR requires the de novo synthesis of proteins (6). In addition, transcriptome analysis following the same treatment reveals an upregulation of many gene products whose functions are unknown (18, 27). Together, these data suggest that a checkpoint exists within *D. radiodurans*, following IR treatment. Those proteins that are upregulated have been one focus of investigation in the *D. radiodurans* field in recent years.

The DdrA protein of *D. radiodurans* is among a group of the most highly upregulated gene products, following IR treatment (13). Previously, we had characterized DdrA as a component of a DNA end protection system in *D. radiodurans* that would prevent nucleolytic degradation of DNA in the absence of nutrients. In the context of a desiccated environment, the DdrA protein may be a critical component in maintaining genome integrity.

Jolivet and colleagues have extended this work, demonstrating that the presence of DdrA is especially important for survival when the essential recombinant RecA is either not present or present at low concentrations (15). RecA actively repairs the genome by the homologous recombination of DNA substrates generated after a phase of extensive DNA synthesis (31). When the amount of RecA protein is reduced, recombination still progresses, albeit at a slower rate. However, without the DdrA protein, the ability to repair is diminished due to the degradation of the chromosomes before recombinational DNA repair can commence. This work supports the idea that DdrA functions to preserve the genome following heavy damage.

In the present work, we have begun an exploration of DdrA structure-function relationships in an attempt to gain a better understanding of this protein's role in DNA repair. We present the identification of a stable protein core consisting of the first 157 residues of the 208-amino-acid protein DdrA. We designated the stable core DdrA157. In vitro, the stable core showed a functional ability to bind and protect DNA from degradation. However, the mutant *ddrA157* gene complemented the wild-type *ddrA* gene in vivo only modestly.

MATERIALS AND METHODS

Cloning, overexpression, and purification of DdrA and DdrA157. Both DdrA and DdrA157 were purified using the same methods. The gene was amplified using the genomic DNA from *Deinococcus radiodurans* strain R1. PCR primers were designed according to the gene sequence annotated in the genomic bank (http://www.ncbi.nlm.nih.gov). The gene was cloned and expressed in *Escherichia coli* plasmid pEAW298 (DdrA) or pDRH2 (DdrA157). Plasmids were transformed into the *E. coli* expression strain BL21(DE3). Ten-liter cultures were grown to the mid-log phase at 37°C (optical density at 600 nm [OD 600], ~0.6), and expression of the protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM, followed by outgrowth for 3 h at 37°C. Cells were collected by centrifugation at 8,000 rpm and 4°C using the Beckman JLA8.1 rotor and the J20-I Avanti centrifuge. The DdrA and mutant proteins were identically purified. Overproducing cells were lysed by the

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addition of lysozyme to 0.5 mg/ml, followed by five 1-min cycles of 0.5-s pulse sonication using the Fisher model 500 Sonic Dismembrator. The protein was precipitated from the supernatant by adding ammonium sulfate to 30% saturation. The proteins were purified with diethylaminoethyl and hydroxyapatite chromatography to >99% purity. Protein samples were concentrated by ammonium sulfate precipitation, resuspended, and dialyzed into elution buffer (25 mM Tris-acetate, 80% cation-10% glycerol-500 mM NaCl-1 mM DTT) and stored at −80°C. Protein concentrations were calculated by using determined (nat) extinction coefficients (DdrA, ε280,nat = 2.8728 × 10−1 cm−1 M−1; DdrA157, ε280,nat = 3.0521 ± 0.0203 × 10−1 cm−1 M−1).

**Native extinction coefficient determination of purified DdrA and DdrA157.** Our procedures for determining the native extinction coefficient have been described previously (13). Briefly, the extinction coefficients for DdrA and DdrA157 proteins were determined using a modification of a published procedure (10). Extinction coefficients were determined in storage buffer by comparing the absorbance spectra of the native protein to the absorbance spectra of the protein denatured in 6 M guanidine hydrochloride in storage buffer. Absorbance spectra of native and denatured protein were scanned at 25°C, from 320 to 240 nm, for five different dilutions and with two different protein preparations. Protein stocks were diluted in storage buffer or storage buffer plus 6 M guanidine HCl (final concentration) in a total volume of 80 μl and were preincubated at 25°C for 15 min before each scan. Each dilution was carried out in triplicate. The absorbance (Abs) levels at 280 nm were averaged. The concentrations of native and denatured (denat) protein were equal to each other in each scan at each dilution. The extinction coefficient of native protein at 280 nm was determined according to the expression $A_{280} = ε_{280,nat} \times C \times V$ (where $A_{280}$ is the Abs, $ε_{280,nat}$ is the extinction coefficient at 280 nm, $C$ is the protein concentration, and $V$ is the volume in cm).

**Limited proteolysis of purified DdrA and DdrA157.** Purified DdrA and DdrA157 proteins were subjected to proteolysis by the nprospec type protease subtilisin (Sigma). In the presence of a molar ratio of 1:100 of protease to protein (0.3 μM) to protein (30 μM), 60-μl samples were incubated at 30°C for a period of 3 h in a buffer containing 40 mM Tris-acetate (80% cation)-10% glycerol (wt/vol)-0.1 mM EDTA-0.1 mM DTT and stored at −80°C. Protein concentrations were calculated by using determined (nat) extinction coefficients (DdrA, ε280,nat = 2.8728 ± 0.1999 × 10−1 cm−1 M−1; DdrA157, ε280,nat = 3.0521 ± 0.0203 × 10−1 cm−1 M−1).

**Fluorescence anisotropy.** Purified DdrA or DdrA157 was serially diluted in dilution buffer (40 mM Tris-acetate [80% cation]-5% glycerol [wt/vol]-0.1 mM NaCl-0.1 mM EDTA-0.1 mg/ml acetylated bovine serum albumin, 1 mM DTT). Diluted proteins were incubated with 1 mM DNA substrate for 30 min at room temperature in a total reaction mixture volume of 100 μl. The fluorescence polarization of each sample was measured at 25°C using a PanVera Beacon 2000 fluorescence polarization system with 490- and 535-nm excitation and emission wavelengths, respectively. The fraction of the substrate bound in each sample was calculated by using the anisotropy value of the fluorescein-labeled substrate in buffer alone as 0% bound and setting the anisotropy value at the highest protein concentration to 100% bound. In all cases, the 100% bound value was easily obtained because the plateau of binding was reached and well surpassed at the concentrations used. Reactions were performed in triplicate. Uncertainty values for the estimated $K_{d}$ (apparent $K_{d}$) were calculated by determining the standard deviation of the average $K_{d}$ from three independent trials.

**EMSA.** EMSA for DNA binding were carried out in 15-μl reaction mixtures containing the reaction buffer (40 mM Tris-acetate, pH 7.5, 10% glycerol [wt/vol], 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT) and 20 mM DNA substrate. The reaction was initiated by adding either DdrA or DdrA157 protein to the indicated concentration. The reaction mixture was incubated at 30°C for 30 min and loaded onto 10% native polyacrylamide gel. When indicated, reaction mixtures were deproteinized by the addition of SDS and 20 mg/ml proteinase K (Sigma) to final concentrations of 0.3% and 2 mg/ml, respectively. The electrophoresis was performed in 1× Tris-borate-EDTA buffer (89 mM Tris-borate, pH 8.3, 2 mM EDTA) at 4°C. After the electrophoresis was complete, the DNA was visualized using the Amersham Typhoon imaging model 9410.

**Exonuclease protection assay.** The duplex with a 3′ extension was prepared as described using the 20-nt ssDNA extension in the 3′ orientation. When DdrA or DdrA157 protein was present, the DNA and protein were preincubated for 10 min at 30°C before exonuclease was added. Reaction mixtures were 15 μl in volume and contained the exonuclease reaction buffer (40 mM Tris-acetate, pH 7.5, 0.1 M NaCl, 10 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 10% glycerol). Exonuclease I (ExoI) was added to 200 U/ml, and the reaction mixture was incubated at 30°C for 30 min. When indicated, reaction mixtures were deproteinized with 0.3% SDS and 2 mg/ml proteinase K at 30°C for 15 min. The DNA-protein complexes were resolved in the native polyacrylamide gel as above.

**Strains, growth conditions, and treatment.** All strains derived from *D. radior- durus* were grown at 30°C in tryptone-glucose-yeast extract (TGY) broth (0.5% tryptone, 0.1% glucose, 0.5% yeast extract) or on TGY agar (1.5% agar). *E. coli* strains were grown in LB broth or on LB agar (1.5% agar) at 37°C. Plasmids were routinely propagated in *E. coli* strain DH5αMCR. Overexpression plasmids were derived from the Novagen PET21A expression vector using the multiple cloning sites.

**D. radiodurus cultures were evaluated for their abilities to survive exposure to gamma radiation in exponential growth (OD595 = 0.15 to 0.20; 5×10^3 to 1×10^5 CFU/ml). All cultures were treated at 25°C. Gamma irradiation was con- ducted using a model 5000 (Fernando, CA) at a rate of 18 Gy/min. The concentration of CFU per milliliter prior to irradiation was determined by serially diluting the culture with 10 mM Tris-acetate, 80% cation (pH 7.5)-10 mM MgSO4 and plating 100 μl on 100-15 mm TGY agar plates. The plates were incubated at 30°C for at least 48 h before counting CFU. For each dilution, three plates were spread and counted to ensure accuracy. Following treatment by gamma radiation, survival was deter- mined by repeating the procedure of diluting and counting (CFU per milliliter), with the exception of extending the incubation of the TGY agar plates to at least 72 h prior to counting. Viability was determined by dividing the CFU per milliliter of the culture after treatment by the CFU per milliliter of the initial culture, as seen in the following equation: Survival = (CFU/mlfinal)/CFU/mlinitial).**

**Construction of strains TNK104, HD31, and HD32.** The construction of TNK104 (ddrA::katAp) has been described previously (13). Strains HD31 (ddrA::katAp::aadA) and HD32 (ddrA157::katAp::aadA) began with the drug cassette capable of conferring spectinomycin resistance on *D. radiodurus*. The cassette was PCR amplified from the pTNK103 vector (as previously described [13]) using primers that overlap the termination region of either the *ddrA* or *ddrA157* sequence, as well as the downstream genomic region in *D. radiodurus*. Genomic DNA from *D. radiodurus* strain RI was used to amplify *ddrA* or *ddrA157* as well as 500 bp of downstream genomic DNA. Subsequently, each set of the PCR fragments was cloned into the PCR-amplified cassette using the extension. Hybrid fragments were cloned into PET21A (Novagen) using the multiple cloning sites, creating pDRH31 (ddrA::katAp::aadA) and pDRH32 (ddrA157::katAp::aadA). Chromosomal replacement of the native *ddrA* gene was accomplished by...
transforming purified pDRH31 or pDRH32, which had been linearized by NdeI and HindIII, into early-log-phase *D. radiodurans* R1 cells. Positives for replacement were selected for resistance to 75 μg/ml spectinomycin. Because of the multiplicity of *D. radiodurans*, isolates were grown to the stationary phase in the presence of spectinomycin and spread again to select single colonies that resist 75 μg/ml spectinomycin. To confirm gene replacement, primers that align to the beginning coding sequence of *ddrA* and to the downstream genomic region were used to generate PCR fragments using genomic DNA from spectinomycin-resistant colonies and R1. The purified PCR products were restricted with BsmI or Acul. The *katAp* promoter contains an Acul site, while the wild-type *ddrA* does not. Moreover, the difference between *ddrA*-katAp::aadA and *ddrA157*-katAp::aadA could be visualized by separation on a 1% agarose gel. Both amplification and digestion of said products resulted in bands corresponding to predicted sizes (see Fig. 4). We concluded that strains DHD31 and DHD32 carried the *ddrA* or *ddrA157* gene, respectively, followed by the marker katAp::aadA and that the strains were homozygous for the replacement.

**Western blot analysis.** *D. radiodurans* strains R1, TNK104, DHD31, and DHD32 were grown in 200 ml of TGY medium and the appropriate antibiotics at 30°C to the log phase (OD600 of 0.17 to 0.2) and centrifuged in a Beckman JLA16.25 rotor at 38,400 × g for 15 min. The pelleted cells were resuspended in fresh TGY medium and transferred to 15-ml conical vials for irradiation. Gamma radiation was applied in a J. L. Shepherd Mark I 137Cs source irradiator for 64 min to 500 Gy. After another round of centrifugation, the cell pellets were resuspended in 500 μl of butanol-saturated phosphate-buffered saline (PBS) and incubated for 20 min at room temperature. The cells were spun down, and the supernatant was removed. They were then resuspended in a solution of 50 μl of 1× PBS and protease inhibitors (leupeptin, pepstatin, and E64; Sigma-Aldrich) and boiled for 10 min with Laemmli buffer. Twenty microliters of the lysate, as well as 17.9 ng of boiled purified DdrA and 22.4 ng of boiled purified DdrA157 as markers, were loaded onto a 4 to 20% gradient Precise Protein gel from Pierce. The gel was transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore) at 350 mA for 2 h and blocked in 5% skim milk in PBS-0.1% Tween solution. The membrane was incubated in a 1:5,000 dilution of rabbit anti-chicken horseradish peroxidase secondary antibody (Genetel Laboratories, Madison, WI) in blocking buffer. The membrane was washed in PBS and 0.1% Tween and then incubated in PBS-Tween and 1:20,000 diluted rabbit anti-chicken horseradish peroxidase secondary antibody (Sigma-Aldrich). The membrane was washed again and visualized with Thermo Scientific SuperSignal West Pico enhanced chemiluminescent substrate on Kodak BioMax light film.

**RESULTS**

**DdrA protein has a stable core consisting of the first 157 residues.** We used proteolysis to study the functional domains of the DdrA protein. Purified DdrA protein was subjected to limited proteolysis by subtilisin, a nonspecific protease. Initially, a time course was taken to observe protein degradation and identify degradation intermediates. Degradation of DdrA protein led to a single protein band corresponding to a stable protein core (Fig. 1). Even under dilute concentrations of protease and incubation on ice, we did not observe other intermediates (data not shown). We used the mass spectrometry facilities of the UW–Madison Biotech Center to identify the products. The single most abundant product strictly correlated to the first 157 residues of the 208-amino-acid protein (*DdrA157* expected mass, 17,428.7 Da; observed mass, 17,432 Da). Hence, the protein core and later construct have been designated DdrA157. The amino-terminal methionine is retained in *DdrA157* expected mass, 17,428.7 Da; observed mass, 17,432 Da). Therefore, the protein core and later construct have been designated DdrA157. The amino-terminal methionine is retained in this protein. No other products were present in significant amounts.

**The purified DdrA157 protein resists degradation by subtilisin and maintains self-association abilities in solution.** The *ddrA157* gene was cloned and expressed in *E. coli*, and then the protein was purified to homogeneity (Fig. 1). The identity of the purified DdrA157 was verified by accurate mass spectrometry and trypsin digest fingerprinting of a protein sample (42% coverage) (UW–Madison Biotech Center).

**To further test DdrA157 as a stable protein core, the purified truncated protein was subjected to the same proteolysis procedure as DdrA. Both DdrA and DdrA157 were subjected to a 3-h incubation with a 1:100 ratio of protease to protein at 30°C.** Mass spectrometry analysis of proteolyzed DdrA and DdrA157 samples in both cases revealed a single protein band correlating to the first 157 residues (Fig. 1). The DdrA157 construct thus represented the stable protein core of the DdrA protein.

In separate experiments not shown, a construct for the first 166 residues was also cloned and expressed into *E. coli* and the protein was purified to homogeneity. The product, designated DdrA166, was also proteolyzed, again yielding a fragment containing the first 157 residues upon subtilisin treatment (data not shown).

During purification of all three proteins (DdrA, DdrA157, and DdrA166), a calibrated high-resolution Sephacryl S-300 column was used. All three proteins eluted from the column at a volume that reflects a mass of approximately 150,000 Da (truncation mutants) or 200,000 Da (wild-type protein), suggesting that the protein exists in solution as a complex of 8 to 10 monomers (data not shown). This indicates that the stable protein core has not lost the ability to self-associate in solution.

**DdrA157 protein is functional for DNA binding, although it possesses a diminished bias for 3’ extensions relative to that of the wild-type DdrA.** The wild-type DdrA protein binds to ssDNA (13). DdrA157 also bound ssDNA substrates (Fig. 2A), Fluorescence anisotropy experiments were carried out to determine relative binding affinities for ssDNA substrates of various lengths. The results have been tabulated in Table 1. The data suggest that DNA length influenced the ssDNA binding affinity for both proteins. Affinity for ssDNA increased (lower $K_{d,app}$) with increased length. Slightly lower $K_{d,app}$ levels were observed in all cases for the mutant DdrA157 protein. Thus, both the DdrA and DdrA157 proteins are capable of binding ssDNA at very similar affinities, and the affinity is proportional
to the length of ssDNA substrate over the range of lengths employed (up to 50 nt).

Binding to DNA substrates was further examined by an EMSA. Binding to dsDNA required the presence of an ssDNA extension for both proteins (Fig. 2B). As previously shown, DdrA preferentially bound to dsDNA with a 3' extension (Fig. 2C and D). DdrA minimally bound to DNA substrates with 5' single-strand extensions only when the extensions were 40 nt in length or longer (Fig. 2E and F).

The mutant DdrA157 also readily bound to dsDNA with a 3' extension with an affinity similar to that of DdrA (Fig. 2C and D). However, when present at sufficient concentrations, DdrA157 shifted 100% of the added dsDNA with longer 5' extensions (Fig. 2E and F). This represents an increased affinity for the 5' extension substrate. The diminished bias for 3' extensions is discussed below.

Both DdrA and DdrA157 bind DNA and protect substrate from degradation by ExoI. ExoI from *E. coli* digests ssDNA from the 3' end (16). The 20-nt 3' tail substrate used in the EMSA experiment (Fig. 2) was incubated with either DdrA or DdrA157, followed by treatment with ExoI. Samples were deproteinized to evaluate the degradation by ExoI. Both DdrA and DdrA157 were able to protect dsDNA with 3' single-strand extensions from degradation by ExoI (Fig. 3). ExoI alone was able to efficiently degrade the substrate. However, in the presence of DdrA or DdrA157, degradation was prevented. This result illustrates that the protein core, DdrA157, is able to protect DNA from degradation by exonucleases.

The *D. radiodurans* strain with the *ddrA157* gene replacing *ddrA* is almost as sensitive to gamma radiation as the strain completely lacking *ddrA* function. To investigate the effect of the mutant DdrA157 protein in vivo, we generated a construct to replace the *ddrA* gene with the *ddrA157* gene using the katAp::aadA drug cassette for spectinomycin resistance, followed by downstream genomic DNA sequence (DHD32). To determine if the drug cassette would affect the survival of *D. radiodurans*, a control construct with the wild-type *ddrA*–katAp::aadA was created and studied as well (DHD33). To investigate the effect of the mutant DdrA157 protein in vivo, we generated a construct to replace the *ddrA* gene with the *ddrA157* gene using the katAp::aadA drug cassette for spectinomycin resistance, followed by downstream genomic DNA sequence (DHD32). To determine if the drug cassette would affect the survival of *D. radiodurans*, a control construct with the wild-type *ddrA*–katAp::aadA was created and studied as well (DHD33). The spectinomycin drug cassette did not affect the survival of *D. radiodurans* R1 (Fig. 5). The strain lacking *ddrA* (TNK104)

![FIG. 2. The mutant DdrA157 retains its DNA binding ability with a diminished bias for 3' extensions. Proteins, either DdrA or DdrA157, were incubated with the indicated DNA substrate for 30 min at 30°C. The asterisk indicates a fluorescein label (Integrated DNA Technologies). Protein concentrations in each experiment were 0, 1, 5, 10, and again 10 μM from left to right. Reactions were stopped, and proteins were removed from DNA when indicated. Samples were loaded onto a native 10% acrylamide gel (1x Tris-borate-EDTA) and visualized using the Amersham Typhoon imaging scanner. DNA bands were quantified using TotalLab software. Each experiment was repeated at least three times and is graphically represented in the left column. For each set of experiments, the EMSA results shown in the second and third panels are plotted in the first panel. The substrates used are single-strand and duplex oligonucleotides (A and B). Duplex substrates with different lengths of 3' extension (C and D) or 5' extension (E and F) were also examined. ProtK, proteinase K.](#)
was radiosensitive relative to the wild-type strain, and results were identical to those achieved previously (13). *Deinococcus radiodurans* containing the mutant gene *ddrA157* (DHD32) and not *ddrA* exhibited sensitivity to gamma radiation that was almost as great as that of the strain lacking a *ddrA* gene (Fig. 5). These data suggest that the stable and functional core of the DdrA protein is not sufficient to supply all requirements for function in vivo.

We assessed the expression of DdrA and DdrA157 in our strains after irradiation to 500 Gy. This was done to ensure that the lack of restoration of radiation resistance by DdrA157 was not due to a lack of expression. As seen in Fig. 5, DdrA and DdrA157 proteins were expressed at comparable levels in each of the two gene replacement strains DHD31 (DdrA) and DHD32 (DdrA157), levels that were also comparable to those observed in the wild-type *Deinococcus radiodurans* R1. The

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**FIG. 3.** The mutant DdrA157 retains the ability to protect 3' ends from degradation by ExoI. Reactions were carried out as described in Materials and Methods. This set of reaction mixtures uses a labeled 30-bp duplex with a 3' extension of 20 nt (as shown in Fig. 2C). Reaction mixtures contained either DdrA (left) or DdrA157 (right). The first and second lanes contain DNA before and after treatment with ExoI from *E. coli*. The third and fourth lanes show the DNA after the addition of DdrA or DdrA157, followed by ExoI. The fifth lane shows the DdrA- or DdrA157-protected DNA after protein removal. ProtK, proteinase K.

**FIG. 4.** Verification of gene replacement. Genomic DNA was isolated from *Deinococcus radiodurans* R1, DHD31, and DHD32. PCR fragments were amplified using primers that anneal to the *ddrA* coding sequence (5'-GTTCGGCATCGGGGT-3') and to downstream genomic DNA (5'-GCCGCTCGGAATGAGG-3'). The same primers were used for all amplifications. Purified fragments were digested by BsmI (R1 strain) or AclI (DHD31 and DHD32 strains). Products were separated on a 1% agarose gel. In each set of three, lane 1 is the PCR product, lane 2 is the gel-purified PCR fragment, and lane 3 is the purified fragment after the indicated digestion. Lane M is a marker of 2-log ladder fragments (New England Biolabs). All fragments corresponded to predicted sizes, indicating successful homozygous replacement of the *ddrA* gene.

**FIG. 5.** The stable and functional core of DdrA, designated DdrA157, cannot complement a *ddrA* deletion mutant in vivo. All strains were subjected to identical levels of IR. These survival curves are for *D. radiodurans* R1 (closed circles), TNK104 ΔddrA (closed squares), DHD31 *ddrA-katAp:aadA* (open circles), and DHD32 *ddrA157-katAp:aadA* (open squares) following gamma radiation. Survival values for the strains are based on at least three independent experiments (n = 9).
DISCUSSION

*Deinococcus radiodurans* resists the lethality of IR by a complex mechanism of damage response and DNA repair. Because of the lack of strong IR sources in the natural environment, it is postulated that the resistance to IR arose from the capacity of *D. radiodurans* to tolerate the equally detrimental effects of desiccation (19, 28).

Desiccation is damaging in at least two ways. First, an arid environment limits the availability of nutrients and raw material for active metabolism and repair. Organisms from many branches of life have adapted to this condition by forming spores, which are metabolically inert (7). Spores can remain inert for long periods of time without an active metabolism and therefore need no nutrients (30). *D. radiodurans* does not form spores and hence requires nutrients for active repair and survival (1, 20).

Secondly, desiccation is damaging to cellular components. The removal of water from the cell can render proteins inactive and distort the shape of nucleic acids (4, 7, 22). As water is removed, reactive oxygen species have much higher propensities to damage the proteins, lipids, and DNA. *D. radiodurans* is no exception. DNA damage is very evident following desiccation (3, 19, 31).

We have come to use survival following gamma radiation as a direct reflection of the DNA repair capacity of *D. radiodurans*. In essence, the damage caused by gamma radiation and desiccation are very similar. However, the presence of nutrients during recovery is of importance. In a desiccated environment, it would be expected that *D. radiodurans* would be starved for nutrients. We have already proposed that in this context DdrA plays a role in maintaining genome integrity (13). By acting as a component in a DNA end-capping mechanism, DdrA would not actively repair the fragmented genome. Instead, DdrA preserves the genetic material for future repair when rehydration makes nutrients available and repair possible.

Here we continued our investigation of DdrA from *D. radiodurans*. In an attempt to characterize domain structure and function, we identified a stable protein core, designated DdrA157 (Fig. 1). Our characterization of the stable protein core revealed that in many ways DdrA157 is functional. In vitro, our experiments revealed only modest differences in the activities between the wild-type DdrA and mutant DdrA157 proteins. Both proteins were able to bind DNA substrates with similar affinities (Table 1 and Fig. 2). In addition, both proteins were able to protect the 3' extension of a dsDNA substrate.
from degradation (Fig. 3). The principal difference between the wild-type and mutant protein was that the mutant DdrA157 had a much higher affinity for dsDNA with a 5′ extension compared to that for the wild-type protein (Fig. 3C to F).

However, in vivo analysis showed that DdrA157 complemented the function of the wild-type DdrA only minimally. Survival data illustrated that a D. radiodurans strain carrying the ddrA157 gene in place of the ddrA gene was almost as sensitive to gamma radiation as a strain of D. radiodurans that completely lacked ddrA function (Fig. 5). The inability of DdrA157 to restore radiation resistance was not due to a lack of expression (Fig. 6).

These results suggest a possible role for the carboxyl terminus of the DdrA protein. Western blot analysis of cell extracts has been complicated by a cross-reacting band at the same position as that of purified DdrA protein. However, we do see production of the mutant DdrA157 protein by comparing both the ddrA knockout and wild-type D. radiodurans to the mutant construct. Neither the presence nor the stability of the DdrA157 protein seems to be a limiting factor, and we propose that the decreased radioresistance is due to altered activities of the DdrA157 protein in vivo.

One plausible explanation is that the diminished bias for 3′ extensions is disruptive to the function of DdrA in vivo. If DdrA157 initiated “promiscuous” DNA binding, two possibilities can be envisioned: (i) the protein is depleted from sites where it is required, or (ii) binding in the secondary location is detrimental to proteins that typically bind the stated location. Either situation could lead to a phenotype where the ability of DdrA to function appears to be lost.

The carboxyl terminus of DdrA could also be required for an interaction with other proteins in preserving genome integrity or repairing DNA. DNA maintenance and repair is a complex process. The ability to regulate the response has been achieved in many organisms by temporal and specialized association and recruitment of protein factors (8, 9, 17, 21, 23, 25, 26). By removing the carboxyl terminus, an undefined role in recruitment by DdrA protein could have been lost. DdrA157 would be able to bind and protect DNA but would not be able to recruit key players required for the active repair of DNA ends.

Finally, DdrA may have a function not yet identified in our in vitro assays, an activity that requires the C-terminal part of the protein. DdrA is distantly but specifically related to the Rad52 family of eukaryotic proteins as well as to a family of phage-associated proteins that mediate single-strand annealing (14). However, among bacteria, the protein appears to be limited to the Deinococccaeae (12). It is possible that DdrA could be a component of a single-strand annealing system and that activities associated with its in vivo abilities have not been identified. For example, DdrA has not been shown to possess strand annealing or strand-exchange activity. However, new interacting protein partners may bring new activities to the complex. In this instance, using the DdrA157 mutant may reveal a compromised capacity in activities not yet identified.

Evidence to date indicates that DdrA is involved in an early and RecA-independent phase of genome reconstitution in members of the Deinococcus (27). In trials to date, we have not found any evidence for an interaction between DdrA and the RecA protein of Deinococcus spp. (data not shown). An extended DNA synthesis-dependent strand-annexing process has been proposed for this early phase (31) and may provide a context for DdrA function.

The role of DdrA in our proposed DNA end-capping mechanism following gamma radiation is critical for preserving the genome integrity. DNA repair in D. radiodurans after radiation damage is biphasic, with RecA-independent pathways, followed by RecA-dependent repair. The ability of RecA protein to actively repair the DNA relies on the presence of recombination substrates, and DdrA has been shown to be involved in the maintenance of such substrates (15). With our mutant DdrA157 protein, we have shown that an ability to bind recombination substrates does not completely restore radioresistance in vivo and suggests that the C-terminal region has a role in either the function or regulation of the DdrA protein in D. radiodurans DNA maintenance. Further work will be needed to identify the function of the C-terminal portion of the protein and to better ascertain the range of functions of this protein in vivo.

While this paper was in revision, an article appeared describing the DdrA protein of Deinococcus deserti, the amino acid sequence of which is 80% identical to that of the DdrA protein of Deinococcus radiodurans (12). A core domain of 160 amino acids was isolated from the D. deserti DdrA protein, with properties that parallel those reported here. Expression of the D. deserti DdrA protein, but not the truncated protein, complemented the reduced radioresistance in a ddrA deletion strain of D. radiodurans. The core domain retains the capacity to form ringlike heptameric oligomers (12).

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REFERENCES


