Effect of a recD Mutation on DNA Damage Resistance and Transformation in Deinococcus radiodurans

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The bacterium Deinococcus radiodurans is resistant to extremely high levels of DNA-damaging agents such as UV light, ionizing radiation, and chemicals such as hydrogen peroxide and mitomycin C. The organism is able to repair large numbers of double-strand breaks caused by ionizing radiation, in spite of the lack of the RecBCD enzyme, which is essential for double-strand DNA break repair in Escherichia coli and many other bacteria. The D. radiodurans genome sequence indicates that the organism lacks recB and recC genes, but there is a gene encoding a protein with significant similarity to the RecD protein of E. coli and other bacteria. We have generated D. radiodurans strains with a disruption or deletion of the recD gene. The recD mutants are more sensitive than wild-type cells to irradiation with gamma rays and UV light and to treatment with hydrogen peroxide, but they are not sensitive to treatment with mitomycin C and methyl methanesulfonate. The recD mutants also show greater efficiency of transformation by exogenous homologous DNA. These results are the first indication that the D. radiodurans RecD protein has a role in DNA damage repair and/or homologous recombination in the organism.

The bacterium Deinococcus radiodurans is one of the most resistant to ionizing radiation (IR) and other DNA-damaging agents of all known organisms (2, 6, 39). D. radiodurans DNA is extensively fragmented after treatment with IR, but the broken chromosomes can be reassembled, and the organism survives except at very high radiation doses (2, 6, 49). D. radiodurans is also resistant to UV light and a variety of chemicals that damage DNA. The ability to survive extensive DNA damage is believed to be a consequence of resistance to desiccation, a condition that also leads to oxidative damage to DNA (35).

Several factors are believed to contribute to the extraordinary DNA damage resistance of D. radiodurans. The organism has multiple genome copies (21, 22), which presumably facilitates DNA repair via homologous recombination (49). In addition, its condensed nucleoid structure may keep broken DNA ends aligned for facile repair (31, 51). D. radiodurans also accumulates Mn^{2+} to high intracellular concentrations and has a low concentration of internal Fe^{2+}. Manganese ion is thought to act as an antioxidant to prevent oxidative damage to cellular components (7, 8, 17).

Much recent research has been directed at identifying and studying the DNA repair enzymes and pathways in D. radiodurans. Repair of double-strand breaks such as those which arise from IR or during replication of a damaged DNA template generally occurs via homologous recombination in bacteria (26–28, 38). The broken DNA ends are first attacked by a helicase/nuclease enzyme (RecBCD in Escherichia coli and AddAB in Bacillus subtilis) (5). The nuclease and helicase activities of the RecBCD enzyme process the broken DNA ends, and the RecBCD enzyme loads the RecA protein onto the resulting 3′-terminated single-stranded DNA strand. The RecA single-stranded DNA filament then binds to a homologous DNA duplex and initiates DNA strand exchange and recombination, leading ultimately to the repair of the DNA break.

Repair of IR-induced DNA damage in D. radiodurans is RecA dependent, since recA mutants are highly sensitive to IR and other DNA-damaging agents (9, 19). Interestingly, there are no identifiable homologues of genes that would encode either the RecB or RecC proteins in the sequenced D. radiodurans genome, nor are there genes related to addAB (48). However, the organism has a gene annotated as recD, which encodes a protein with amino acid sequence similarity to the RecD protein from E. coli and other bacteria. The function of the D. radiodurans RecD protein is unknown, since RecD proteins in other organisms have no known function except as part of the RecBCD enzyme.

RecD proteins share amino acid sequence motifs that are found in the enzymes grouped as superfamily I helicases (18). Genes that encode proteins closely related to RecD from E. coli have been identified in a number of bacterial genomes and are grouped in a single group of orthologues in the Clusters of Orthologous Groups database (accession number COG0507) (see http://www.ncbi.nlm.nih.gov/COG/) (45). Further analysis of these RecD protein sequences shows that they can be divided into two subfamilies based on the amino acid sequences N terminal to the conserved helicase motifs (42, 47). One subfamily (referred to as RecD1 in reference 42) includes RecD subunits of RecBCD enzymes. The second subfamily (RecD2 in reference 42) includes the D. radiodurans RecD protein and closely related proteins from a number of other bacteria including B. subtilis, Lactococcus lactis, Streptococcus pyogenes, and Chlamydia species (42, 47). Most of these bac-
teria lack a RecBCD enzyme and instead have the AddAB enzyme. *D. radiodurans* is unusual in that it has a RecD-like protein but neither RecBCD nor AddAB.

In previous work, we purified the *D. radiodurans* RecD protein and found that it is a DNA helicase capable of efficient unwinding of short double-stranded DNA (dsDNA) substrates (47). In this work, we have generated recD mutations in the *D. radiodurans* chromosome by insertional mutagenesis. The recD mutants are sensitive to irradiation with gamma rays and UV light and to treatment with hydrogen peroxide, but they are resistant to mitomycin C (MMC) and to methyl methanesulfonate (MMS). A recD mutant is also more transformable than the wild type in assays involving the uptake of DNA from the medium and incorporation into the chromosome by homologous recombination. These results indicate that RecD has a role in the extraordinary DNA damage resistance of *D. radiodurans*, and they provide the first indication of a role for any member of this RecD protein family in DNA metabolism.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Wild-type *D. radiodurans* strains R1 and BAA-816 were obtained from the ATCC, Manassas, VA, and from Michael Daly, Uniformed Services University of the Health Sciences, respectively. *D. radiodurans* strain LS18 (streptomycin resistant) was a gift from John Battista, Louisiana State University. *E. coli* strain DH5α was used for routine cloning procedures. TGY medium contains 0.5% tryptone, 0.3% yeast extract, and 0.1% dextrose. LB medium contains 1% tryptone, 0.5% yeast extract, and 1% NaCl. Antibiotics were added to *D. radiodurans* cultures as follows: 8 μg/ml kanamycin, 3 μg/ml chloramphenicol, and 50 μg/ml streptomycin unless stated otherwise. Antibiotics were added to *E. coli* cultures as follows: 30 μg/ml kanamycin and 100 μg/ml ampicillin.

**PCRs.** PCRs (50 μl) contained 100 ng genomic DNA template or 0.1 ng plasmid DNA template, 0.4 μM primers, and 200 μM deoxynucleoside triphosphates with Pfu DNA polymerase (Stratagene Corp.). Other conditions were as recommended by the DNA polymerase supplier. Primer sequences were listed in Table S1 in the supplemental material.

**Southern blotting.** Southern blotting was performed using genomic DNA isolated from wild-type *D. radiodurans* or recD mutants as described previously (13). The DNA was digested with appropriate restriction endonucleases (see figure legends) and subjected to agarose gel electrophoresis. DNA was transferred from the gel to uncharged nitrocellulose membranes (Optitran membrane; Schleicher & Schuell) by capillary transfer and fixed by baking at 120°C for 30 min. Digoxigenin-labeled probes were created with the PCR DIG Probe Synthesis kit (Roche). Hybridization was done at 42°C with shaking for 60 min after blocking with salmon sperm DNA, followed by several washes at 60°C. Detection was done using anti-digoxigenin-AP Fab fragments (Roche) and ECF substrate (catalog no. RPN5785; Amersham).

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**FIG. 1.** recD::kan mutant construction and verification. (A) Map of the recD gene region in the wild-type *D. radiodurans* chromosome (top) and after disruption by insertion of the kanamycin resistance (kan) gene (bottom). The kan gene replaced a 48-bp PshAI-SacI fragment within the recD gene. The recD gene is transcribed left to right, while kan is transcribed right to left, in this diagram. (B) PCRs were done with primers Prom1 and DRecD2 (lanes 1 and 2) and Prom1 and KanFor (lanes 3 and 4) using genomic DNA isolated from either wild-type (wt) or recD::kan mutant (mut) cells. Primer sequences are given in Table S1 in the supplemental material, and their annealing locations are indicated in A. (C) Southern blots. Genomic DNA isolated from the recD::kan mutant was digested with EagI (lane 1), EcoRI (lane 2), Apal (lane 3), and NcoI/PstI (lane 4) and displayed on a 0.9% agarose gel. The DNA was transferred onto a nitrocellulose membrane, hybridized to a kan gene probe (left-hand panel), stripped, and rehybridized to an undisrupted recD gene probe (right-hand panel). Molecular weights were determined from the signal due to nonspecific hybridization of the digoxigenin-labeled probe to molecular weight markers run in an adjacent lane (see Fig. S1 in the supplemental material). The expected band sizes are 2.3 and 2.5 kb (lane 1), 4.7 kb (lane 2), 4.4 kb (lane 3), and 6.1 and 2.7 kb (lane 4).
sites at the upstream and downstream ends, respectively. A 662-bp \textit{D. radiodurans} cam resistance gene (\textit{gene}). The \textit{gene} was am-
confirmed by PCR and by Southern blotting (see Results).

\textbf{recD} mutation. The \textit{gene} was am-

\textbf{Southern blots were visualized using a Storm PhosphorImager (GE Health-

\textbf{Creation of \textit{D. radiodurans} recD mutants.} Disruption of the \textit{recD} gene was accomplished by insertional mutagenesis (16). The kanamycin resistance gene and promoter region (\textit{gene}) were PCR amplified from the plasmid pCR-Blunt (Invitrogen Corp.) using primers that introduced SacII and PshAI sites. The \textit{D. radiodurans} \textit{recD} gene in plasmid pDr-RecD.pzt (47) and the \textit{kan} PCR product were digested with PshAI and SacII and ligated to create plasmid pRecDKanDis. Primers complementary to the ends of the \textit{recD} sequence (DRecD1 and DRecD2) (Fig. 1A and see Table S1 in the supplemental material) were used to PCR amplify the disrupted \textit{recD} gene (\textit{recD}:\textit{kan} gene) using pRecDKanDis as the template. The PCR product was used to transform \textit{D. radiodurans} strain R1 to kanamycin resistance as described previously (34) (\textit{recD}:\textit{kan} mutant 1).

We generated several versions of the \textit{recD}:\textit{kan} mutation to ensure that the phenotypic effects were due to the \textit{recD} disruption and not other mutation. First, we transferred kanamycin resistance to fresh wild-type cells by transforming \textit{D. radiodurans} strain BAA-816 with genomic DNA from \textit{recD} mutant 1 to make \textit{recD}:\textit{kan} mutant 2. We then PCR amplified the \textit{recD}:\textit{kan} gene using genomic DNA from \textit{recD}:\textit{kan} mutant 2 and again transferred it to fresh BAA-816 wild-type cells (\textit{recD}:\textit{kan} mutant 3). The results shown in Fig. 2 were obtained with \textit{recD}:\textit{kan} mutant 1, while the results in Fig. 3 to 6 were obtained using \textit{recD}:\textit{kan} mutant 3. The structure of each \textit{recD}:\textit{kan} disruption mutant was confirmed by PCR and by Southern blotting (see Results).

The \textit{gene} was also deleted completely and replaced by a chloramphenicol resistance gene (\textit{gene}). The \textit{gene} was in plasmid p11530 (29) was PCR amplified using Tag DNA polymerase (Fermentas) and ligated into the T/A cloning vector pCR2.1 (Invitrogen Corp.) to make pCR2.1-\textit{gene}. A 558-bp \textit{D. radiodurans} chromosomal DNA fragment upstream of the \textit{recD} gene was am-

\textbf{Fig. 2.} Growth of wild-type and \textit{recD}:\textit{kan} mutant \textit{D. radiodurans} strains. Cells were grown overnight (with kanamycin for the \textit{recD}:\textit{kan} mutant), diluted 1:200 into fresh TGY medium with no antibiotic, and shaken at 30°C. Samples were removed at the indicated times after dilution, and the OD\textsubscript{600} was measured with a Perkin-Elmer Lambda Bio spectrophotometer in a 1-cm-path-length cuvette. Closed circles, wild-type strain R1; inverted open triangles, \textit{recD} mutant.

\textbf{Southern blots were visualized using a Storm PhosphorImager (GE Health-

\textbf{Fig. 3.} Sensitivity of the \textit{recD}:\textit{kan} mutant to gamma and UV irradiation and to hydrogen peroxide. Closed circles, wild-type strain BAA-816; open inverted triangles, \textit{recD} mutant. (A) Gamma irradiation was from a \textit{60}Co source at 100 Gy/min on ice. (B) Cells were spread onto TGY plates and irradiated with UV light from a germicidal lamp at 90 J/m\textsuperscript{2}/min. (C) Cells were treated with hydrogen peroxide (0.03%; 8.8 mM) for the indicated times and then spread onto TGY plates. The results for gamma and UV irradiation (A and B) are from two independent experiments (\textit{n} = 6), while the results for hydrogen peroxide are from a single experiment (\textit{n} = 3).
used as described previously (34). Transformed cells were spread onto plates containing streptomycin (5 or 50 μg/ml) and scored as described above.

**Complementation experiments.** The *D. radiodurans recD* gene, along with 948 bp of DNA upstream of the *recD* start codon, was PCR amplified from genomic wild-type DNA and ligated into BamHI-cleaved pRAD1 (36) to make pRAD1- *recD*. The *recD* gene and upstream DNA inserted into this plasmid were sequenced, and no differences from the sequence in GenBank (accession number AE000513) were found. The pRAD1- *recD* plasmid was transformed into *D. radiodurans* cells (wild-type or *recD::kan* mutant strains) as described above, and transformants were selected for their ability to grow on chloramphenicol and kanamycin. Sensitivity of the *recD::kan* (pRAD1- *recD*) cells to UV irradiation was tested as described above.

**RESULTS**

Verification of *recD::kan* gene disruption. The presence of the desired *recD* gene disruption in the *recD::kan* mutant, and the absence of undisrupted wild-type *recD* genes, was verified by PCR and Southern blotting. Chromosomal DNA was isolated from wild-type and kanamycin-resistant colonies, and PCRs were done using primers Prom1 and DRecD2, which should anneal to both the wild-type and *recD::kan* mutant strains (Fig. 1A). The wild-type DNA gave the expected 2.2-kb fragment, while the *recD::kan* mutant gave the expected 3.2-kb fragment due to the insertion of the *kan* gene (Fig. 1B). The mutant gave the expected 1.6-kb fragment with primers Prom1 and KanFor, a primer specific for the *kan* gene, while no fragment was made from wild-type DNA with these primers (Fig. 1B).

The structure of the *recD::kan* mutant was confirmed by Southern blotting. Genomic DNA from the *recD::kan* mutant was digested with four different restriction endonucleases and analyzed with a *kan* gene probe. DNA fragments of the expected size for the desired *recD* gene disruption were detected for each enzyme used (Fig. 1C). The same membrane was stripped and reprobed with a *recD* gene probe. The fragments detected were as expected for the disrupted *recD* gene (and the same as those detected with the *kan* gene probe), with no detectable undisrupted, wild-type *recD* gene (Fig. 1C).

Growth of the *recD::kan* mutant. The *recD::kan* mutant strain grew at the same rate as the wild type and reached essentially the same final cell density (Fig. 2). Retention of the disrupted gene in the mutant cells, which were grown without kanamycin, was verified by PCR using genomic DNA isolated from the *recD::kan* cells at the end of the experiment (data not shown).

DNA damage sensitivity of the *recD::kan* mutant. The *recD::kan* mutant cells are more sensitive to UV and gamma irradiation than the wild type (Fig. 3A and B). The mutant exhibits a “shoulder” of resistance to moderate doses that is characteristic of wild-type *D. radiodurans*, but the mutant is more sensitive to higher doses of these types of radiation than the wild type. The *recD::kan* mutant is also more sensitive to treatment with hydrogen peroxide than the wild type (Fig. 3C). These results are the first indication that any member of the RecD2 protein subfamily (see the introduction) (42) is involved in DNA metabolism in any bacterium in which they have been identified. In contrast to the DNA-damaging agents shown in Fig. 3, *recD::kan* mutant cells are no more sensitive to MMC and to MMS than are wild-type cells (Fig. 4).

UV sensitivity of the Δ*recD::cam* deletion mutant. We attempted to generate a complete deletion of the *recD* gene starting with wild-type cells but were unsuccessful for unknown reasons. However, we were able to replace the disrupted *recD::kan* gene with a *cam* gene and, in the process, delete the entire *recD* gene sequence (see Materials and Methods). The structure of the *ΔrecD::cam* deletion mutation was verified by Southern blotting. A *cam* gene probe hybridized with chromosomal DNA fragments of the expected size from the deletion mutant, while no fragments were detected with either a *kan* gene or a *recD* gene probe (see Fig. S1 in the supplemental material) (data not shown). The *ΔrecD::cam* deletion mutant is as sensitive to gamma and UV irradiation as the *recD::kan* disruption mutant (Fig. 5).

Complementation of the *recD::kan* mutation. We next tested whether the UV sensitivity of the *recD::kan* mutant strain could be complemented by a plasmid-borne copy of the wild-type *recD* gene. We inserted the entire *recD* coding region, as well as a large portion of genomic DNA upstream of *recD*, into plasmid pRAD1, which replicates at low copy number in *D. radiodurans* cells. The *recD* gene in this plasmid should be expressed from its natural promoter, which we presume is present in the upstream DNA. The pRAD1-*recD* plasmid partially complements the UV sensitivity of the *recD::kan* mutant (Fig. 6).

We are uncertain why there is reproducibly partial complementation of the *recD::kan* mutation by plasmid pRAD1-*recD*. We considered the possibility that the observed phenotype of the *recD::kan* mutant could be the combined effect of the *recD* disruption mutation as well as an additional mutation in some gene other than *recD*. However, we believe that this is very unlikely for the following reasons: (i) the PCR analysis and Southern blots (Fig. 1) indicate that the *kan* gene insertion is...
It is conceivable that the insertion into the recD gene could have a polar effect on neighboring genes. However, an examination of the *D. radiodurans* genome sequence indicated that there are no other genes in the vicinity of recD that are likely to be involved in DNA metabolism. The nearest annotated open reading frame in the genome downstream of recD begins 229 bp after the predicted recD stop codon. This open reading frame, and the one that follows, would encode proteins of unknown function and with no significant similarity to other proteins in the GenBank database. It is thus very unlikely that the observed phenotype arises from an effect on expression of these putative proteins. The nearest gene upstream of recD is predicted to be transcribed in the opposite direction from recD and thus would be unlikely to feel a polar effect of recD gene disruption.

**Transformation efficiency of the recD::kan mutant.** Finally, we tested the ability of recD::kan mutant cells to take up and be transformed by exogenous DNA. *D. radiodurans* is naturally competent to take up DNA from its environment and integrate the transforming DNA into its chromosome via homologous recombination (41). The transformation efficiency of the recD::kan mutant was greater than that of the wild type with transforming DNA from *D. radiodurans* strain LS18, which carries streptomycin resistance. Using a protocol in which wild-type *D. radiodurans* is transformed relatively efficiently (12), the recD::kan mutant was transformed with a three- to sevenfold-greater efficiency than the wild type (Table 1). The difference in transformation efficiency between the mutant and wild type was even greater (30- to 100-fold) when cells were treated as described previously (34) (Table 1).

**DISCUSSION**

The RecD protein is well known as a subunit of the RecBCD enzyme and has been studied extensively in that enzyme from *E. coli*. There has been much less work on the proteins in the RecD2 subfamily that includes homologues from *D. radiodurans*, *B. subtilis*, *Chlamydia*, and other bacteria (see the introduction). The results reported in this paper indicate that RecD serves some function in DNA damage repair and/or

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**TABLE 1. Transformation efficiency of the recD::kan mutant**

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<thead>
<tr>
<th>Streptomycin conc (μg/ml)</th>
<th>Transformation efficiency (transformants/μg DNA/total viable cells)</th>
<th>No. of determinations</th>
<th>Method</th>
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<tr>
<td>Wild type</td>
<td>recD::kan</td>
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<tr>
<td>5</td>
<td>14.7 (3–7.8)</td>
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<td>5</td>
<td>20, 80</td>
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<td>50</td>
<td>97, 130</td>
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* A Competent cells were transformed with genomic DNA from *D. radiodurans* strain LS18, and transformants were selected by the ability to grow in the presence of the indicated concentration of streptomycin.

* Competent cells were prepared and transformed by a method reported previously (12) and in Materials and Methods. Transformation efficiency of the wild-type strain (BAA-816) was about 6 × 10^-5 transformants/μg DNA/viable cell.

* Range of experimental values.

* Competent cells were prepared and transformed by a method described previously (34) and in Materials and Methods. Transformation efficiency of the wild-type strain (BAA-816) was about 2 × 10^-5 transformants/μg DNA/viable cell on 5 μg/ml streptomycin and 2 × 10^-3 on 50 μg/ml streptomycin.
recombination, at least in *D. radiodurans*. A study similar to ours, in which a recD disruption mutation was constructed in the *D. radiodurans* chromosome, has been reported recently (50). Those workers found, as we did, that the recD mutant is sensitive to treatment with hydrogen peroxide. However, those authors reported that the recD cells are less sensitive to UV than we have found and that they are not sensitive to IR. The reason for this difference between the two sets of results is not clear at this time.

**Role of RecD in DNA repair in *D. radiodurans***. It is not yet possible to specify a precise function for RecD in DNA damage resistance in *D. radiodurans*. The DNA-damaging agents used in this work give rise to a variety of forms of damage, and the phenotype of the recD mutant is somewhat different from those of other *D. radiodurans* mutants studied previously, as elaborated below. Thus, much further work is required before RecD can be placed in a specific DNA repair pathway.

A significant effect of gamma irradiation is the production of dsDNA breaks, and a mechanism by which *D. radiodurans* can repair thousands of double-strand breaks was proposed recently (49). Double-strand ends are thought to be resected by an unknown nuclease (10, 14, 30), and the resulting single-stranded dsDNA breaks, and a mechanism by which *D. radiodurans* RecD can be placed in a specific DNA repair pathway. The elevated level of transformation in the recD::kan mutant is similar to the effect of mutations in genes encoding several different helicases in other organisms. Interestingly, *E. coli* recD mutants exhibit three- to sixfold-greater levels of recombination than wild-type cells in some assays (4), and *E. coli* recD mutants are more readily transformable than the wild type with linear dsDNA (43). The loss of the RecD subunit in *E. coli* disables the nuclease activity of RecBCD (4), and therefore, transforming DNA is not degraded in the cell. Further work will be needed to see whether RecD is involved in DNA degradation in *D. radiodurans*. The *D. radiodurans* recD mutants differ from *E. coli* recD mutants in that the latter mutants are not sensitive to DNA-damaging agents such as UV light (4, 44).

Several other helicases, including UvrD, its eukaryotic homologue Srs2, and several RecQ homologues, also suppress the levels of homologous recombination. UvrD and Srs2 can displace the RecA protein from DNA to suppress recombination (1, 3, 40, 46), while the RecQ homologues unwind recombination intermediates and prevent their resolution to recombinant products (24, 32, 33). Interestingly, a *D. radiodurans* uvrD mutant shows lower levels of recombination than does the wild type (37). It could be that RecD serves the antirecombination function in *D. radiodurans* that is served by the UvrD helicase in *E. coli*.

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