Analysis of *Deinococcus radiodurans*'s Transcriptional Response to Ionizing Radiation and Desiccation Reveals Novel Proteins That Contribute to Extreme Radioresistance

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ABSTRACT

During the first hour after a sublethal dose of ionizing radiation, 72 genes were upregulated threefold or higher in *D. radiodurans* R1. Thirty-three of these loci were also among a set of 73 genes expressed in R1 cultures recovering from desiccation. The five transcripts most highly induced in response to each stress are the same and encode proteins of unknown function. The genes (*ddrA, ddrB, ddrC, ddrD,* and *pprA*) corresponding to these transcripts were deleted, both alone and in all possible two-way combinations. Characterization of the mutant strains defines three epistasis groups that reflect different cellular responses to ionizing radiation-induced damage. The *ddrA* and *ddrB* gene products have complementary activities and inactivating both loci generates a strain that is more sensitive to ionizing radiation than strains in which either single gene has been deleted. These proteins appear to mediate efficient RecA-independent processes connected to ionizing radiation resistance. The *pprA* gene product is not necessary for homologous recombination during natural transformation, but nevertheless may participate in a RecA-dependent process during recovery from radiation damage. These characterizations clearly demonstrate that novel mechanisms significantly contribute to the ionizing radiation resistance in *D. radiodurans*.

*D*EINOCOCCUS *radiodurans* R1 is the type species of a bacterial family distinguished by its ability to tolerate exposure to ionizing radiation (BATTISTA *et al.* 1999); exponential phase cultures survive doses to 5 kGy without loss of viability. A 5-kGy dose causes massive DNA damage, cleaving the genome of every *D. radiodurans* cell into multiple, subgenomic fragments (BATTISTA *et al.* 1999). For most species, this level of DNA damage is lethal, but *D. radiodurans* has the capacity to reform its genome from these fragments in what appears to be an error-free process. The biochemical details of *D. radiodurans*'s ionizing radiation resistance are poorly understood, but it is clear that proteins needed for cell survival are synthesized in cultures exposed to ionizing radiation. Irradiated cultures cannot recover in the presence of chloramphenicol; this antibiotic prevents resti-

tution of ionizing radiation-induced single-strand (DEAN *et al.* 1969) and double-strand DNA breaks (KITAYAMA and MATSUYAMA 1971). We have made identifying the proteins that mediate ionizing radiation resistance a priority in our efforts to better explain *D. radiodurans*'s extreme radioresistance.

In this report we have described the genomic expression profile of *D. radiodurans* R1 cultures as they recover from a sublethal dose of ionizing radiation and compare that profile with R1 cultures recovering from desiccation (MATTIMORE and BATTISTA 1996) to define the overlap in the *D. radiodurans* response to these stresses. MATTIMORE and BATTISTA (1996) established a link between the desiccation resistance and the radiotolerance of *D. radiodurans* by demonstrating that a collection of ionizing radiation-sensitive strains were also sensitive to desiccation. The process of desiccation is inherently DNA damaging and dried bacterial cells exhibit a substantial number of DNA double-strand breaks, single-strand breaks, and DNA crosslinks (DOSE *et al.* 1992), DNA damage that is also observed following exposure to ionizing radiation (WARD 1975). *D. radiodurans*'s ability to repair extensive DNA damage renders this organism resistant to both stresses.

Three key results have come from this comparison. First, we find a significant overlap in the genomic expres-

sion programs of *D. radiodurans* cultures recovering from ionizing radiation and desiccation. Second, this organism's remarkable ability to repair ionizing radiation-induced DNA damage does not appear to be related to massive alterations in gene expression or large-magnitude changes in transcript abundance. Third, half of the loci that respond to ionizing radiation and desiccation encode proteins of unknown function, and the five genes most highly induced in response to each stress are the same.

To establish the utility of this comparative study, the genes encoding the five most abundant transcripts were deleted and the resulting strains evaluated for their ability to tolerate exposure to ionizing radiation. None of the genes is essential and all confer enhanced radioresistance on *D. radiodurans* R1. In addition, all possible pairs of double mutants were generated using the five alleles generated by deletion. Analysis of these strains revealed previously unrecognized RecA-dependent and RecA-independent processes that significantly contribute to the ionizing radiation resistance of this species.

MATERIALS AND METHODS

Strains, growth conditions, and treatment: All strains and plasmids used in this study are listed in Table 1. *D. radiodurans* R1 ATCC13939 and all derivatives were grown at 30° in TGY (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) broth (MAT-TIMORE *et al.* 1995; EARL *et al.* 2002) or on TGY agar (1.5% agar). *Escherichia coli* strains were grown at 37° in Luria-Bertani broth. Only cultures in exponential growth (OD_{600} = 0.08–0.15, $5 \times 10^6 - 1 \times 10^7$ cfu/ml) were evaluated for their ability to survive ionizing radiation and desiccation. All cultures were treated at 25°. γ-Irradiation was conducted using a Model 484R ^{60}Co irradiator (J. L. Shepherd & Associates, San Fernando, CA) at a rate of 30 Gy/min. Cultures were desiccated and held at 5% relative humidity for 2 weeks as previously described (MATTIMORE and BATTISTA 1996). Survival was determined by plating serial dilutions of irradiated cultures in triplicate on TGY plates and incubating at 30°. Most strains were scored for survivors 3 days after plating. Strains TNK103, TNK106, TNK107, TNK108, TNK109, TNK110, and TNK111 form small or slow-growing colonies. These strains were scored 4 days after plating. TNK113 was not scored until 7 days after plating.

Gene descriptions: All genes are identified as described in the published genome sequence (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gdr). Genes encoding proteins of unknown function that are induced in response to ionizing radiation and desiccation were assigned the designation *ddr* (*DNA damage response*), individual genes being differentiated by the letters A–P as indicated in Table 2.

RNA isolation: Total RNA was extracted from 1-liter cultures of irradiated and unirradiated *D. radiodurans* cultures using TRI Reagent (Molecular Research Center, Cincinnati), following the manufacturer's instructions after disrupting cells with glass beads. Total RNA derived from each sample condition was treated with 10 units DNase I (Ambion, Austin, TX) and purified using RNeasy Minikit columns (QIAGEN, Valencia, CA). RNA quality and quantity were evaluated by determining UV absorbance at 260 and 280 nm.

Microarray design and construction: PCR primers were designed to amplify each open reading frame present in the fully

sequenced R1 genome (WHITE *et al.* 1999). PCR products represent internal portions of annotated sequences with a size range between 100 and 800 bp. Primer pairs were designed for 3180 ORFs. PCR products were generated by combining 20 ng of genomic DNA from strain R1 with oligonucleotide primer pairs (0.2 μmol each, average T_m = 55°) and 0.1 units *Taq* DNA polymerase (Perkin-Elmer, Wellesley, MA) in a total volume of 100 μl. The other reaction components were as specified by the manufacturer except that 0.3 M betaine was included in the reaction to aid in denaturing *D. radiodurans* genomic DNA. PCR amplification successes were scored (single band, correct size, >50 ng/ul). Failed reactions were repeated with an additional 2% success, for an overall efficiency of 93%. PCR products were spotted onto Superamine glass slides (Telechem, Sunnyvale, CA) using an MD Gen III Array Spotter (Molecular Dynamics, Sunnyvale, CA) at a redundancy of either 1.5 or 3.0. PCR products were immobilized to the slide surface using a Stratalinker UV crosslinker (Stratagene, La Jolla, CA). All slides were stored in a desiccator at room temperature.

Probe preparation, microarray hybridization, and data acquisition: Hybridization probes were made as described in PETERSON *et al.* (2000). The cDNAs were purified with QIAGEN QIAquick PCR purification columns (QIAGEN, Valencia, CA). NHS-Cy5 and NHS-Cy3 were coupled to amino-allyl containing cDNA (Amersham Biosciences, Piscataway, NJ) as described (http://www.derisilab.ucsf.edu).

Between one and four DNA microarray slides were prehybridized by incubation in 50 ml of 5× SSC, 0.1% SDS, and 1.0% bovine serum albumin for 1 hr at 42°. Slides were washed four times with vigorous agitation in distilled water followed by slide washing and dried with compressed air.

All slides were scanned at both 532 and 635 nm visible light using a Genepix 4000 imager (Axon, Union City, CA). TIGR Spotfinder (http://www.tigr.org/software/tm4/spotfinder.html) was used to quantify hybridization signals that were three times above the local background in one of the two channels (Cy3 or Cy5). The data were normalized using total intensity normalization. Due to pervasive low-magnitude gene induction values associated with *D. radiodurans*'s response to IR and since independent biological replicates varied by as much as threefold, we set conservative criteria for categorizing gene induction. Only genes with an average expression ratio greater than threefold in at least two independent experiments were considered induced. Averages were based on at least four hybridization experiments and included at least two independent biological samples. Aberrant values were removed from consideration in calculating averages. In most cases this occurred due to weak signal intensity in one channel resulting in ratios with high variability.

Quantitative real-time PCR: Two micrograms of each DNase I-treated, purified RNA sample were converted to cDNA using SUPERSCRIPT II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA) combined with 25 pmol of random hexamers to initiate synthesis. Conditions for this reaction followed the manufacturer's instructions.

Approximately 100 bp of unique sequence from the genes of interest was amplified using the following primer sets: DR2340up and DR2340dwn, DR1343up and DR1343dwn, DR0003up and DR0003dwn, DR0070up and DR0070dwn, DR0326up and DR0326dwn, DR0423up and DR0423dwn, and DRA0346up and DRA0346dwn (supplemental Table 1 at http://www.genetics.org/supplemental/). The PCR reaction (50 μl) for amplifying these genes contained the appropriate primers at a final concentration of 0.2 μM, 1 μl of the cDNA template, and SYBR Green PCR core reagents (Applied Biosystems, Foster City, CA). Amplifications were carried out by incubating reactions at 95° for 3 min prior to 40 cycles of 30 sec at 95° followed by 30 sec at 65° and 30 sec at 72°. Data were collected

TABLE 1

Strains and plasmids used in this study

Genes	Description	Reference
DR0003	*ddrC* (*DNA damage response C*)	This study
DR0070	*ddrB*	This study
DR0326	*ddrD*	This study
DR0423	*ddrA*	HARRIS *et al.* (2004)
DRA0346	*pprA*	Accession no. O32504
Strains		
Deinococcus radiodurans R1	ATCC13939	
TNK101	As R1 but Δ*ddrC*::p*kat-aadA*	This study
TNK102	As R1 but Δ*ddrB*::p*kat-cat*	This study
TNK103	As R1 but Δ*ddrD*::p*kat-kan*	This study
TNK104	As R1 but Δ*ddrA*::p*kat-hyg*	HARRIS *et al.* (2004)
TNK105	As R1 but Δ*pprA*::p*kat-aadA*	This study
TNK106	As R1 but Δ*recA*::p*kat-cat*	HARRIS *et al.* (2004)
TNK107	Δ*ddrC*::p*kat-aadA*, Δ*recA*::p*kat-cat*	This study
TNK108	Δ*ddrB*::p*kat-aadA*, Δ*recA*::p*kat-cat*	This study
TNK109	Δ*ddrD*::p*kat-kan*, Δ*recA*::p*kat-cat*	This study
TNK110	**Δ***ddrA*::p*kat-hyg*, Δ*recA*::p*kat-cat*	HARRIS *et al.* (2004)
TNK111	Δ*pprA*::p*kat-aadA*, Δ*recA*::p*kat-cat*	This study
TNK112	Δ*ddrB*::p*kat-cat*, Δ*ddrC*::p*kat-aadA*,	This study
TNK113	Δ*ddrC*::p*kat-aadA*, Δ*ddrD*::p*kat-kan*	This study
TNK114	Δ*ddrA*::p*kat-hyg*, Δ*ddrC*::p*kat-aadA*	This study
TNK115	Δ*ddrC*::p*kat-aadA*, Δ*pprA*::p*kat-hyg*	This study
TNK116	Δ*ddrB*::p*kat-cat*, Δ*ddrD*::p*kat-kan*	This study
TNK117	Δ*ddrA*::p*kat-hyg*, Δ*ddrB*::p*kat-cat*	This study
TNK118	Δ*ddrB*::p*kat-cat*, Δ*pprA*::p*kat-hyg*	This study
TNK119	Δ*ddrA*::p*kat-hyg*, Δ*ddrD*::p*kat-kan*	This study
TNK120	Δ*ddrD*::p*kat-kan*, Δ*pprA*::p*kat-aadA*	This study
TNK121	Δ*ddrA*::p*kat-hyg*, Δ*pprA*::p*kat-aadA*	This study
TNK122	As R1 but Δ*ddrB*::p*kat-aadA*	This study
TNK123	As R1 but Δ*pprA*::p*kat-hyg*	This study
DH5α-MCR	F *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ*80 lacZ*Δ*15* Δ*lacX74 endA1 recA1 deoR* Δ(*ara-leu*) *7697 araD139 galU galK nupG rpsL*	Invitrogen (Grand Island, NY)
Plasmids		
pGEM-T	TA cloning vector	Promega (Madison, WI)
pTNK101	pGEM-T::p*katA-cat*	This study
pTNK102	pGEM-T::p*katA-kan*	This study
pTNK103	pGEM-T::p*katA-aadA*	This study
pTNK104	pGEM-T::p*katA-hyg*	This study
pTNK201	pGEM-T::Δ*ddrC*::p*katA-aadA*	This study
pTNK202	pGEM-T::Δ*ddrB*::p*katA-cat*	This study
pTNK203	pGEM-T::Δ*ddrB*::p*katA-aadA*	This study
pTNK204	pGEM-T::Δ*ddrD*::p*katA-kan*	This study
pTNK205	pGEM-T::Δ*ddrA*::p*katA-hyg*	HARRIS *et al.* (2004)
pTNK207	pGEM-T::Δ*pprA*::p*katA-aadA*	This study
pTNK208	pGEM-T::Δ*pprA*::p*katA-hyg*	This study
pTNK210	pGEM-T::Δ*recA*::p*katA-cat*	HARRIS *et al.* (2004)

and analyzed at each 72° interval. Amplification was followed by melting-curve analysis consisting of 80 cycles of 55° at 10-sec intervals with 0.5° increments per cycle. Reactions were then held at 23° until analysis.

Each 96-well plate consisted of standard curves for each primer set run in duplicate. Standard curves were constructed using cDNA obtained from the unirradiated wild-type orga-nism. A dilution series ($1 - 1 \times 10^{-4}$) of each experimental sample was generated and run in duplicate. Negative controls without cDNA template were run on every plate analyzed.

All assays were performed using the iCycler iQTM real-time detection system (Bio-Rad, Hercules, CA). All data were PCR baseline subtracted before threshold cycle values were desig-nated and standard curves were constructed. Mean concentra-

TABLE 2

**The overlap in loci that respond as exponential phase populations of *D. radiodurans* R1
recover from ionizing radiation and desiccation**

Accurate mass tag	Identifier	Gene name/annotation
		DNA metabolism
−	DR0596	*ruvB*/Holliday junction DNA helicase
+	DR2340	*recA*/recombinase
+	DR1771	*uvrA*/exinuclease ABC, subunit A
+	DR2275	*uvrB*/exinuclease ABC, subunit B
+	DR1913	*gyrA*/DNA gyrase, subunit A
+	DR0906	*gyrB*/DNA gyrase, subunit B
		Adaptation to oxidative stress
+	DR2220	*terB*/tellurium resistance protein
−	DR2224	*terZ*/tellurium resistance protein
		Putative regulatory proteins
+	DR2338	*cinA*/competence-inducible protein
		RNA metabolism
−	DR2339	*ligT*/2′-5′ RNA ligase, putative
−	DR1262	*rsr*/ribonucleotide Ro/SS-A-related protein
		Protein fate
−	DR1114	Heat-shock protein, HSP20 family
		Transport
+	DR1709	NRAMP protein
		Unknown function
−	DR0003	*ddrC*/hypothetical
+	DR0070	*ddrB*/hypothetical
+	DR0194	*ddrE*/conserved hypothetical
+	DR0219	*ddrF*/hypothetical
+	DR0227	*ddrG*/hypothetical
+	DR0326	*ddrD*/hypothetical
+	DR0423	*ddrA*/hypothetical
−	DR0438	*ddrH*/hypothetical
−	DR0659	frnE/predicted dithiol-disulfide isomerase
−	DR0997	*ddrI*/predicted cyclic nucleotide-binding domain/CRP family
+	DR1263	*ddrJ*/conserved hypothetical
−	DR1264	*ddrK*/hypothetical
+	DR1439	*ddrL*/hypothetical
−	DR1440	*ddrM*/hypothetical
−	DR2441	*ddrN*/hypothetical
−	DR2574	*ddrO*/predicted helix-turn-helix XRE-family
+	DRA0346	*pprA*/DNA damage repair protein
+	DRB0100	*ddrP*/hypothetical
−	DRB0141	*hicB*/uncharacterized

tions of each transcript in each sample were calculated from the standard curves generated using each primer set. Induction levels were determined by dividing the calculated concentration of the irradiated sample by the concentration of the unirradiated sample for each strain. The mean concentration of the glyceraldehyde 3-phosphate dehydrogenase (*gap*) transcript, a housekeeping gene whose expression is unaffected by ionizing radiation, was also determined before and after irradiation for each strain.

Antibiotic resistance cassettes: Four antibiotic resistance cassettes were constructed by separately fusing (i) the *aadA* gene

(accession no. AF458479), (ii) the *cat* gene of pBC (Stratagene Cloning Systems, La Jolla, CA), (iii) the *kan* gene of pGPS3 (New England Biolabs, Beverly, MA), and (iv) the *hyg* gene of *Streptomyces hygroscopicus* (accession no. X03615) to the 120 bp of DNA sequence immediately upstream of the *katA* (DR1998) gene of *D. radiodurans* R1. Genes spliced downstream of this sequence, which is designated p*katA*, are constitutively expressed in R1 (FUNAYAMA *et al.* 1999). Each antibiotic resistance gene was spliced to p*katA* by overlap extension *in vitro* during a polymerase chain reaction (HORTON *et al.* 1989). The primers used in these reactions are listed in supplemental Table 2 at http://

www.genetics.org/supplemental/. The fused fragments were amplified and cloned into pGEM-T (Promega, Madison WI) by T-A cloning. The sequence of pGEM-T can be found at http://wheat.pw.usda.gov/~lazo/methods/pro/tb150.html#ix. The resulting plasmids (pTNK101, pTNK102, pTNK103, and pTNK104) were propagated in the *E. coli* strain DH5α-MCR.

Strain construction: Genes were deleted using techniques described previously (FUNAYAMA *et al.* 1999; RUAN *et al.* 2004). Fragments corresponding to ~1000 bp of DNA sequence immediately upstream and downstream of each gene to be deleted were amplified and spliced to the 5′and 3′ ends of an antibiotic resistance cassette by overlap extension during a polymerase chain reaction. The resulting fragment was transformed into exponential phase cells, and recombinants were selected on TGY plates containing an appropriate antibiotic. Since *D. radiodurans* is multigenomic, individual antibiotic resistant colonies were screened to establish whether they were homozygous for the deletion. Pairs of primers that anneal outside each gene's coding sequence (supplemental Table 2) were used to amplify diagnostic PCR fragments, establishing whether the strain was homozygous for the marker that replaced the gene of interest. The details of each construction are given below.

TNK101 Δ*ddrC* (ΔDR0003) was created by splicing the p*katA-aadA* (Str^R) cassette to the 1.1 kbp of genomic DNA sequence immediately upstream and the 1.1 kbp immediately downstream of DR0003. This Δ*ddrC*::p*katA-aadA* hybrid fragment was cloned into pGEM-T (Promega), creating pTNK201. pTNK201 was propagated in *E. coli* DH5α-MCR. The hybrid fragment was PCR amplified using primers tnk0003FW2 and tnk0003RV3 and used to transform an R1 culture by standard methods (RUAN *et al.* 2004). Recombinants were selected on TGY plates containing 8 μg/ml streptomycin. The deletion of *ddrA* was confirmed by PCR using the primers that anneal to sequences flanking DR0003 (supplemental Table 2). These primers generate a 0.9-kbp fragment when R1 genomic DNA is used as a template in the reaction and a 1.2-kbp fragment if *ddrC* is replaced by the p*katA-aadA* cassette. Amplification of genomic DNA isolated from the strain designated TNK101 produced only the 1.2-kbp fragment. The Δ*ddrB* (ΔDR0070) strains TNK102 and TNK122, the Δ*ddrD* (ΔDR0326) strain TNK103, the Δ*ddrA* (ΔDR0423) strain TNK104, the Δ*pprA* (ΔDRA0346) strains TNK105 and TNK123, and the Δ*recA* (ΔDR2340) strain TNK106 were constructed in the same manner as TNK101.

To permit the generation of all possible pairs of double mutants with these alleles, it was necessary to create two Δ*ddrB* strains. In TNK102 *ddrB* is replaced with the p*katA-cat* cassette and in TNK122 *ddrB* is replaced with the p*katA-aadA* cassette. In each construction the cassette was spliced to PCR fragments derived from the sequence 0.8 kbp upstream and 0.8 kbp downstream of *ddrB*. The hybrid fragment Δ*ddrB*::p*katA-cat* or Δ*ddrB*::p*katA-aadA* was cloned into pGEM-T to generate pTNK202 or pTNK203, respectively. The fragments used to create the deletions were amplified by PCR using tnk0070FW2 and tnk0070RV3 and transformed into exponential phase cultures of R1. Recombinants were obtained on TGY plates containing 3 μg/ml chloramphenicol for Δ*ddrB*::p*katA-cat* candidates or TGY plates with 8 μg/ml streptomycin for Δ*ddrB*::p*katA-aadA* candidates. Genomic DNA isolated from putative recombinants was PCR screened to establish that the disruption was homozygous, using primers listed in supplemental Table 2.

TNK103 Δ*ddrD* (ΔDR0326) was deleted using a Δ*ddrD*::p*katA-kan* cassette built into pTNK204. The appropriate fragment was PCR amplified using tnk0326FW2 and tnk0326RV3 (supplemental Table 2) and used to transform into exponential phase cultures. This deletion cassette is a hybrid fragment in which the p*katA-kan* cassette is spliced to the sequence 0.9 kbp up-

stream and 1.3 kbp downstream of the *ddrD* coding region. Recombinants were selected on TGY plates containing 10 mg/ml kanamycin. Primers (supplemental Table 2) flanking *ddrD* were used to verify the deletion. These primers amplify a 2.8-kbp fragment from R1 and a 3.1-kbp fragment corresponding to p*katA-npt* when *ddrD* is deleted.

Deletion of *ddrA* (DR0423) was performed as described previously (HARRIS *et al.* 2004). The Δ*ddrA*::p*katA-hyg* cassette from pTNK205 was used to transform exponential phase cells. The Δ*ddrA*::p*katA-hyg* cassette is a hybrid fragment in which p*katA-hyg* is joined to the 1.0-kbp upstream region and 0.9-kbp downstream region of *ddrA*. Recombinants were selected on TGY agar containing 37.5 μg/ml hygromycin. Primers were designed to anneal outside the coding sequence of *ddrA* (Table 2). These primers amplify a 0.85-kbp fragment from R1 and a 1.3-kbp fragment corresponding to the *katA-npt* fusion when *ddrD* is deleted.

The Δ*pprA* (ΔDRA0346) strains, TNK105 and TNK123, were generated using the Δ*pprA*::p*katA-aadA* cassette from pTNK207 or the Δ*pprA*::p*katA-hyg* cassette from pTNK208, respectively. For each cassette, the sequence 0.85 kbp upstream and 0.85 kbp downstream of *pprA* was fused to p*katA-aadA* or p*katA-hyg*. Recombinants were selected on TGY agar containing 8 or 37.5 μg/ml streptomycin. Deletions were confirmed using primers annealing to flanking sequences to *pprA* (supplemental Table 2). Amplification with tnkA0346FW2 and tnkA0346RV3 generates a 0.9-kbp fragment from R1 genomic DNA and a 1.2-kbp fragment corresponding to the *katA-aadA* cassette. Amplification with tnkA0346FW5 and tnkA0346RV7 produces a 2.6-kbp product from R1 genomic DNA and a 2.8-kbp fragment if *pprA* is replaced by the *katA-hyg* cassette.

Deletion of *recA* (DR2340) was accomplished using a Δ*recA*::p*katA-cat* cassette from pTNK210, as described previously (HARRIS *et al.* 2004). This hybrid fragment was constructed by joining p*katA-cat* to the 1.6 kbp upstream and 1.2 kbp downstream of the *recA* coding region. Recombinants were selected on a TGY plate containing 3 μg/ml chloramphenicol and the deletion was screened for using primers annealing to sequences flanking to *recA* (supplemental Table 2). These primers amplified a 1.5-kbp fragment if the *recA* coding sequence remained and a 1.3-kbp fragment corresponding to the *katA-cat* cassette that was used to replace *recA*.

Double mutants were created in the same manner as that of single mutants. TNK101, TNK102, TNK103, TNK104, TNK105, and TNK122 were used as the parental strains and these strains were transformed with another allele carrying a compatible antibiotic resistance marker.

RESULTS

Identification of loci in *D. radiodurans* R1 induced in response to 3 kGy ionizing radiation: We compared unirradiated R1 cultures in exponential phase growth with age-matched R1 cultures during the first hour after exposure to a nonlethal 3 kGy dose of γ radiation. This time course was deemed appropriate on the basis of data shown in Figure 1, which documents restitution of the R1 genome following 3 kGy γ-radiation. In agreement with previous studies (UDUPA *et al.* 1994; MATTIMORE *et al.* 1995; EARL *et al.* 2002), there is no loss of viability in R1 cultures receiving a 3-kGy dose (Figure 1A). The titer of the cultures prior to irradiation was $1.7 \pm 0.5 \times 10^7$ cfu/ml, and that determined immediately after irradiation (zero time) was $1.66 \pm 0.6 \times 10^7$
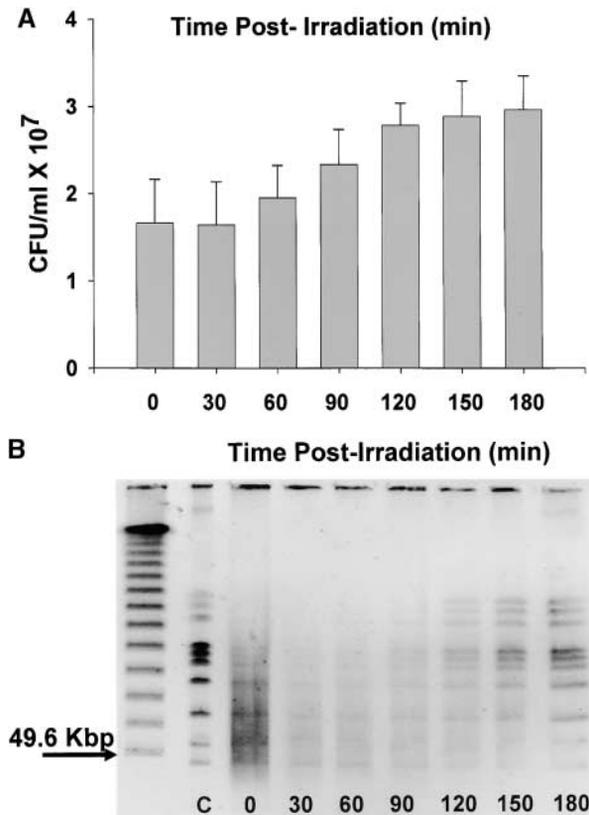
FIGURE 1.—(A) Growth of R1 cultures as a function of time following exposure to 3000 Gy γ-radiation. Titers were determined immediately after irradiation (zero time) and at the times indicated. (B) Pulsed-field gel illustrating the restitution of the R1 genome following 3000 Gy γ-radiation. The unlabeled lane corresponds to a lambda ladder standard. Lane C is a Not 1 digest of genomic DNA obtained from the culture prior to irradiation. The remaining lanes contain a Not 1 digest of genomic DNA isolated from irradiated cultures at the times indicated.

cfu/ml. After irradiation there is evidence of DNA double-strand breaks, indicated by the reduction in high-molecular-weight DNA and an accompanying increase in low-molecular-weight fragments (Figure 1B). Within 90 min, the high-molecular-weight fragments become more intense, signaling that genome reassembly is well underway. After 1 hr delay, there is a significant increase in cell numbers in the irradiated population ($t = -2.52$, $P = 0.014$, d.f. = 58) relative to the number of cells present immediately before irradiation, and the titer of the irradiated population doubles within 3 hr postirradiation, suggesting that all repairs have been completed. On the basis of this time course, it was decided that isolating total RNA immediately after and at 30 and 60 min postirradiation provided the best opportunity to characterize the cell's response to ionizing radiation.

To determine how global transcription in *D. radiodurans* R1 changes in response to ionizing radiation, competitive hybridizations were repeated for six independent experimental trials. Those studies are summarized in supplemental Table 3 at http://www.genetics.

org/supplemental/, which identifies the mean Cy5/Cy3 ratios obtained for those genes in the irradiated population that exhibited a threefold or higher increase in expression relative to the unirradiated population. Seventy-two genes (2.2% of the genome) respond with increased expression within the first hour after exposure. A detectable protein product, an accurate mass tag (LIPTON *et al.* 2002), has been reported for 65% of these loci, verifying that these open reading frames encode protein products.

To help establish the validity of the ratios recorded in supplemental Table 3, gene expression was also measured by quantitative RT-PCR for seven loci. Total RNA was isolated from exponential phase cultures of R1 before irradiation and at the three time points following exposure to 3 kGy irradiation (IR). Changes in transcript abundance for *recA* (DR2340), *gap* (DR1343), and five genes encoding hypothetical proteins (DR0003, DR0070, DR0326, DR0423, and DRA0346) were determined. An increase in *recA* gene expression served as a positive control as this deinococcal gene's response to IR had already been established (EARL *et al.* 2002), and *gap*, which encodes glyceraldehyde 3-phosphate dehydrogenase, was included among these genes because it was not, on the basis of our microarray data, expected to be a radiation-responsive gene. The relative change in expression for each gene is provided as the mean ratio in supplemental Table 4 at http://www.genetics.org/supplemental/. With one exception, there was remarkable concordance between induction ratios obtained by these methods. The relative change observed for the DR0070 transcript measured by quantitative (Q)-RT-PCR was two to six times higher compared to relative abundance measurements derived from microarray hybridization. Overall the uniformity of measurements from the Q-RT-PCR and microarray analyses suggests that the changes in transcription reported by this microarray are reliable.

The genes induced in response to ionizing radiation were grouped into nine categories on the basis of their similarity to known proteins (supplemental Table 3) with 54 of the 72 loci falling into one of three categories. The largest group (44%) encodes proteins of unknown function. These loci are among the most highly induced, 20 being induced greater than fivefold in the irradiated population. Six genes (*recA*, *ruvB*, *uvrA*, *uvrB*, *gyrA*, and *gyrB*) make up a second group that encodes proteins associated with DNA metabolism. The third group consists of five loci (*katA*, *terB*, *terZ*, *mrsA*, and *dps*) that encode proteins that are associated with alleviating the effects of oxidative stress in other species. The *katA* gene product (DR1998p) is similar to the KatE catalase of *E. coli*. TerB (DR2220p) and TerZ (DR2224p) are homologs of *E. coli* proteins that appear to play a role in maintaining the intracellular reducing environment possibly by directly reversing disulfide bonds (TURNER *et al.* 1999). MsrA (DR1849p) shares similarity with *E. coli*'s

methionine sulfoxide reductase. Loss of MsrA sensitizes *E. coli* to hydrogen peroxide (MOSKOVITZ *et al.* 1995). Dps (DRB0092p) is related to an *E. coli* protein that nonspecifically binds to DNA, protecting it from oxidative damage (ALMIRON *et al.* 1992; MARTINEZ and KOLTER 1997).

Molecular dissection of *D. radiodurans*'s response to ionizing radiation through an examination of transcription in cells recovering from desiccation: There is never a perfect correlation between genes that are essential under a given condition and genes that are induced under the same condition (BADARINARAYANA *et al.* 2001; WINZELER *et al.* 1999). Many prokaryotic genes are organized into operons and stress-induced gene expression may generate a polycistronic mRNA, which will be detected as multiple signals on an array, even if only one of the encoded proteins is needed to combat the stress. Therefore, supplemental Table 3 undoubtedly identifies gene products whose expression is incidental and that do not directly participate in the repair of ionizing-radiation-induced intracellular damage. To better characterize *D. radiodurans*'s response to ionizing radiation, we subjected R1 cultures to desiccation and compared the expression pattern to that obtained after ionizing radiation. For a vegetative species, *D. radiodurans* is quite resistant to desiccation, exhibiting ~90% viability after being stored dry for 6 weeks (MATTIMORE and BATTISTA 1996). Since the process of desiccation and rehydration introduces DNA damage (DOSE *et al.* 1992), we assumed that some of the proteins needed to repair ionizing-radiation-induced damage, including double-strand breaks, would be identical to proteins used to mend DNA damage introduced following desiccation. The overlap in the cell's response to each stress should specify gene products that directly participate in repair of common DNA damage, potentially identifying novel proteins critical to this process.

The transcriptome of R1 in liquid culture was compared with a culture recovering from 2 weeks desiccation at 5% relative humidity. Samples were obtained from six independent cultures over a three-point time course (0, 0.5, and 1 hr) following rehydration. Under these nonlethal conditions, 73 genes were induced during this time course (supplemental Table 5 at http://www.genetics.org/supplemental/). Although *D. radiodurans* maintains distinct inducible responses to each stress, there is substantial overlap (Table 2) in the gene expression profiles observed. Thirty-two (45%) of the 73 loci responding to desiccation were also observed during R1's recovery from ionizing radiation.

Deletion of the locus designated *pprA* (DRA0346) sensitizes *D. radiodurans* R1 to ionizing radiation: The overlap in *D. radiodurans*'s response to ionizing radiation and desiccation described by Table 2 is significant because it suggests something long suspected: *D. radiodurans* radioresistance is the consequence of unprecedented mechanisms of DNA damage tolerance (BATTISTA 1997, 2000;

BATTISTA *et al.* 1999). The preponderance of hypothetical proteins on this list, coupled with only a limited number of well-defined DNA repair proteins, argues that novel processes are facilitating this species' capacity to survive genetic insult. To test the validity of this inference, the five hypothetical genes that were induced to highest level in response to each treatment, *ddrA* (DR0423), *ddrB* (DR0070), *ddrC* (DR0003), *ddrD* (DR0326), and *pprA* (DRA0346), were deleted and replaced by an antibiotic resistance marker. The ionizing radiation resistance of each strain was then compared to that of the R1 parent.

With the exception of TNK103 (Δ*ddrD*), the resulting strains were identical in size and appearance to *D. radiodurans* R1. Like its parent, TNK103 cultures consist of spherical cells that form pairs and tetrads in liquid media, but these cells are approximately one-half the diameter of the parent strain. Deletion of *ddrA*, *ddrB*, *ddrC*, and *ddrD* does not alter the growth rate of the resulting strains (~1.5-hr doubling time) in rich liquid media relative to the parent strain, but deletion of *pprA* results in a strain with a 2.4-hr doubling time (mean of 10 independent measurements). None of the mutant strains exhibits a decrease in the efficiency of natural transformation (~5 × 10^{-5} rifampicin-resistant transformants per colony-forming unit) relative to R1, indicating that none of the gene products encoded by these loci is essential for RecA-dependent homologous recombination, which is required for natural transformation in this species.

Strains TNK101 (Δ*ddrC*) and TNK103 (Δ*ddrD*) were as resistant to ionizing radiation as their R1 parent, but the other three mutants displayed varying degrees of sensitivity to ionizing radiation (Figure 2). As shown in earlier work, TNK102 (Δ*ddrB*) and TNK104 (Δ*ddrA*) exhibit a reduced capacity to survive these exposures (LIU *et al.* 2003; HARRIS *et al.* 2004), but this is apparent only at doses >2.5 and 5 kGy, respectively. Cultures of TNK105 (Δ*pprA*) are much more sensitive to ionizing radiation than are TNK102 and TNK104. The shape of the survival curve for TNK105 is atypical. The reduction in viability observed at the lowest doses is not sustained as higher doses of ionizing radiation are applied. At doses above 5 kGy, the slope changes notably, and the rate at which viability declines is reduced. The shoulder of resistance that characterizes R1 cultures is not apparent in this strain; the viability of the irradiated population drops off rapidly at 1 kGy, the lowest dose examined. Very few single mutations have been characterized that result in similar reductions in R1 resistance. To our knowledge only deletions of *recA* and *polA* have similar effect (GUTMAN *et al.* 1993, 1994). Even so, TNK105 is not as sensitive to γ-radiation (Figure 2) as TNK106, an isogenic Δ*recA* strain created for this study.

Deletions of the loci designated *ddrA* (DR0423) or *ddrB* (DR0070) decrease the ionizing radiation resistance of the Δ*pprA* strain TNK105: Figure 3 illustrates the sur-
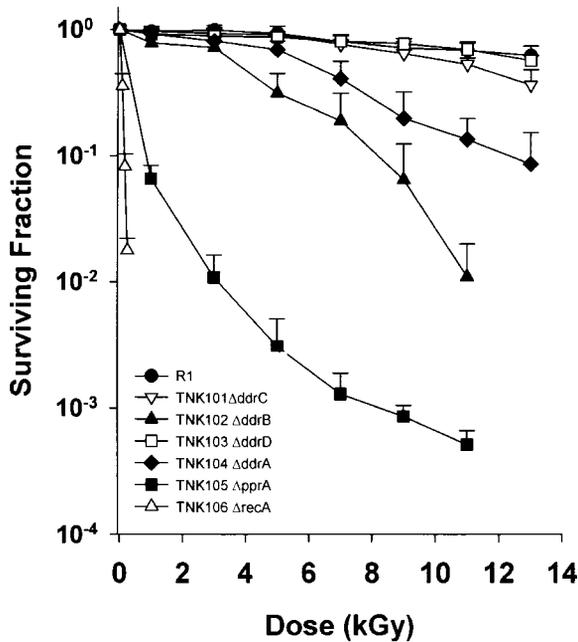
FIGURE 2.—Representative survival curves for *D. radiodurans* strains TNK101 Δ*ddrC*, TNK102 Δ*ddrB*, TNK103 Δ*ddrD*, TNK104 Δ*ddrA*, TNK105 Δ*pprA*, TNK106 Δ*recA*, and *D. radiodurans* R1 following exposure to γ-radiation. Values are the means ± standard deviations of three independent experiments. *n* = 9.

vival curves for all combinations of double mutants possible in a Δ*pprA* background using the genes examined in this study. TNK118 Δ*ddrB* Δ*pprA* and TNK121 Δ*ddrA*
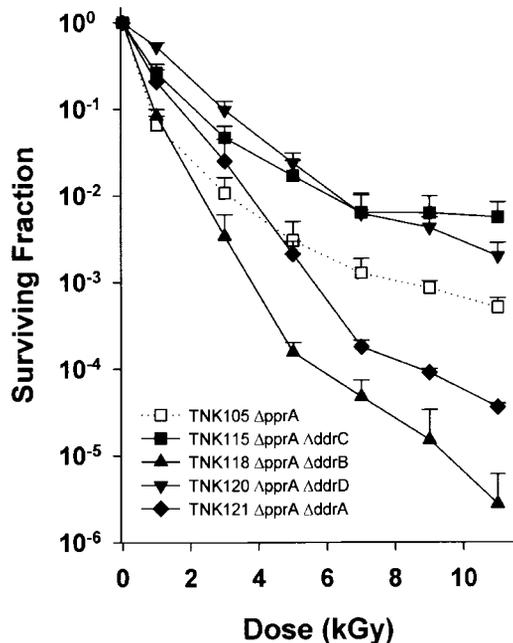
FIGURE 3.—Representative survival curves for *D. radiodurans* strains TNK105 Δ*pprA*, TNK115 Δ*ddrC* Δ*pprA*, TNK118 Δ*ddrB* Δ*pprA*, TNK120 Δ*ddrD* Δ*pprA*, and TNK121 Δ*ddrA* Δ*pprA* following exposure to γ-radiation. Values are the means ± standard deviations of three independent experiments. *n* = 9.
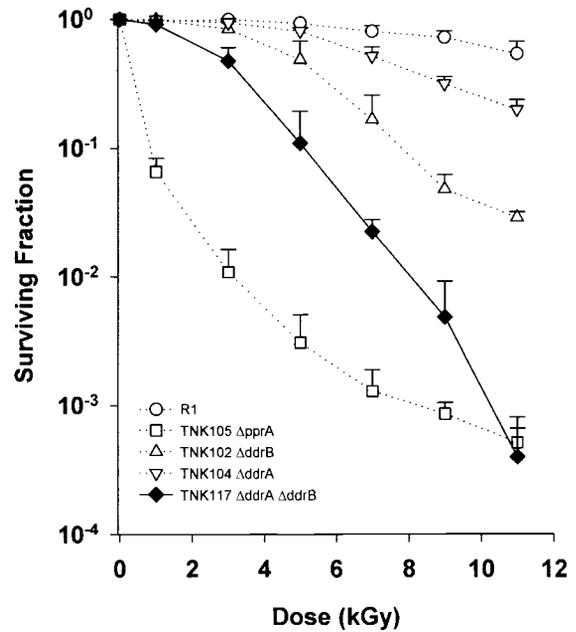

FIGURE 4.—Representative survival curve for *D. radiodurans* strain TNK117 Δ*ddrA* Δ*ddrB*. For comparison, the survival curves of TNK102 Δ*ddrB*, TNK104 Δ*ddrA*, TNK105 Δ*pprA*, and *D. radiodurans* R1 from Figure 1 are included as dotted lines. Values are the means ± standard deviations of three independent experiments. *n* = 9.

Δ*pprA* exhibit increased sensitivity to ionizing radiation relative to TNK105 Δ*pprA*. The loss of DdrA and DdrB is most apparent at doses >3 kGy, largely eliminating the biphasic appearance of the TNK105 survival curve (dotted line in Figure 3). TNK115 Δ*ddrC* Δ*pprA* and TNK119 Δ*ddrD* Δ*pprA* appear to decrease the sensitivity of the Δ*pprA* single mutant to ionizing radiation.

Deletion of the locus designated *ddrA* (DR0423) decreases the ionizing radiation resistance of the Δ*ddrB* strain TNK102: At doses >3 kGy, TNK117 Δ*ddrA* Δ*ddrB* is more sensitive to ionizing radiation than TNK102 Δ*ddrB* or TNK104 Δ*ddrA* (Figure 4). TNK117 displays a small shoulder of resistance, noted at 1 kGy, but above this dose survival is substantially reduced relative to each single mutant. At 9 kGy, for example, TNK117 is 10-fold more sensitive than TNK102 and 60-fold more sensitive than TNK104.

Deletion of the locus designated *ddrC* (DR0003) decreases the ionizing radiation resistance of the Δ*ddrB* strain TNK102: A family of double mutants carrying Δ*ddrC* and Δ*ddrD* was also evaluated for its ability to survive ionizing radiation (Figure 5). In general, the deletion of *ddrC* and *ddrD* had little effect on the ionizing radiation resistance of the resulting strain. TNK113, a double mutant lacking *ddrC* and *ddrD*, did exhibit a slight (twofold) increase in sensitivity to ionizing radiation, but this phenotype is apparent only at the highest applied doses. In addition, an increase in ionizing radiation sensitivity was noted when TNK112 Δ*ddrC* Δ*ddrB* was compared to TNK102 Δ*ddrB*.

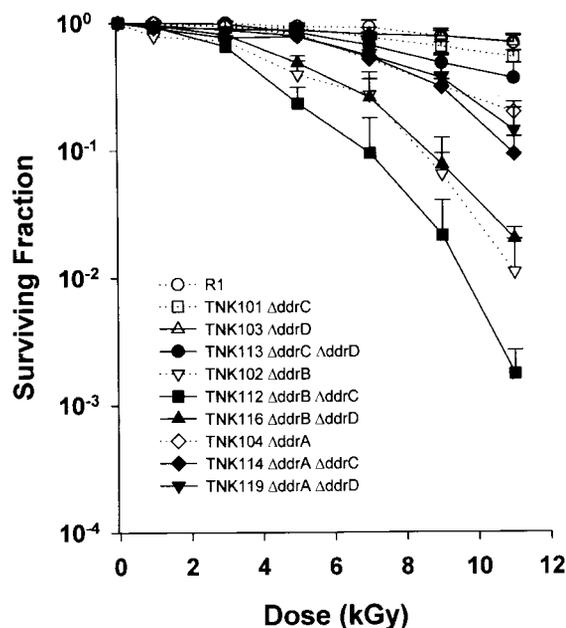FIGURE 5.—Representative survival curves for *D. radiodurans* strains TNK112 Δ*ddrB* Δ*ddrC*, TNK113 Δ*ddrC* Δ*ddrD*, TNK114 Δ*ddrA* Δ*ddrC*, TNK116 Δ*ddrB* Δ*ddrD*, and TNK119 Δ*ddrA* Δ*ddrD* following exposure to γ-radiation. For comparison, the survival curves of TNK101 Δ*ddrC*, TNK102 Δ*ddrB*, TNK103 Δ*ddrD*, TNK104 Δ*ddrA*, and *D. radiodurans* R1 from Figure 1 are included as dotted lines. Values are the means ± standard deviations of three independent experiments. $n = 9$.

Deletions of loci designated *ddrA* (DR0423), *ddrB* (DR0070), *ddrC* (DR0003), or *ddrD* (DR0326) decrease the resistance of the *recA* strain TNK106 to ionizing radiation: In addition to creating double mutants of all pairwise combinations of deletions of *ddrA*, *ddrB*, *ddrC*, *ddrD*, and *pprA*, we also moved a Δ*recA* allele into the five strains carrying each deletion. As depicted in Figure 6 inactivation of *recA* dramatically sensitizes *D. radiodurans* to ionizing radiation, but the *recA* strain is made even more sensitive when *ddrA*, *ddrB*, *ddrC*, or *ddrD* is also inactivated. This increase is statistically significant for each of these double mutants. For example, the slope of the line describing TNK106 (Δ*recA*) survival is significantly different ($t = 5.5475$, $P < 0.01$, d.f. = 16) from that obtained for TNK109 (Δ*recA* Δ*ddrD*), the strain that exhibits the smallest increase (fourfold at 260 Gy) in sensitivity relative to TNK106. TNK108 (Δ*recA* Δ*ddrB*) is 50 times more sensitive than TNK106 at 260 Gy. The Δ*recA* Δ*pprA* double mutant (TNK111) is no more sensitive to ionizing radiation than the TNK106 Δ*recA* strain (Figure 6).

DISCUSSION

D. radiodurans is one of the most DNA damage tolerant organisms known. The species' ability to withstand what is a sterilizing dose of ionizing radiation for almost all other microbes has led to a great deal of superficial
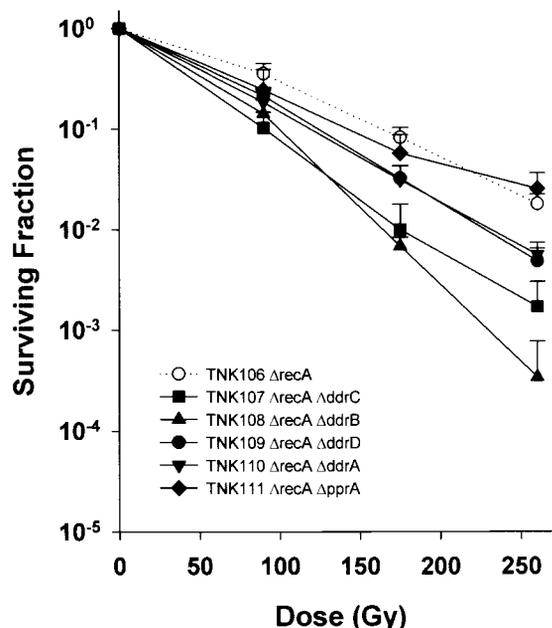

FIGURE 6.—Representative survival curves for *D. radiodurans* strains TNK106 Δ*recA*, TNK107 Δ*ddrC* Δ*recA*, TNK108 Δ*ddrB* Δ*recA*, TNK109 Δ*ddrD* Δ*recA*, TNK110 Δ*ddrA* Δ*recA*, and TNK111 Δ*pprA* Δ*recA* following exposure to γ-radiation. Values are the means ± standard deviations of three independent experiments. $n = 9$.

speculation about the origins of the Deinococcaceae and the mechanisms they employ to repair DNA damage. Despite the fact that the genome has been sequenced and annotated (WHITE *et al.* 1999; MAKAROVA *et al.* 2001), and analyses of the transcriptome (LIU *et al.* 2003) and proteome published (LIPTON *et al.* 2002), there is very little specific information available that characterizes the unique properties of deinococcal species (EDWARDS and BATTISTA 2003; NARUMI 2003). It has been suggested that the extreme radioresistance of this family is a consequence of unprecedented DNA repair mechanisms, and that the absence of well-defined explanations for the radioresistance of the Deinococcaceae reflects our lack of knowledge about DNA repair in this species (BATTISTA *et al.* 1999; BATTISTA 2000).

As discussed by EISEN and HANAWALT (1999), our perception of what qualifies as a DNA repair protein in a prokaryote is determined by our knowledge of DNA repair in *E. coli*. Since prokaryotes are remarkably diverse, occupying every environmental niche, it is all but certain that the *E. coli* paradigm for DNA repair does not apply to all species. Among characterized prokaryotes, *D. radiodurans*'s repair functions seem very likely to deviate from that of *E. coli*. *D. radiodurans* tolerates levels of DNA damage (with little or no loss of viability) that are capable of completely eradicating an *E. coli* population. Genome analysis (WHITE *et al.* 1999; MAKAROVA *et al.* 2001) revealed that *D. radiodurans* encodes the same complement of DNA repair proteins found in *E. coli*, suggesting that there are unidentified

features of *D. radiodurans* physiology that facilitate its resistance to DNA damage. Since almost half of the open reading frames described for *D. radiodurans* encode proteins of unknown function, it is reasonable to assume that some of these uncharacterized proteins are critical for DNA damage tolerance. In this study, we have described phenotypes associated with the inactivation of five "hypothetical" proteins encoded by *D. radiodurans* R1 that clearly indicate that the action of novel proteins contributes to this species' capacity to tolerate exposure to ionizing radiation.

D. radiodurans's transcriptional responses to ionizing radiation and desiccation overlap: We have defined the genomic expression profile of *D. radiodurans* R1 cultures as they recover from a sublethal dose of ionizing radiation, comparing that profile with R1 cultures recovering from desiccation (Mattimore and Battista 1996). Table 2 lists 32 loci that are induced as cultures recover from ionizing radiation and desiccation. This group includes only four genes (*recA*, *ruvB*, *uvrA*, and *uvrB*) that encode proteins that have been previously associated with DNA repair in other species, indicating that increased transcription from most genes encoding identifiable DNA repair proteins is not necessary for *D. radiodurans*'s radioresistance. The reasons for this are not apparent from this study, but two possible explanations seem likely. Either constitutive expression of these proteins is sufficient for the repair of DNA damage or the cell does not depend on these proteins to repair ionizing radiation-induced DNA damage. At present, neither possibility can be excluded. These data argue that *D. radiodurans*'s ability to repair the damage caused by ionizing radiation and desiccation is not related to dramatic shifts in gene expression or large-magnitude changes in transcript abundance.

This result differs from a previous study that monitored ionizing-radiation-induced global gene expression in *D. radiodurans*. Liu *et al.* (2003) reported that 832 genes were induced during a 24-hr recovery period in *D. radiodurans* cultures exposed to 15 kGy γ-radiation. These numbers are an order of magnitude larger than those we are reporting, and we believe there are two principle reasons for the difference. First, in the earlier study stationary-phase cultures of *D. radiodurans* were irradiated and this culture was transferred to fresh media to recover (Liu *et al.* 2003). These authors compared the RNA isolated from the recovering cultures to RNA isolated from the unirradiated stationary-phase control culture. The ratios obtained were, therefore, detecting transcription occurring in response to ionizing radiation as well as the transcription needed to transition the cell from stationary phase to exponential-phase growth. The cell's need to deal with both conditions simultaneously may help explain the large number of differentially expressed transcripts reported relative to this study. Second, we have chosen to stringently exclude any transcript from this analysis that does not exhibit at least a

threefold differential relative to the unirradiated control. The previous study included all changes in transcripts that were twofold or higher (Liu *et al.* 2003).

The RecA and RuvB proteins function in homologous recombination, and UvrA and UvrB are subunits of the endonuclease responsible for nucleotide excision repair in prokaryotes. Of these proteins only RecA is necessary for ionizing radiation resistance in *D. radiodurans*. Genetic inactivation of RuvB (Kitayama *et al.* 1997) and UvrA (Agostini *et al.* 1996) does not substantively alter the radioresistance of R1, suggesting that if these proteins participate in protecting *D. radiodurans* from ionizing radiation-induced DNA damage, their activities are redundant. In contrast, deletion of *recA* from R1 results in a strain that is extremely sensitive to ionizing radiation (Narumi *et al.* 1999), a result reiterated in Figure 2. Experimental evidence has identified two physiological functions that are RecA dependent, homologous recombination (Daly and Minton 1995, 1996) and cleavage of LexA repressor (Satoh *et al.* 2002), and arguments have been made that both activities are necessary for the ionizing radiation resistance of this species.

Genes encoding the two subunits of the DNA gyrase, *gyrA* (DR1913) and *gyrB* (DR0906), are also induced in response to ionizing radiation. DNA gyrase forms negative supercoils in the DNA helix to alleviate excess positive supercoiling caused by transcription, DNA replication, and repair processes. In *E. coli*, treatments that reduce negative supercoiling increase expression of *gyrA* and *gyrB* (Menzel and Gellert 1983), and the introduction of DNA double-strand breaks will trigger their expression.

Sixty percent of the loci that are part of *D. radiodurans*'s common response to ionizing radiation and desiccation encode proteins of unknown function (Table 2). As indicated in Table 2, some of the predicted proteins share motifs with characterized proteins, but the degree of similarity is not sufficiently high to assign a putative function to the gene product. Five of these hypothetical genes (designated *ddrA*, *ddrB*, *ddrC*, *ddrD*, and *pprA*) were, on average, induced >10-fold when cells were irradiated.

The hypothetical proteins DdrA, DdrB, and PprA confer resistance to killing by ionizing radiation: The coding sequences of *ddrA*, *ddrB*, *ddrC*, *ddrD*, *pprA*, and *recA* were deleted and replaced with an antibiotic resistance marker. In addition, all possible combinations of double mutants with these six alleles were constructed in an attempt to establish genetic evidence for potential interactions between the encoded gene products. All of these genes appear to contribute to *D. radiodurans*'s capacity to survive exposure to ionizing radiation. Pending further investigation, we are assuming that the increases in ionizing radiation sensitivity are due to the loss of the gene product encoded by the gene that has been deleted. We note that the *ddrA*, *ddrC*, *ddrD*, and *pprA* genes are located within small clusters of genes, and in each of

these situations the downstream gene also encodes a hypothetical protein. Although we did not observe an increase in transcription from these downstream genes when R1 was exposed to ionizing radiation, there is a formal possibility that the gene replacement might affect expression of the downstream coding sequence.

Our analyses of the effect of how combining alleles influences the ionizing radiation resistance of the resulting strain have allowed us to operationally define the founding members of three epistasis groups involved in the *D. radiodurans* response to ionizing radiation, which we have chosen to name the *recA*, *ddrA*, and *ddrB* groups. The *recA* epistasis group includes *recA* and *pprA*. The effect of the combined loss of *recA* and *pprA* on the radioresistance of *D. radiodurans* is quantitatively the same as the loss observed when only *recA* has been deleted (Figure 6). Deletion of *ddrA* and *ddrB* increases the sensitivity of the *recA* strain, indicating that their respective gene products contribute to radioresistance by mechanisms that are distinct from that of RecA. Consistent with this grouping, *pprA* is not epistatic to *ddrA* or *ddrB*. The shape of the survival curves for the *ddrA pprA* and *ddrB pprA* strains and that of the *pprA* strain are dissimilar, the double mutants exhibiting substantially greater sensitivity to doses of ionizing radiation in excess of 5 kGy (Figure 3).

The *ddrA* and *ddrB* genes are clearly part of separate epistasis groups. The combined effect of inactivation of both loci results in a decrease in ionizing radiation resistance comparable to that observed when the *pprA* locus is inactivated (Figure 4). Taken together, the survival data in Figures 3, 4, and 6 argue that three genetically separable processes significantly contribute to the ionizing radiation resistance of *D. radiodurans* and that the level of resistance observed in this species is a consequence the combined effect of all three activities.

At present, it is not possible to definitively place *ddrC* or *ddrD* in one of these groups. Since the *ddrC* and *ddrD* single mutants do not exhibit any increased susceptibility relative to R1 (Figure 2), the failure of double mutants, generated using these alleles, to express a phenotype different from that of a sensitive single mutant cannot be taken as proof of epistasis. Thus, while there is evidence that DdrC and DdrD participate in ionizing radiation resistance (Figures 3, 5, and 6), the relationships between these proteins and DdrA, DdrB, and PprA cannot be unequivocally defined.

The *ddrA* and *ddrB* gene products: Previous studies established that DdrA (Harris *et al.* 2004) and DdrB (Liu *et al.* 2003) contribute to the ionizing radiation resistance of *D. radiodurans* R1, and those results are confirmed here. DdrA is distantly, but specifically, related to the Rad52 protein of eukaryotes and the Erf protein of some cryptic phage (Iyer *et al.* 2002), and *in vitro* studies have established that purified DdrA binds to single-stranded DNA with affinity for 3′ ends, protecting those ends from nuclease degradation (Harris *et al.* 2004).

In contrast, DdrB has no obvious similarity to any characterized protein, and its biochemical function has not been elucidated. Since TNK117 Δ*ddrA* Δ*ddrB* is much more sensitive to ionizing radiation than TNK102 Δ*ddrB* or TNK104 Δ*ddrA* (Figure 4), it appears that the loss of one of these proteins is partially compensated for by the activity of the other, suggesting that DdrA and DdrB have overlapping functions. We have proposed that DdrA is part of a DNA end-protection system that helps to preserve genome integrity following exposure to ionizing radiation (Harris *et al.* 2004). On the basis of this interpretation, we predict that DdrB may also act to protect the genome of the irradiated cell possibly by preventing degradation of genomic DNA postirradiation by an alternate mechanism.

The *pprA* gene product: Narumi *et al.* described PprA as a DNA repair protein when they deposited the coding sequence in 1997 (NCBI accession no. O32504), but with the exception of anecdotal reference to this peptide in two recent articles (Gao *et al.* 2003; Hua *et al.* 2003), there has not been a published description of the protein's activity or effect on the DNA damage tolerance of *D. radiodurans*. A search for homologs of PprA (DRA0346p) using its predicted amino acid sequence failed to identify any significant matches between this protein and any other listed in current databases or to any described sequence motif. In this study, we demonstrate that the *pprA* gene product is required for ionizing radiation resistance (Figure 2), and that it is epistatic to RecA (Figure 6). Also, we have determined that PprA is not necessary for natural transformation. The Δ*pprA* strain TNK105 takes up and integrates genetic markers as efficiently as R1, indicating that this protein functions to facilitate DNA repair and not homologous recombination *per se*. This observation suggests that PprA modifies the process of homologous recombination in a way that enhances the cell's capacity to deal with ionizing-radiation-induced DNA damage. The nature of this enhancement is unknown, but any mechanism that improves the efficiency of recombinational repair would also improve cell survival. PprA could, for example, protect the substrates for recombination (such as free ends generated by DNA double-strand breaks) from exonuclease digestion. In many species, including *D. radiodurans*, exposed free ends serve as substrates for intracellular exonucleases that begin degrading DNA from these sites. If it is genomic DNA that is being degraded, the loss of genetic information will ultimately lead to cell death. *D. radiodurans* appears to have the ability to control DNA degradation postirradiation by synthesizing proteins that prevent extensive digestion of the genome (Dean *et al.* 1966; Lett *et al.* 1967; Vukovic-Nagy *et al.* 1974). Perhaps PprA is one of these proteins.

A RecA-independent contribution to the radioresistance of *D. radiodurans*: As illustrated in Figures 2 and 6, a *recA* strain of *D. radiodurans* is very sensitive to ionizing radiation when compared with the R1 parent. Clearly,

RecA-mediated homologous recombination is necessary for ionizing radiation resistance in this species, but the Δ*recA* strain TNK106 can be made more sensitive by also deleting *ddrA, ddrB, ddrC,* or *ddrD.* This result provides genetic evidence that *D. radiodurans* utilizes RecA-independent mechanisms that enhance cellular resistance to ionizing-radiation-induced damage. The significance of these mechanisms is apparent when one compares the sensitivity of the TNK117 Δ*ddrA* Δ*ddrB* with the other strains evaluated in this study. At the highest dose tested this strain was as sensitive as the Δ*pprA* strain TNK105 (Figure 4). The only strains showing higher sensitivity were the double mutants that combined Δ*ddrA* or Δ*ddrB* with Δ*pprA* (Figure 3) and those strains that lack *recA.* Thus, it appears that at least two *recA*-independent processes contribute to ionizing radiation resistance in *D. radiodurans,* one dependent on DdrA and the other dependent on DdrB.

Evidence for *recA*-independent repair of DNA double-strand breaks has been provided previously. Daly and Minton (1996) reported that as much as 30% of the genome of *D. radiodurans* could be reassembled by a mechanism that did not require RecA. These authors argued that single-strand annealing was responsible for the limited restitution observed, in part because, despite the damage caused by high-dose irradiation, *D. radiodurans* cultures recover without evidence of mutation. As opposed to other known RecA-independent processes, which can repair double-strand DNA breaks, such as nonhomologous end joining (Lees-Miller and Meek 2003), single-strand annealing is a largely error-free process. However, direct evidence for single-strand annealing participating in the repair of DNA damage in *D. radiodurans* has never been provided.

The radioresistance of *D. radiodurans*: The results presented here indicate that part of the explanation for the radioresistance of *D. radiodurans* is found among the proteins of unknown function encoded by this species. As discussed above, this result was not unexpected. Analysis of the *D. radiodurans* genome sequence established that most of the DNA repair proteins, found in *E. coli,* are also present in *D. radiodurans* (White *et al.* 1999). Since *E. coli* is far more sensitive to ionizing radiation than *D. radiodurans,* it has been considered likely that *D. radiodurans* expresses unique mechanisms for dealing with ionizing-radiation-induced DNA damage. Here, we have provided evidence that at least five of these hypothetical proteins contribute to the radioresistance of this species, and in the process we have taken the preliminary steps toward defining what appears to be a complex interaction between RecA-dependent and RecA-independent mechanisms of DNA repair in this species.

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