

# Genetic Characterization of Forty Ionizing Radiation-Sensitive Strains of *Deinococcus radiodurans*: Linkage Information from Transformation

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**Natural transformation was used to help define a collection of ionizing radiation-sensitive strains of *Deinococcus radiodurans*. Three putative *rec* mutations were identified, as were three *pol* alleles. Forty of the ionizing radiation-sensitive strains were placed into 16 linkage groups, and evidence obtained indicates that each linkage group consists of a cluster of mutations not more than 1,000 bp apart. In addition, a new class of *D. radiodurans* mutant was described that, although radioresistant, appears to recover from ionizing radiation-induced DNA damage slowly relative to other strains of *D. radiodurans*.**

*Deinococcus radiodurans* is the most-studied member of the family *Deinococcaceae*, a bacterial family characterized by extreme resistance to ionizing radiation (11, 13, 17). Well-aerated, exponential-phase cultures of *D. radiodurans* survive 5,000 Gy of  $\gamma$  radiation without loss of viability (16), and there are reports of other deinococcal strains surviving as much as 50 kGy (3) of  $\gamma$  radiation. Despite this remarkable property and the potential advantages such an organism offers to those studying ionizing radiation-induced DNA damage and its repair, the mechanisms responsible for the extreme radioresistance of the deinococci are poorly understood.

While the capacity to survive the massive DNA damage inflicted by extreme doses of ionizing radiation certainly suggests that the deinococci have evolved sophisticated DNA repair systems to deal with that damage, there is little direct evidence to support this conclusion. Only four deinococcal proteins have been associated with ionizing radiation resistance. They are the *rec* and *pol* gene products, homologs of *Escherichia coli* RecA (8) and DNA polymerase I (9), respectively, and the *irrB* and *irrI* gene products, whose biochemical functions have yet to be determined (20).

During previous studies of *D. radiodurans*, we isolated 49 putative ionizing radiation-sensitive (IRS) strains with the intention of using these strains to define the enzymatic components involved in deinococcal ionizing radiation resistance. We further characterize this collection of strains here by demonstrating (i) that the mutations responsible for the IRS phenotype fall into 16 different linkage groups, (ii) that all but three of the IRS strains are recombination proficient as evidenced by their ability to undergo natural transformation, (iii) that three of the IRS strains carry mutations in the *D. radiodurans pol* locus, and (iv) that five of the strains in this collection recover from  $\gamma$  radiation-induced DNA damage at a significantly slower rate than does their parent, *D. radiodurans* 302.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. All *D. radiodurans* strains were grown at 30°C in TGY broth (0.5% tryptone, 0.3% yeast extract, 0.1% glucose) or on TGY agar (1.5%

agar). Plasmids were routinely propagated in *E. coli* DH5 $\alpha$ -MCR. Plasmids were isolated by alkaline extraction (4).

***N-Methyl-N'-nitro-N-nitrosoguanidine* mutagenesis and isolation of IRS strains.** Exponentially growing cultures of *D. radiodurans* 302 were treated with 20  $\mu$ g of *N-methyl-N'-nitro-N-nitrosoguanidine* per ml and incubated for 2 h at 30°C with shaking (18, 20). A 100- $\mu$ l aliquot of mutagenized cells was diluted in 25 ml of TGY and incubated for an additional 18 h. The resulting culture was diluted and plated at 200 to 300 CFU per plate. The mutagenized population was screened for ionizing radiation sensitivity by patching individual colonies onto TGY plates and exposing the plates to 5,000 Gy of  $\gamma$  radiation ( $^{60}\text{Co}$  source, 14.4 Gy/min, 22°C). Colonies of untreated *D. radiodurans* 302 patched onto each plate served as a control. IRS mutants were identified by comparing the growth of mutagenized colonies with that of strain 302 48 h after irradiation. Putative mutants were streaked to isolation, and the IRS phenotype was confirmed by irradiating cultures grown from isolated colonies.

**Quantification of ionizing radiation resistance.** Only *D. radiodurans* cultures in the exponential growth phase were evaluated for the ability to survive ionizing radiation. Cultures were divided into 1-ml aliquots, placed in Eppendorf tubes, and exposed to a  $^{60}\text{Co}$  source. Cultures were removed from the source after an accumulated dose of 5,200 Gy was achieved. Irradiated cells were diluted, plated in triplicate on TGY agar plates, and incubated for 5 days at 30°C before being scored for survivors. The size of the population irradiated was determined by measuring the titer of each culture immediately prior to irradiation. Percent survival for individual IRS strains was calculated by dividing the number of survivors, expressed as CFU per milliliter, by that strain's titer prior to irradiation and multiplying the resulting fraction by 100.

**Dot transformation.** The dot transformation protocol used in this study is a modification of that described previously (20). A 100- $\mu$ l aliquot of a *D. radiodurans* culture in the exponential growth phase was spread onto a TGY plate and incubated for 4 to 6 h at 30°C. Transformations were performed by dotting 3 to 7  $\mu$ g of either chromosomal or plasmid DNA onto the plate. Twenty-four hours later, the bacterial lawn was replica plated onto TGY agar and selective pressure was applied. Plates were exposed to 7,500 Gy of  $\gamma$  radiation to select for ionizing radiation resistance. Plates were examined each day for the next 5 days and scored by noting colony formation within the area where the DNA had been dotted.

**Chromosomal DNA isolation.** TGY broth (500 ml) was inoculated with a 2-ml overnight culture (approximately  $2 \times 10^7$  CFU/ml) of *D. radiodurans*. After 18 h, the 500-ml cultures were harvested by centrifugation at 4°C at  $3,000 \times g$  for 15 min. Pellets were resuspended in 25 ml of absolute ethanol to remove the *D. radiodurans* outer membrane. The ethanol-stripped cells were collected by centrifugation at 4°C at  $3,000 \times g$  for 15 min, and the resulting pellet was resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Two milligrams of lysozyme was added to the stripped cells, and the mixture was incubated at 37°C for 30 min. Five milliliters of a pronase E solution (2% sodium dodecyl sulfate, 0.1 M EDTA [pH 8.0], 4 mg of pronase E) was added to the lysozyme-treated cells, and the mixture was incubated for 3 h at 50°C. Lysed cells were transferred to a centrifuge tube and extracted twice with an equal volume of phenol-chloroform (1:1). The DNA was precipitated by adding 1 ml of 3 M sodium acetate (pH 7.0) and 20 ml of ice-cold absolute ethanol to the extracted material. The DNA was spooled out with a curved glass rod and washed twice with 70% ethanol. The DNA was air dried and dissolved in 4 ml of TE buffer (pH 8.0) and stored at 4°C. This procedure yielded approximately 400 mg of chromosomal DNA per 500-ml culture. When analyzed by pulsed-field gel electrophoresis, this preparation routinely generated 40- to 50-kb DNA fragments.

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain(s)	Relevant description	Source or reference(s)
<i>Deinococcus radiodurans</i>		
R1	ATCC 13939	2
302	Same as R1 but <i>uvrA1</i> ( <i>mtcA</i> ) <sup>a</sup>	15, 16
IRS1-IRS49	Same as 302 but IRS	20; this study
IRS26	Same as 302 but nontransformable	20; this study
IRS27	Same as 302 but nontransformable	20; this study
IRS46	Same as 302 but nontransformable	20; this study
IRS7	Same as 302 but <i>pol-1</i>	20; this study
IRS33	Same as 302 but <i>pol-2</i>	20; this study
IRS38	Same as 302 but <i>pol-3</i>	20; this study
SLR1	Formerly IRS1	20; this study
SLR2	Formerly IRS2	20; this study
SLR3	Formerly IRS5	20; this study
SLR4	Formerly IRS16	20; this study
SLR5	Formerly IRS17	20; this study
LS18	Same as R1 but streptomycin resistant	20
<i>Escherichia coli</i>		
DH5 $\alpha$ -MCR	F <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>dlacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>endA1</i> <i>recA1</i> <i>deoR</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>araD139</i> <i>galU</i> <i>galK</i> <i>nupG</i> <i>rpsL</i>	Life Technologies Inc., Gaithersburg, Md.
Plasmids		
pUE58	pAT153: <i>uvrA</i> <sup>+</sup> ( <i>mtcA</i> <sup>+</sup> ) <i>irrB</i> <sup>+</sup> ; 5.6-kb subclone of <i>D. radiodurans</i> genomic DNA carrying part of wild-type <i>uvrA</i> gene that restores <i>uvrA1</i> ( <i>mtcA</i> ) strains to mitomycin C resistance and <i>irrB</i> gene	1
pPG9	pBC SK <sup>+</sup> derivative with 2.3-kb insert of <i>D. radiodurans</i> genomic DNA carrying 1.9-kb fragment of <i>pol</i> gene	9
pPG11	pBluescript SK <sup>+</sup> derivative with 5.2-kb insert of <i>D. radiodurans</i> genomic DNA carrying 1.89-kb fragment of <i>pol</i> gene	9
pPG12	pBC SK <sup>+</sup> derivative with 1.2-kb insert of <i>D. radiodurans</i> genomic DNA carrying 0.9-kb fragment of <i>pol</i> locus	9

<sup>a</sup> The *mtcAB* region of the *D. radiodurans* chromosome has recently been sequenced by Minton and colleagues (11, 12) and shown to contain a single gene whose product is homologous with the UvrA protein of *E. coli*. In light of this new information, these investigators have recommended that the *mtcA* mutation of *D. radiodurans* 302 be designated *uvrA1*.

**Growth measurement.** A five-hundred-microliter aliquot of an overnight culture of each strain was transferred to 50 ml of TGY broth in a 250-ml flask. The culture was grown at 30°C with vigorous shaking (200 rpm). At 2 ( $t_1$ ) and 4 ( $t_2$ ) h postinoculation, an aliquot of the growing culture was removed and serially diluted in 10 mM MgSO<sub>4</sub>, and the dilutions were spread onto TGY agar. Spread plates were incubated at 30°C for 5 days, and the number of CFU was determined. The doubling time,  $g$ , was calculated from the equation  $g = \ln 2/k$ . The growth rate constant,  $k$ , was calculated from the equation  $k = (\log_{10} N_2 - \log_{10} N_1) / 2.303/\Delta t$ , where  $N_1$  is CFU per milliliter at  $t_1$  and  $N_2$  is CFU per milliliter at  $t_2$ .

## RESULTS

**IRS mutants of *D. radiodurans* 302.** Isolation of IRS strains was described in an earlier report (20). To confirm the IRS phenotype, each IRS strain's ability to survive exposure to 5,200 Gy of  $\gamma$  radiation was compared with that of the parent strain. Exponential-phase cultures of each putative IRS strain were irradiated, appropriately diluted, plated in triplicate on TGY agar, and scored for survivors 5 days after plating. Figure 1 depicts the results of this assessment. Each value is the mean percent survival  $\pm$  the standard deviation of duplicate experiments. Strain 302 displayed approximately 84% survival. Forty-three of the 49 putative IRS strains exhibited reduced survival relative to strain 302 in this assay. IRS46 was the most ionizing radiation resistant of the IRS strains, with 65% survival. Twenty-nine of the IRS strains demonstrated survival of 10% or less. IRS26, IRS27, IRS33, IRS34, IRS38, and IRS41 were the most sensitive strains, with less than 0.1% survival at the dose administered. The survival of *D. radiodurans* R1, the type strain for this species, did not differ from that of strain 302.

Six of the strains that we originally designated IRS failed to exhibit enhanced sensitivity to  $\gamma$  radiation in this assay and are not included in Fig. 1. We could not confirm the IRS phenotype for the strain originally designated IRS3, and this strain was not investigated further. The other five strains displayed a novel phenotype that explained their misclassification as IRS strains.

**Mutants of *D. radiodurans* that recover from DNA damage more slowly than strain 302.** Five of the original 49 IRS strains evaluated in this study displayed an unusual phenotype. They recovered from the detrimental effects of  $\gamma$  radiation more slowly than the parent strain. When the survival of these strains, originally designated IRS1, IRS2, IRS5, IRS16, and IRS17, was determined 2 days postirradiation, the strains appeared to be highly susceptible to  $\gamma$  radiation (Fig. 2), with none of the five strains exhibiting more than 5% survival. However, when the plates used to quantify survival were returned to the incubator and recounted 5 days postirradiation, the strains did not exhibit enhanced ionizing radiation sensitivity (Fig. 2). Surviving cells could not form detectable colonies within 2 days of irradiation, but visible colonies did form within 5 days. The low level of survival calculated after 2 days was therefore an artifact of the slow appearance of survivors. We have reclassified these five strains, referring to them as slow-recovery or SLR strains. The new strain designations are listed in Table 1.

The slow-recovery phenotype was not observed when evaluating the survival of R1, 302, or the majority of the IRS strains following exposure to  $\gamma$  radiation (data not shown). Three IRS strains did, however, exhibit slow recovery. There was an ap-

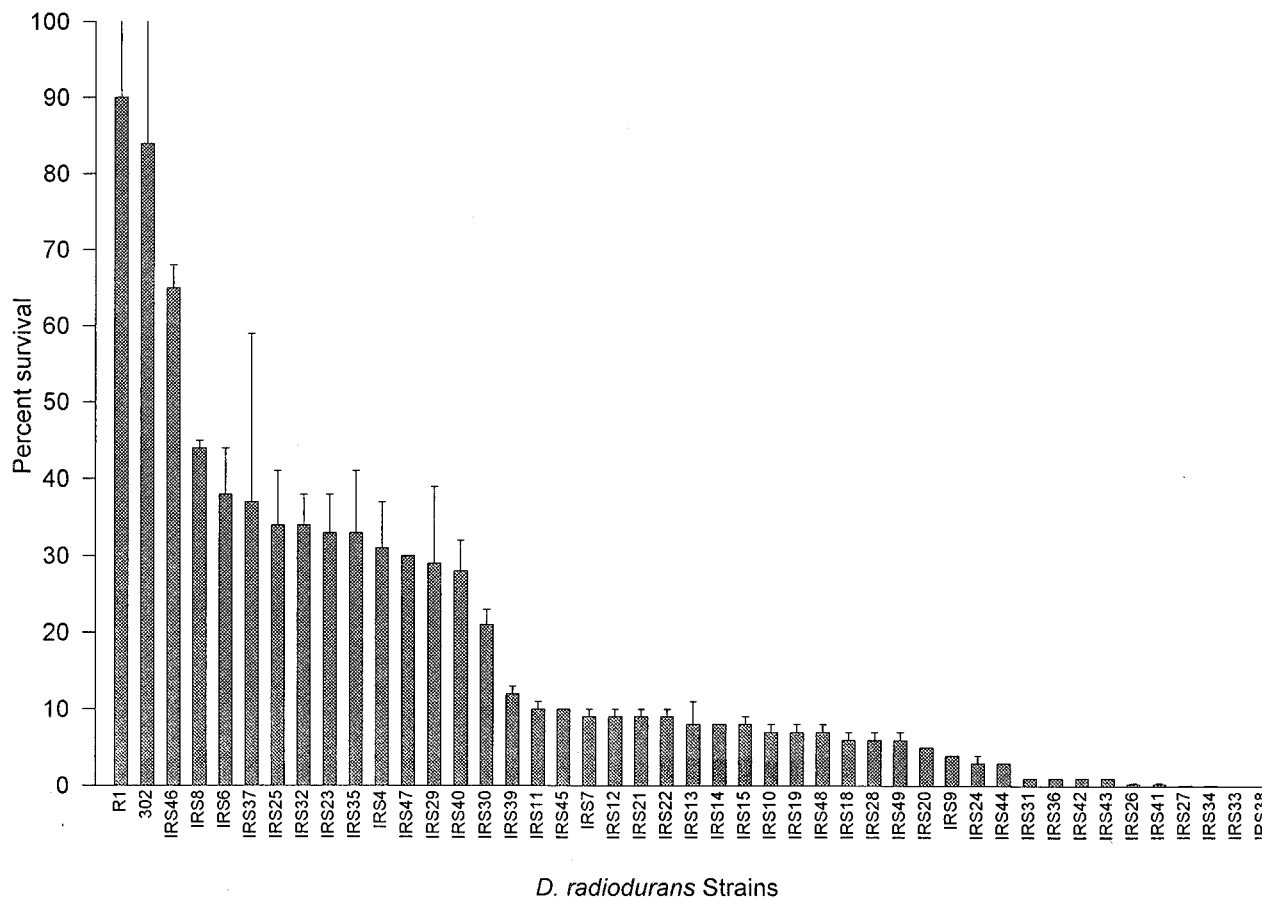


FIG. 1. Survival of IRS strains following exposure to 5,200 Gy of  $\gamma$  radiation. Each value is the mean  $\pm$  the standard deviation of six replicates from two experiments.

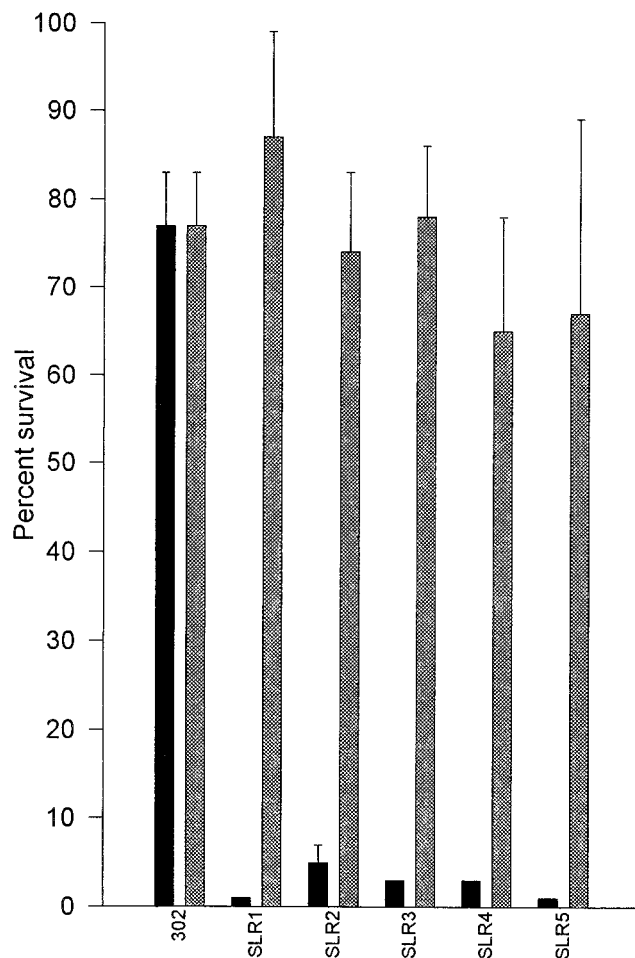
proximately 30-fold increase in the number of detectable survivors between days 2 and 5 postirradiation in cultures of IRS6, IRS35, and IRS37.

Two possibilities seemed likely to account for the slow-recovery phenotype: (i) the SLR strains acquired mutations that resulted in slow growth, and (ii) the SLR strains acquired mutations that slowed some aspect of the repair process needed to recover from ionizing radiation-induced DNA damage. We evaluated the first possibility by determining the generation time of the SLR strains and comparing that value with the generation time of strains R1 and 302. Those results are presented in Table 2. The growth rate of SLR2, SLR4, and SLR5 did not differ from that of 302. In contrast, SLR1 and SLR3 grew from 1.5 to 2 times more slowly than 302.

**Forty of the IRS strains are naturally transformable.** The IRS strains were screened for possible loss of RecA activity by determining if they could be naturally transformed. Exponential-phase cultures of *D. radiodurans* readily take up and incorporate homologous DNA into their chromosome (19), and functional deinococcal RecA is required for this process (8, 14). An attempt was made to transform each IRS strain to streptomycin resistance by using chromosomal DNA isolated from LS18, a streptomycin-resistant isolate of *D. radiodurans* R1 (20). All but three of the IRS strains were successfully transformed to streptomycin resistance with an efficiency of approximately 0.1%. It was therefore assumed that all transformable strains expressed a functional RecA protein and were proficient in homologous recombination.

IRS26, IRS27, and IRS47 could not be transformed by this protocol. IRS26 and IRS27 were found to be quite sensitive to  $\gamma$  radiation, being 100- and 300-fold more sensitive than 302, respectively, to a 5,200-Gy dose (Fig. 1). In contrast, IRS47 was only fourfold more sensitive than 302.

**Three of the recombination-proficient IRS strains are *pol* mutants.** The recombination-proficient IRS strains were examined to determine if they carried a mutation in the *D. radiodurans pol* gene since mutations at this locus have been shown to adversely affect ionizing radiation resistance (9). An attempt was made to restore radioresistance to the 40 recombination-proficient IRS strains by transforming them with subclones of the wild-type *pol* gene found on three plasmids: pPG9, pPG11, and pPG12 (9). pPG9 carries a 2,300-bp subclone of the *D. radiodurans* chromosome that includes the first 1,900 bp of the *pol* gene and approximately 400 bp of the upstream sequence. pPG11 carries a 5,200-bp cloned insert that covers the terminal 1,890 bp of the *pol* coding sequence with 3,200 bp of the downstream sequence. pPG12 has a 1,200-bp insert consisting of 990 bp of the 3' end of the *pol* gene and 210 bp of the downstream sequence. A 155-bp region of the *pol* coding sequence is common to all three plasmids (Fig. 3). These plasmids were linearized and used to dot transform each of the 40 transformable IRS strains. Strains IRS7, IRS33, and IRS38 were transformed to ionizing radiation resistance by two or more of these plasmids (Table 3). IRS7 and IRS38 were restored with pPG11 and pPG12, but not with pPG9, indicating that the mutations found in IRS7 and IRS38 were located



#### *D. radiodurans* Strains

FIG. 2. Survival of SLR strains following exposure to 5,200 Gy of  $\gamma$  radiation. Solid bars represent percent survival based on the number of detectable survivors 48 h postirradiation. Hatched bars represent percent survival based on the number of detectable survivors 120 h postirradiation. Each value is the mean  $\pm$  the standard deviation of 12 replicates from three experiments.

within the terminal 990 bp of the *pol* coding sequence. All three plasmids restored ionizing radiation resistance to IRS33, localizing this mutation to the 155-bp region of the *pol* gene common to the three plasmids. The mutations found in IRS7, IRS33, and IRS38 were designated *pol-1*, *pol-2*, and *pol-3*, respectively. The assignment of separate allele numbers is justified because (i) the location of the mutation in IRS33 is distinct from that of the mutations of IRS7 and IRS38 (Table 3) and (ii) IRS7 and IRS38 are phenotypically distinct, displaying different degrees of sensitivity to ionizing radiation (Fig. 1).

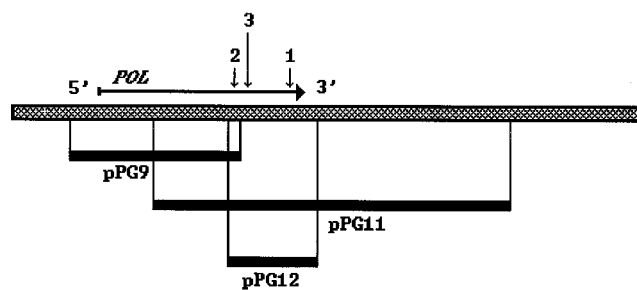


FIG. 3. Representation of the relative positions of the *pol-1*, *pol-2*, and *pol-3* alleles within the *D. radiodurans pol* coding sequence. The numbers 1, 2, and 3 indicate the positions of the respective mutations. Those portions of the *pol* gene associated with plasmids pPG9, pPG11, and pPG12 are represented diagrammatically below the coding sequence.

IRS38 is approximately 100-fold more sensitive to  $\gamma$  radiation than is IRS7. In addition, genomic DNA from IRS7 restored ionizing radiation resistance to IRS38 in a dot transformation assay, indicating that mutation in IRS7 affects a site within the *pol* coding sequence different from that affected by the mutation in IRS38. This result is discussed in greater detail in the following section.

**Identification of 16 linkage groups among the recombination-proficient IRS strains.** Linkage between mutations represented within the recombination-proficient IRS strains was determined by using transformation. An aliquot from an exponential-phase culture of each IRS strain was spread onto a TGY agar plate, and 5  $\mu$ g of genomic DNA from 8 to 12 other IRS strains was dotted in separate locations on the plate. After 48 h of incubation at 30°C, the lawn that formed was replica plated onto fresh TGY plates and  $\gamma$  irradiated at 7,500 Gy to select for cells transformed to wild-type levels of ionizing radiation resistance. Restoration of ionizing radiation resistance in the recipient strain indicates allelic substitution. The mutation responsible for the recipient strain's ionizing radiation sensitivity is replaced with the wild-type sequence provided by the donor DNA in a recombinational event. Chromosomal DNA from each IRS strain was tested for the ability to restore a radioresistant phenotype to all other IRS strains. The results of those analyses are summarized in Table 4. We grouped those strains that failed to restore radioresistance to each other together and assigned a letter designation to each group. For example, the five strains in group K cannot restore radioresistance to each other but do restore resistance to all other recombination-proficient IRS strains. By this analysis, the IRS strains were divided into 16 different linkage groups.

The physical distance separating the mutations that define each linkage group appears to be small. This fact was most evident when linkage groups C and D were examined. As noted previously (Table 3), IRS7, IRS33, and IRS38 carry different mutations within the *pol* gene. IRS33 and IRS38 form a linkage group that does not include IRS7. This suggests that

TABLE 2. Generation times of SLR strains of *D. radiodurans*

Mean generation time (h) $\pm$ SD of strain <sup>a</sup> :						
R1	302	SLR1	SLR2	SLR3	SLR4	SLR5
1.0 $\pm$ 0.03	1.5 $\pm$ 0.3	2.2 $\pm$ 0.3 <sup>b</sup>	1.5 $\pm$ 0.2	2.9 $\pm$ 0.7 <sup>b</sup>	1.5 $\pm$ 0.04	1.2 $\pm$ 0.02

<sup>a</sup> See Table 1 for descriptions of the strains used in this analysis. Each value is the mean  $\pm$  the standard deviation of four experiments.

<sup>b</sup> With Student's *t* test, the mean generation times calculated for strains SLR1 and SLR3 were found to differ significantly from that calculated for strain 302 at a 95% level of confidence.

TABLE 3. Restoration of ionizing radiation resistance in IRS7, IRS33, and IRS38 with subclones of the *D. radiodurans* *pol* gene<sup>a</sup>

Strain	Restoration of ionizing radiation resistance by plasmid <sup>b</sup> :		
	pPG9	pPG11	pPG12
IRS7	–	+	+
IRS33	+	+	+
IRS38	–	+	+

<sup>a</sup> See Table 1 for detailed descriptions of the plasmids used in this analysis.

<sup>b</sup> +, restoration of IRS strain to a radioresistant phenotype following transformation with donor DNA; –, no restoration.

the *pol-2* and *pol-3* mutations found in IRS33 and IRS38 are closer to each other than they are to the *pol-1* mutation found in IRS7. Genomic DNA isolated from IRS7 transformed IRS38 to wild-type levels of ionizing radiation resistance with an efficiency of approximately 0.1%. This efficiency is identical to that obtained when chromosomal DNA isolated from wild-type *D. radiodurans* R1 was used to transform IRS38 to ionizing radiation resistance. In contrast, DNA isolated from IRS33 did not transform IRS38 to ionizing radiation resistance at a detectable frequency. Presumably, the sites of the *pol-2* and *pol-3* mutations are so close that replacement of the *pol-3* mutation with the wild-type sequence derived from IRS33 would result in simultaneous incorporation of the *pol-2* mutation. The transformant remains IRS. The relative positions of the *pol-1*, *pol-2*, and *pol-3* mutations are represented diagrammatically in Fig. 3.

## DISCUSSION

We previously reported the isolation of 49 putative IRS derivatives of *D. radiodurans* 302 (20). In this report, we further characterize these isolates. Evaluation of the 49 strains revealed that 43 were truly IRS compared with the parent strain (Fig. 1) and that the other 6 strains had been misclassified as IRS in our initial study.

TABLE 4. Linkage groups identified among IRS strains by dot transformation

Group	Strain(s)	Group	Strain(s)
A	IRS9	L	IRS8, IRS43, IRS44
B	IRS18		
C	IRS7	M	IRS14, IRS15, IRS21, IRS29, IRS48
D	IRS33, IRS38		
E	IRS24	N	IRS4, IRS20, IRS30, IRS40
F	IRS34		
G	IRS32		
H	IRS25, IRS42, IRS49	O	IRS12, IRS39, IRS45
I	IRS41		
J	IRS6, IRS13, IRS46		
K	IRS10, IRS11, IRS19, IRS22, IRS36	P	IRS23, IRS28, IRS31, IRS35, IRS37

Five of the six misclassified strains exhibited a mutant phenotype that gave the appearance of ionizing radiation sensitivity. These strains, which were originally designated IRS1, IRS2, IRS5, IRS16, and IRS17, recover from  $\gamma$  irradiation more slowly than does the parent strain. For this reason, we chose to rename these strains SLR1 to SLR5 (Table 1). Two of the strains, SLR1 and SLR3, grow approximately twice as slowly as strain 302, and this may account for the slow appearance of colonies postirradiation. This explanation, if correct, suggests that the mutations found in SLR1 and SLR3 affect growth without affecting the cell's ability to recover from radiation-induced DNA damage. In contrast, SLR2, SLR4, and SLR5 exhibit normal growth rates, indicating that their slow recovery is a consequence of the effects of  $\gamma$  radiation. These three strains, while obviously able to repair ionizing radiation-induced damage, appear to be defective in temporal control of the repair process. This defect could be as simple as the failure of an enzyme to excise DNA damage as rapidly as in the parent strain, or it could involve a more complex regulatory network that controls the timing of events associated with damage repair.

Among the 43 IRS strains identified in Fig. 1, 40 were found to be recombination proficient as judged by the ability to take up an antibiotic resistance marker. Only IRS26, IRS27, and IRS47 could not be transformed to streptomycin resistance with chromosomal DNA from LS18. Since defects at any stage in the uptake or incorporation of DNA during natural transformation would result in this failure, it is not possible to state with certainty that IRS26, IRS27, and IRS47 are recombination defective. It does, however, seem likely that they are *rec* mutant strains. Each, in addition to being nontransformable, is also IRS, a characteristic typical of *rec* mutant strains of *D. radiodurans* (8, 14). Also, there is no reason to believe that the cell's loss of non-RecA functions related to transformation affects the cell's ability to cope with DNA damage. *D. radiodurans* is the only species in the family *Deinococcaceae* that is naturally transformable, and the other members of this family are as resistant to DNA damage as is *D. radiodurans* (11).

Three *pol* mutant strains (IRS7, IRS33, and IRS38) were found in the collection of IRS strains. This was expected, since *pol* mutants of *D. radiodurans* are known to be very sensitive to the effects of UV and  $\gamma$  radiation (9). The evidence presented indicates that each strain carries a different *pol* allele and that all of these alleles are located in the terminal 1,890 bp of the *pol* coding sequence. We have designated the *pol* mutants in IRS7, IRS33, and IRS38 as *pol-1*, *pol-2*, and *pol-3*, respectively. The data presented in Tables 3 and 4 permit the *pol* mutations to be ordered within the *pol* coding sequence. The *pol-2* allele is the only one of the three that can be transformed to radioresistance with pPG9, pPG11, and pPG12 (Table 3), making it the most 5' of the *pol* alleles. Further, chromosomal DNAs from *pol-2* and *pol-3* mutant strains cannot transform each other to radioresistance (Table 4), placing them closer to each other than to *pol-1*. The three alleles should, therefore, be arranged as follows within the *pol* coding sequence: 5'–*pol-2*–*pol-3*–*pol-1*–3' (Fig. 3).

By using natural transformation, we identified 16 linkage groups among the mutations represented in the IRS strains (Table 4). Strains that could not restore each other to radioresistance were grouped together and assigned a letter designation. The failure of chromosomal DNA from one IRS strain to restore radioresistance to another IRS strain indicates one of two alternative possibilities: (i) that the mutations found in the donor and recipient strains affect the same site, or (ii) that these mutations affect different sites closely linked to each other. In either circumstance, restoration of the radioresistant

phenotype is prevented but for different reasons. When the mutations of the donor and recipient are at the same site, the wild-type sequence needed to replace the recipient's mutation is not present in the donor DNA, making restoration of radioresistance impossible. When the mutations of the donor and recipient are at different but closely linked sites, successful transformation of the recipient with the wild-type sequence results in cotransformation with the donor's mutation. The transformants remain IRS because they carry the donor strain's mutation.

Cotransformation of linked markers is expected during natural transformation. In studies conducted with other transformable species, such as *Bacillus subtilis*, it is assumed that if two markers cotransform they are near enough to each other to be carried on the same DNA fragment and that there is a strong probability that both genes will be incorporated into the chromosome simultaneously during recombination (10). The cotransformation of linked markers in *D. radiodurans* is remarkable, however, in that the physical distance between markers appears to be very small. This was demonstrated by using the *pol* mutants. Chromosomal DNA from *pol-1* mutant strain IRS7, unlike DNA from *pol-2* mutant strain IRS33, efficiently transforms *pol-3* mutant strain IRS38 to radioresistance, even though the *pol-1* mutation is no more than 990 bp from the *pol-3* mutation. In other words, the *pol-1* allele is not incorporated into the *pol-3* recipient at a detectable frequency despite the proximity of these alleles to each other within the *pol* coding sequence. This indicates that the size of the piece of DNA incorporated into the recipient strain during transformation of this region of the deinococcal chromosome is less than 1,000 bp. In comparison, the average size of the DNA fragment incorporated into *B. subtilis* during natural transformation has been estimated to be 4,300 bp (6), and markers 3,000 bp from each other are incorporated with a cotransformation frequency of greater than 75% (10). The fragment sizes incorporated in *Streptococcus pneumoniae* and *Haemophilus influenzae* are even larger, with estimates of 5 kb (7) and 18 kb (5), respectively. Whether this small fragment size is representative of all fragments incorporated during natural transformation in *D. radiodurans* remains to be determined.

During our characterization of this collection of IRS strains, we identified five different genes involved in the radioresistance of *D. radiodurans*. In this study, we found *pol* mutants and evidence of at least one other locus whose inactivation affects ionizing radiation resistance and natural transformation. Previously, IRS18, IRS34, and IRS41 were shown to carry mutations in unique loci designated *irrB*, *irrF*, and *irrI*, respectively (20). The number of loci inactivated in the other 34 IRS strains has not been determined. These 34 strains make up 12 of the linkage groups defined in Table 4. Given the possibility that a linkage group consists of mutations found within 1,000 bp of each other, this linkage information cannot be used to estimate the number of loci represented among these 34 strains. There is a strong probability that, as we found with the *pol* gene, different mutations within a single locus occupy more than one linkage group. Pending further investigation, each linkage group should be viewed as a cluster of mutations in

close proximity to each other that affect a gene or operon necessary for ionizing radiation resistance.

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