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Interchromosomal Recombination in the Extremely Radioresistant Bacterium *Deinococcus radiodurans*

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Deinococcus radiodurans **and other members of the genus** ***Deinococcus*** **are remarkable for their extreme resistance to ionizing radiation and many other agents that damage DNA. We have recently shown that recombinational processes participate in interplasmidic repair following in vivo irradiation. We now present direct studies on interchromosomal recombination among chromosomes irradiated in vivo during stationary phase (four chromosomes per cell). Following an exposure to 1.75 Mrad (the dose required to achieve a survival of 37%, which degrades the cells' four chromosomes into about 500 fragments), we determined that there may be as many as 175 crossovers per chromosome (700 crossovers per nucleoid) undergoing repair. In addition, these studies suggest that many of the crossovers occurring during repair are nonreciprocal.**

The aspect of DNA double-strand break (DSB) metabolism addressed in this report is the repair of ionizing radiation-induced DSBs in the extremely radioresistant bacterium *Deinococcus* (formerly *Micrococcus*) *radiodurans*. Radiation-induced DSBs pose a lethal challenge to the cellular DNA repair machinery because the lesion is noninformative; i.e., it affects both strands of the DNA double helix at the same site, precluding excision repair. *Escherichia coli* is able to repair and survive ionizing radiation-induced DSBs when (i) breaks are few (about two or three DSBs per chromosome), (ii) the *recA* gene is functional, and (iii) there are two or more identical chromosomes (*E. coli* possesses up to five chromosomal copies) (15). Like *E. coli*, most other prokaryotic and eukaryotic cells are killed if there are more than just a few radiation-induced DSBs per chromosome (30).

An exception to this rule is the anomalously high radioresistance of the deinobacterial species. These bacteria are the most ionizing radiation-resistant organisms discovered to date (23). The first of the five deinococcal species to be discovered and the most studied is *D. radiodurans*, isolated in Oregon from X-ray-sterilized canned meat that was found to have undergone spoilage (1, 25). Culture yielded a red-pigmented, nonsporulating, gram-positive bacterium that was extremely resistant to ionizing and UV radiation and many chemical agents that damage DNA; these features are characteristic of all the deinobacteria (23, 25). Full survival of the deinobacteria has been reported following ionizing and UV radiation exposures of 0.5 to 3 Mrad and 600 to 1,000 J/m^2, respectively (3, 4, 14, 23, 27).

Even though it is known that extremely efficient chromosomal DNA repair is central to the resistance of *D. radiodurans* to DNA-damaging agents (for reviews, references see 21 and 23), the mechanisms of its DNA repair pathway(s) have not been studied in any detail. Following an exposure to 1.5 Mrad of γ radiation, about 100 DSBs per chromosome are observed, and these DSBs are repaired within 12 to 24 h without bacterial lethality (4). *D. radiodurans* contains 4 chromosomes per cell during stationary phase and up to 10 chromosomes per cell during exponential phase (13, 36); thus, substrate for recombinational repair is available at every stage of its growth cycle. However, the mere presence of redundant information is insufficient to account for the radioresistance of *D. radiodurans* (2, 33). Polyploidy is common among prokaryotes and does not confer extreme resistance to DNA damage. For example, *Micrococcus luteus* and *M. sodonensis*, which contain multiple chromosomes per cell, are radiosensitive (23). *Azotobacter vinelandii*, which contains 40 to 80 chromosomes (28, 31), is sensitive to UV (18), unlike *D. radiodurans*. Thus, it is likely that *D. radiodurans* uses redundant information in a manner that other organisms do not.

Although postreplication repair is often referred to as recombinational repair, the latter is in fact only a means of tolerating a limited amount of DNA damage involving the two new daughter duplexes just behind the semiconservative replication fork. This process fills in any single-stranded gaps left behind by the replication fork at sites of template strand damage (12). Whereas postreplication repair is limited to repair of single-strand gaps and occurs only behind the replication fork, interchromosomal recombination can repair double-strand gaps or breaks efficiently, as demonstrated in a few eukaryotic model systems, such as *Saccharomyces cerevisiae* (11). We suggest that it is an efficient *recA*-dependent DSB-mediated recombinational repair pathway by which *D. radiodurans* can reconstitute its chromosome from many hundreds of DNA fragments; this is a process that does not necessarily require concurrent semiconservative replicative DNA synthesis (15).

In previous studies on the recovery of a single shuttle plasmid following γ irradiation in vivo, we determined that such a plasmid is repaired with the same extraordinary efficiency as the chromosomal DNA (4). We subsequently evaluated homologous recombination between two distinct plasmids maintained together in the same cells after being irradiated to very high doses in vivo. Plasmidic recombination frequencies were determined and found to support a high frequency of crossovers between plasmids (5).

These studies are advanced here by analyzing DSB-induced chromosomal recombination in real-time studies of *D. radiodurans* cultures recovering from irradiation, using two related plasmid-derived sequences inserted at different locations in the *D. radiodurans* chromosome. The two integrated sequences are alike except for small regions designed to provide several indicators of chromosomal recombination. Studies reported

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TABLE 1. *D. radiodurans* strains used

Strain	Pertinent genotype[a]	Reference or source
R1	Wild type	1
rec30	*recA*	8, 24
MD360	rec30(*recA*) Ω[pS11Ω[*aphA*$^+$::*tet*(*Xho*I-5)]]	This work
MD367	R1 Ω[pS11Ω[*aphA*$^+$::*tet*(*Xho*I-5)]]	This work
MD399	R1 Ω[pS11Ω[*cat*$^+$::*tet*(*Xho*I-2)]]	This work
MD401	R1 Ω[pS14Ω[*aphA*$^+$::*tet*(*Xho*I-5)]	This work
MD420	R1 Ω[pS14Ω[*aphA*$^+$::*tet*(*Xho*I-5)]] Ω[pS11Ω[*cat*$^+$::*tet*(*Xho*I-2)]]	This work
MD429	rec30(*recA*) Ω[pS14Ω[*aphA*$^+$::*tet*(XhoI-5)]] Ω[pS11Ω[*cat*$^+$::*tet*(*Xho*I-2)]]	This work
MD373	rec30(*recA*) Ω[pS11Ω[*aphA*$^+$::*tet*(*Xho*I-5)]] rec30(*recA*) Ω[pS11Ω[*cat*$^+$::*tet*(*Xho*I-2)]]	This work
MD389	R1 Ω[pS11Ω[*aphA*$^+$::*tet*(*Xho*I-5)]] R1 Ω[pS11Ω[*cat*$^+$::*tet*(*Xho*I-2)]]	This work

[a] pS11 and pS14 are integrative vectors in *D. radiodurans* but replicate as plasmids in *E. coli*. Ω indicates an insertion; :: indicates a novel joint. For example, the description of MD429 as rec30(*recA*) Ω[pS14Ω[*aphA*::*tet*(*Xho*I-5)]] Ω[pS11Ω[*cat*::*tet*(*Xho*I-2)]] indicates that this strain is derived from rec30 (hence *recA*) and has two different insertions. The first insertion is of the tandem duplication insertion vector pS14, which in turn contains the *aphA*$^+$ gene (Km^r) linked to the *Xho*I-5 allele of *tet*. The second insertion is at a distant locus, using a direct integration vector derived from pS11, which in turn contains an insertion of the *cat*$^+$ gene (Cm^r) linked to a different mutant allele of *tet*, namely, the *Xho*I-2 allele. The *aphA*$^+$ or *cat*$^+$ gene was linked to the mutant *tet* heteroalleles and inserted into pS14 or a pS11-derived vector in vitro prior to transformation of the integrative vector into *D. radiodurans*, as described in Materials and Methods. The broken line separating the two different genotypes in MD373 and MD389 indicates that both of these insertions were present on two different but otherwise identical chromosomes. This represents a heterozygosity in the otherwise homozygous haploid chromosomes. *D. radiodurans* contains at least 4 identical chromosomes per cell in stationary phase and up to 10 copies in exponential phase (see text).

here, using these chromosomal polymorphic indicators, support the hypothesis that radiation-induced *recA*-dependent interchromosomal recombination is extensive and occurs at levels consistent with the repair of >100 DSBs per chromosome.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and transformation. *D. radiodurans* R1 is wild type (1), and the *D. radiodurans* R1 derivative rec30 is a *recA* strain obtained by chemical mutagenesis using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (8, 24). *D. radiodurans* was grown in TGY broth (0.8% Bacto Tryptone, 0.1% glucose, 0.4% Bacto Yeast Extract [Difco Laboratories]) at 32°C with aeration or on TGY plates solidified with 1.5% agar. *E. coli* DH10B (Life Technologies, Gaithersburg, Md.), was used in the deinococcal plasmid constructions and was grown in Luria-Bertani broth or on Luria-Bertani plates solidified with 1.5% agar. Selective drug concentrations for *D. radiodurans* were 3 μg of chloramphenicol per ml, 8 μg of kanamycin per ml, or 2.5 μg of tetracycline per ml. For *E. coli*, selective drug concentrations were 50 μg of ampicillin per ml or 30 μg of tetracycline per ml. Plasmid transfer in *E. coli* was done by the $CaCl_2$ technique. Chromosomal transformation in *D. radiodurans* was done with $CaCl_2$-treated cells as detailed previously (20).

DNA isolation and manipulation. Isolation of plasmid and chromosomal DNA from *E. coli* and *D. radiodurans*, use of enzymatic reagents, pulsed-field gel electrophoresis (PFGE) and conventional gel electrophoresis, radiolabeling of DNA, blotting, hybridization, washing of blots, and autoradiography were as described previously (4, 16, 35). The blots were probed with the pBR322 *tet* gene as a purified fragment extending from the *Eco*RI site to base 1268 of pBR322, which is the location of the *Xho*I linker at the end of *tet* in pRDK39, a pBR322 derivative (4–6).

Strain constructions. The various *D. radiodurans* strains used in these studies are summarized in Table 1. They are identical except for small chromosomal regions designed to provide several indicators of recombination. Strains were constructed by natural chromosomal transformation of *D. radiodurans*. Some plasmids used in generating the current strains were previously derived, and their constructions are described in earlier reports (4, 5). The relevant regions of the chromosomal DNA of strains used in this work are depicted in Fig. 5, and their various functions are described in the corresponding figure legend. The two chromosomal regions that were targeted for integration were chosen from an *E. coli* plasmid library, plasmids pS11 and pS14 (35), denoted in Fig. 5 by ABCDEF and WXYZ, respectively. These regions in the deinococcal chromosome are referred to as locus 11 and locus 14, respectively. All constructions were verified by Southern blots probed by the appropriate radiolabeled fragments. In addition, since *D. radiodurans* contains multiple identical chromosomes, transformants required prolonged growth under selective pressure to yield homozygotes, which were verified by Southern blotting.

Construction of strains MD360 and MD367. Strains MD360 and MD367 are the same except that MD360 is derived from *recA* strain rec30 (8), while MD367 is a derivative of wild-type R1. The structure of the chromosomal region designed for detection of recombination following DNA damage is identical in these strains and is shown at the top of Fig. 1. MD360 was generated by chromosomal transformation of rec30 with a linearized portion of the donor plasmid pMD345, resulting in a single-copy direct insertion in the *D. radiodurans* chromosomal locus 11. pMD345 was derived from pMD66 (4, 5), a *D. radiodurans-E. coli* shuttle vector that employs the *D. radiodurans* SARK pUE10-derived autonomous origin of replication. We have previously observed that chromosomal insertion of this plasmid origin of replication results in chromosomal rearrangements and deletions (16). Hence, in designing the current chromosomal insertions, it was necessary to remove the pUE10 origin without losing the flanking deinococcal promoter of the *tet* gene characteristic of the pMD66 series vectors (4, 5). This was accomplished by eliminating the 10-kbp *Sac*II fragment from pMD66 and inserting an *Xba*I linker, thereby generating pMD209. pS11 has been useful for tandem duplication in *D. radiodurans* (8, 9, 35). To utilize the vector sequences for direct (not tandem) insertion into the chromosome, the following maneuver was undertaken. From pS11, the *E. coli* portion (the 4.0-kbp vector pMK20) was excised by using *Eco*RI, leaving 12 kbp of deinococcal chromosomal sequence. This 12-kbp deinococcal sequence is uninterrupted and can be thought of as ABCDEF (Fig. 1). This 12-kbp deinococcal fragment was isolated by gel electrophoresis and circularized in vitro at the *Eco*RI site. Approximately midway in the vector sequence (between ABC and DEF) is a unique *Xba*I site, which was cleaved and inserted into the *Xba*I site of pMD209 (noted above). This vector could then be opened at the *Bgl*II site (adjacent to the *Eco*RI site, as noted above) to yield a linear molecule with the configuration ABC-pMD209-DEF (pMD292), which upon transformation should yield a direct single-copy insertion. First, however, the 3.5-kbp *Bam*HI-*Sca*I fragment of pMD292 was exchanged with the 2.8-kbp *Bam*HI-*Sca*I fragment of pMD280 (5), thereby generating pMD297 (equivalent to ABC-pMD280-DEF). Because of the swapping of the *Bam*HI-*Sca*I fragment with pMD280, pMD297 acquired two critical markers in this region: (i) the *Xho*I-5 *tet* mutation and (ii) the physical polymorphism generated by the deletion of the 695-bp *Sty*I-*Pvu*II region downstream from the *tet* gene. Because of host *D. radiodurans* restriction cleavage of DNA derived from *E. coli* (4, 5), it was first necessary to introduce pMD297 to *D. radiodurans* as a plasmid to acquire restriction-methylation. Consequently, a plasmid origin cassette that conferred Tc^r was made from *Eco*RI-cleaved pBR322 ligated to the *Eco*RI large fragment of pI3 (19). The latter fragment contains the pUE10 plasmid origin of replication and a strong promoter that expresses the pBR322 *tet* gene. The origin cassette was completed by removal of a small fragment by cleavage at the *Hpa*I and *Sca*I sites, and the large fragment was circularized by using a *Bgl*II linker, yielding the *D. radiodurans* plasmid pMD344. pMD344 was inserted into pMD297 at the *Bgl*II site that flanked the direct insertion construct (*Bgl*II-ABC-pMD280-DEF-*Bgl*II), yielding pMD345, which transformed rec30 with low efficiency. DNA purified from a Tc^r transformant was able to plasmid transform rec30 with high efficiency. After large-scale preparation of pMD345 from *D. radiodurans*, the origin cassette was removed from pMD345 by *Bgl*II cleavage, leaving the direct insertion construction, ABC-pMD280-DEF, intact and potent (due to alleviation of restriction) for transformation. This linear plasmid construction transformed strain rec30 to Km^r giving *D. radiodurans* MD360. After confirming the predicted

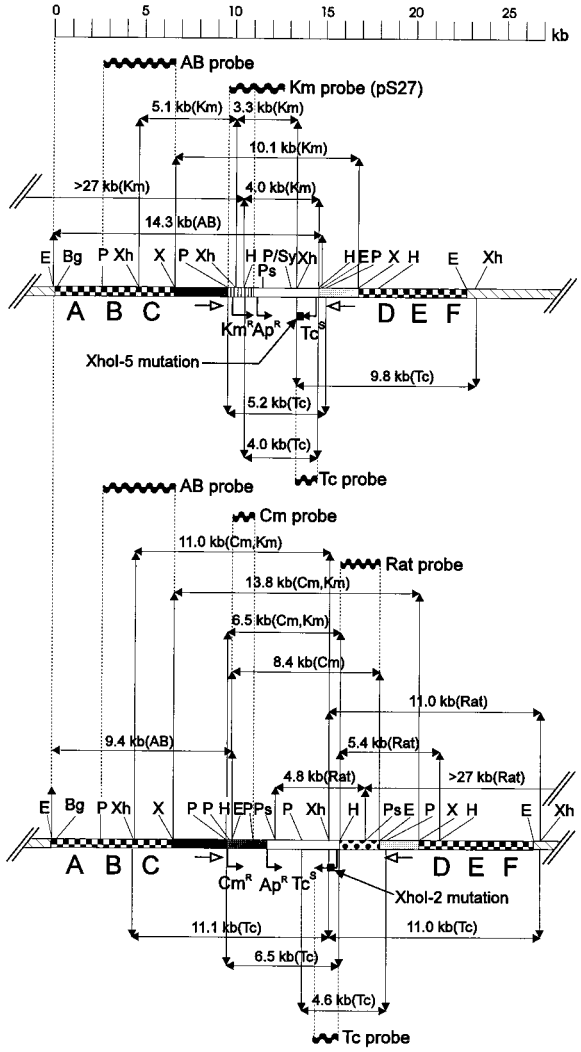

FIG. 1. Regional maps of two different chromosomes both present in MD389 (*recA*$^+$) and MD373 (*recA*). The top part shows a detail of that region of locus 11 containing the Km^r cassette (*Xho*I-5::*aphA*), while the lower part illustrates the Cm^r cassette (*Xho*I-2::*cat*). ABC and DEF represent flanking chromosomal DNA. Five different probes (indicated by wavy bars) were hybridized against Southern blots of numerous different chromosomal digestions indicated by restriction sites on the maps. These designated probes included (i) the kanamycin probe (*E. coli* vector pMK20 derivative pS27 [34]), (ii) the tetracycline probe (1,268-bp *Eco*RI-*Xho*I fragment of pRDK39), (iii) the rat DNA probe (2.2-kbp *Eco*RI fragment of pMD289 [5], (iv) the locus 11-specific AB probe (4.0-kbp *Pvu*II-*Xba*I fragment of pS11), and (v) the chloramphenicol probe (1,278-bp *Pvu*II fragment of pKK232-8). The Southern blot results of the probings are shown in Fig. 4. Restriction site abbreviations: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; Ps, *Pst*I; P/Sy, *Pvu*II-*Sty*I fusion; X, *Xba*I; Xh, *Xho*I. The key to the variously shaded segments of the chromosomal insertions is given in the legend to Fig. 5.

chromosomal structure of MD360, total DNA from this strain was transformed into wild-type R1 to Km^r giving strain MD367.

Construction of strain MD399. The relevant region in this strain, shown both in Fig. 1 (bottom) and Fig. 5A, was derived from the above-described strain MD367 by replacing the region ABC-pMD280-DEF (pMD292) with ABC-pMD300-DEF. The pMD280 chromosomal region was replaced by transformation of this strain with *Hpa*I-linearized pMD300 (5) and selecting for Cm^r for a prolonged period, followed by Southern blot mapping of Km^s Cm^r clones to verify the predicted structure and homozygosity. Linearized pMD300 is able to replace pMD280 by direct insertion because the deinococcal plasmid sequences in these two derivatives are identical (5).

Construction of strains MD420 and MD429. Strains MD420 and MD429 were constructed to investigate whether homologous genomic fragments from different sites could successfully encounter and recombine with one another following irradiation. The *D. radiodurans* chromosomal locus that contains the same chromosomal sequences as pS14 is termed locus 14. The deinococcal sequence at locus 14 can be thought of as the uninterrupted chromosomal sequence WXYZ. To use the vector sequences for tandem duplication insertion of pMD280 (see above) into the *D. radiodurans* chromosome at locus 14, the following manipulations were done. The 6.0-kbp *Eco*RI-*Sac*I (XY) region of pS14 was isolated, blunt ended with T4 DNA polymerase and Klenow fragment, and ligated to *Xba*I linkers. This 6.0-kbp *Xba*I fragment was ligated to *Xba*I-cleaved pMD297 (see above) to produce pMD397. pMD397 was used to transform wild-type R1 followed by Km^r selection, which yielded MD401 (Table 1). Strain MD401 contains the structure illustrated in Fig. 5A. Genomic DNA from MD401 was then transformed into MD399 and subjected to double selection on chloramphenicol and kanamycin, and a clone (MD420) that contains both MD401 and MD399 genomic sequences was isolated. The *recA* equivalent of MD420, strain MD429, was obtained by transformation of genomic DNA from MD401 and MD399 into strain rec30 with double selection for Cm^r and Km^r.

Construction of strains MD389 and MD373. To investigate the ability of homologous genomic fragments located at the same position (locus 11) on two different chromosomes to recombine with one another, MD389 was constructed. Strain MD373 is identical to MD389 except that MD373 is *recA* (Table 1). Strain MD367 (Table 1) was transformed with genomic DNA prepared from MD399 (Table 1), and selection was with chloramphenicol. Double selection on chloramphenicol plus kanamycin was not possible on solid media. Although MD389 does not grow on solid media containing both chloramphenicol and kanamycin, it could be propagated slowly in liquid culture containing both antibiotics. The *recA* strain MD373 was produced by transformation of genomic DNA from MD399 into strain MD360 (see above) to give Cm^r clones.

RESULTS

***D. radiodurans* requires homozygosity for propagation.** Two different Tc^s alleles, *XhoI*-2 and *Xho*I-5, contain mutations consisting of two *Xho*I linkers located 929 bases apart within the *tet* gene of pBR322 (5, 6). These two different Tc^s alleles were inserted into the same randomly selected site, termed locus 11, on two different chromosomes by transformation, yielding heterozygous strains MD389 (*recA*$^+$) and MD373 (*recA*) (Fig. 1 and Materials and Methods). These alleles and their associated polymorphic markers were temporarily retained by the host because one allele was associated with Km^r (*Xho*I-5::*aphA*) and the other was associated with Cm^r (*Xho*I-2::*cat*). Growth did not occur on solid media in the presence of both kanamycin and chloramphenicol, but very slow growth could be obtained in doubly selective liquid media (0.2 generation per h, as opposed to the normal 1 generation per h) (Fig. 2A). If only one drug resistance was selected (either Km^r or Cm^r), the cells grew at wild-type rates on solid and in liquid media. However, this was accompanied by the loss of the chromosome that was not selected. Tc^r recombinants from *recA* or *recA*$^+$ strains could not be obtained from irradiated or nonirradiated cultures in the presence or absence of selective drugs. Consistent with this observation, we found no evidence for recombination postirradiation on Southern blots in *recA*$^+$ or *recA* cells, which would have shown itself by the appearance of new diagnostic restriction fragments (Fig. 3). In the case of *Xho*I digestion, these new bands would be 4.9, 4.2, and/or 0.9 kbp; in the case of *Pvu*II digestion, they would be 2.35 and/or 7.3 kbp (e.g., Fig. 5). Since this is a negative result (i.e., failure to demonstrate allelic recombination), chromosome structures were documented with extreme care. The presence of only two chromosome types, each containing one of the two polymorphisms at the same site, was verified by Southern blot analysis using five different radiolabeled probes and numerous restriction cleavages of chromosomal DNA (Fig. 1 and 4). The results of these studies shows that the chromosomal structures are bona fide and imply that *D. radiodurans* tolerates heterozygosity poorly.

Characteristics and chromosomal locations of polymorphisms. To circumvent the problem associated with maintaining two heterozygous sequences at the same site on two dif-

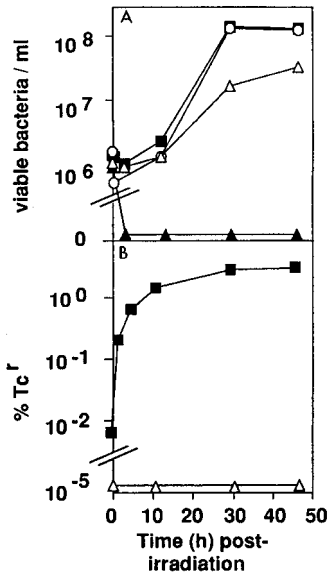

FIG. 2. Viable cells and Tc^r recombinants. (A) Cells were inoculated into TGY medium at 10^6/ml and grown at 32°C to the early plateau phase (10^8 cells per ml) after overnight growth either in the presence of double kanamycin and chloramphenicol drug selection to retain the desired chromosomes in MD420, MD429, MD389, and MD373 (Table 1) or no drug selection in the case of R1 (wild type) and rec30 (*recA*). The cells were then irradiated without change of broth on ice with ^{60}Co at 1.0 Mrad/h to a total of 1.75 Mrad. The cells were diluted 1/50 into fresh TGY medium with or without selective drugs (see below) and incubated at 32°C with vigorous aeration for the times indicated. Viable cell counts were determined by plating appropriate cell dilutions for colony formation on TGY agar without selection. (B) Tc^r recombinants were determined by plating *D. radiodurans* cells taken at the indicated postirradiation intervals on TGY agar that contained 2.5 μg of tetracycline per ml. Symbols: circles, wild-type, no drug selection; squares, MD420, no drug selection; open triangles, MD389, double chloramphenicol and kanamycin selection; closed triangles, MD429 (*recA*), no drug selection. Growth of MD389, no drug selection, was identical to that of MD420 without drug selection (squares). Growth of rec30 (*recA*) and MD373 (*recA*) was the same as that of MD429 (*recA*) (closed triangles).

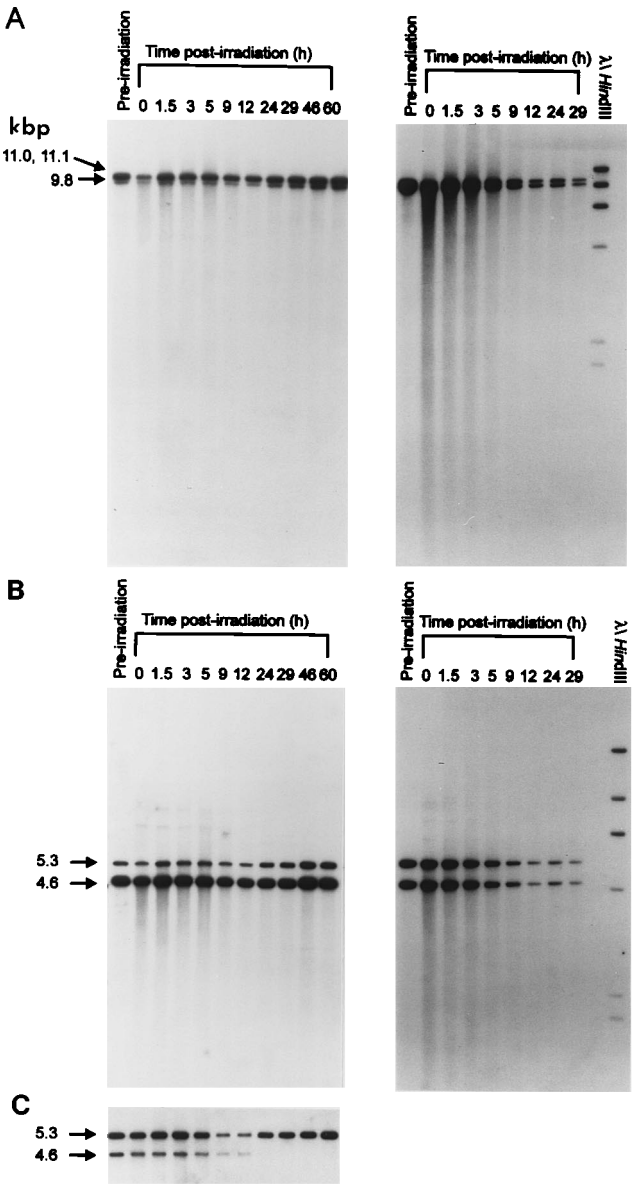

FIG. 3. Two different Tc^s alleles (*Xho*I-5 and *Xho*I-2) each associated with different drug markers, *Xho*I-5::*aphA* (Km^r) and *Xho*I-2::*cat* (Cm^r), were inserted into the same chromosomal site, locus 11, on two different chromosomes. After an exposure to 1.75 Mrad, *recA*$^+$ (MD389) and *recA* (MD373) cells were diluted into fresh TGY medium containing both kanamycin and chloramphenicol and were incubated with aeration for 60 h at 32°C. DNA was prepared from samples (3×10^8 cells) taken at the indicated times by the miniprep technique. (A) Results of Southern blot hybridization using an *Xho*I digestion of genomic DNA from *recA*$^+$ (MD389) cells (left) and *recA* (MD373) cells (right) demonstrating only the 11.0-, 11.1-, and 9.8-kbp predicted parental bands that hybridize to the *tet* (Tc^r) probe. The Southern blots in panel B show the results of *Pvu*II digestion of genomic DNA time point samples prepared from *recA*$^+$ (MD389) cells (left) and *recA* (MD373) cells (right), followed by hybridization with the *tet* probe, demonstrating the 5.3- and 4.6-kbp parental bands. (C) *Pvu*II digest of DNA samples prepared from a *recA*$^+$ (MD389) 1.75-Mrad recovery time course without drug selection. The 4.6-kbp *Pvu*II parental band is indicative of the chromosome containing *Xho*I-2::*cat* (Cm^r) and is lost during the course of repair, while the other chromosome, containing *Xho*I-5::*aphA* (Km^r), is retained.

ferent chromosomes, we used an ectopic recombination approach in which two sites (locus 11 and another randomly chosen distant site, termed locus 14) on the same chromosome contained the two different *tet* alleles. Strains MD420 and MD429 represent two such constructions and are shown in Fig. 5. Strains MD420 and MD429 differ in that MD429 is *recA*. All chromosomes of MD420 and MD429 contain both of two different insertional cassettes: (i) a Cm^r-conferring *cat* gene cassette forming a direct insertion (no flanking repeats) at locus 11 and (ii) a Km^r-conferring *aphA* gene cassette inserted into the chromosome by tandem duplication at locus 14. The retention of both drug markers and their associated polymorphisms during growth in liquid or solid culture could be done without difficulty by the use of double drug selection. The Km^r and Cm^r gene cassettes are the same in those portions derived from the *D. radiodurans* SARK natural plasmids pUE10 and pUE11 and have no appreciable homology with the strain R1 genome. However, these two cassettes differ within their *E. coli*-derived sequences in two ways. (i) The Km^r and Cm^r cassettes are physically polymorphic in the region of the *tet* gene, containing flanking sequences that differ both upstream of *tet* (the 2.2-kbp insert of rat cDNA in the Cm^r cassette) and downstream of *tet* (the 695-bp *Sty*I-*Pvu*II deletion of pBR322 DNA in the Km^r cassette) (Fig. 5A). These polymorphisms allow for detection of crossovers in the recombination region by appropriate restriction cleavage and Southern blot analysis. (ii) The Km^r cassette is Tc^s as a result of the *Xho*I-5 mutation in the *tet* gene, consisting of an 8-bp *Xho*I linker inserted at nucleotide position 1268 of pBR322. The Cm^r cassette is Tc^s as the result of the *Xho*I-2 mutation, consisting of the same linker

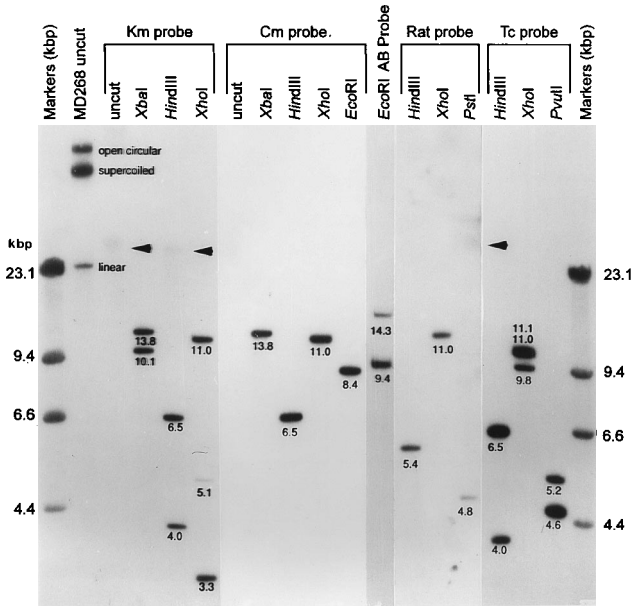

FIG. 4. Hybridizing restriction fragments that were used to construct the two regional maps shown in Fig. 1. DNA was prepared from MD389 and cut with the indicated restriction enzymes. The five probes used are listed at the top and are the same as those described in the legend to Fig. 1. The absence of circular forms of the Cm^r or Km^r cassette is indicated in uncut DNA lanes, compared with a 27.5-kbp plasmid (pMD66; six plasmid copies per cell [4]) present in the genomic sample labeled MD268 uncut. The locations of the open circular, supercoiled, and linear forms of pMD66 are illustrated. The three arrowheads indicate the positions of very large DNAs migrating at the exclusion limit of the gel. Superimposed over each band is its size in kilobase pairs. The markers are λ phage DNA cleaved with *Hin*dIII.

inserted at pBR322 nucleotide position 339 in the *tet* gene. These mutations are 929 bp apart, allowing for detection of recombination in *D. radiodurans* by *Xho*I restriction cleavage and Southern blot analysis (both crossover and gene conversion) and also by selecting for Tc^r colonies postirradiation.

A Southern blot of the product of PFGE of a strain MD420 chromosomal digestion by the 8-bp-specific restriction enzyme *Not*I shows that the locus 11-specific Cm^r gene probe hybridized to the 180-kbp band. The locus 14-specific Km^r gene probe hybridized to the 322-kbp band (Fig. 6). PFGE and Southern analysis of MD429 (*recA*) chromosomal DNA gave the same result. Because the two different *tet* alleles and their associated polymorphic markers are located at two different sites on the same chromosome, these cells could become homozygous and were found to be proficient at ectopic recombination following irradiation, as discussed below.

DNA damage and recovery of *D. radiodurans*. A dose of 1.75 Mrad (17.5 kGy in units of the Système International d'Unités) was used in this study because it is the D_{37} of *D. radiodurans* under our conditions (Materials and Methods). The D_{37} is the dose required to achieve a survival of 37%, statistically representing one lethal hit per CFU, and is therefore closely related to the maximum repair capacity of the cell, while at the same time resulting in minimal detrimental effects on growth curves, since the initial population (100%) is regenerated in only 1.4 cell divisions. The growth lag seen in deinococcal cells after DNA damage (Fig. 2A) is a well-described phenomenon in *D. radiodurans* and is of finite duration, depending on the amount of damage inflicted (for a review, see reference 23). In the present case, the growth lag was 10 to 12 h followed by a 20-h growth phase (Fig. 2A), only 1.4 h of which could be attributed to replacement of cells killed during the original D_{37} radiation exposure.

Recovery in *recA*$^+$ cells depends on the mending of many small DNA fragments. While *recA* cells showed little chromosomal repair by PFGE following exposure to 1.75 Mrad (4), full repair was found to occur in strain MD420 (*recA*$^+$) by 29 h (Fig. 7), and this result is identical to that reported for R1 (4). To measure in vivo mending of *D. radiodurans* DNA by PFGE (Fig. 7), we prepared all DNAs gently in liquid, as described previously (4), rather than purifying DNA in agarose plugs. The reason for liquid DNA purification is to avert the retention of DNA in the agarose plugs during PFGE, which can occur unpredictably in DNA from any organism in any lane (e.g., see reference 29). While such retention is not problematic in chromosomal mapping, it can be a severe liability for strand break rejoining studies. The chromosome size of *D. radiodurans* is about 3×10^6 bp (13), and the average size of DNA fragments in the unirradiated sample is about 1.4×10^5 bp (Fig. 7), indicating that purification in liquid introduced about 21 DSBs per chromosome. The postirradiation time zero sample shows DNA fragments that are about 2.3×10^4 bp or less (Fig. 7), as reported previously (5), indicating at least 130 DSBs per chromosome. Since the maximum-sized fragments after irradiation are smaller than the minimum-sized fragments generated by shearing of DNA from unirradiated cells, it is likely that few or no DSBs (caused by shearing) are generated in the postirradiation time zero samples, because the starting material is already shorter than what is generated by shear forces in the unirradiated samples.

Hence, essentially all of the DNA breaks (130 DSBs per chromosome) at the postirradiation time zero point are introduced by irradiation. This extensive fragmentation would be expected to reduce drastically chromosomal continuity, thereby allowing fragments from one chromosome to interact with fragments from the others. The number of DNA-DNA contacts among fragments occurring after irradiation could be many hundreds, even thousands, of exploratory events just to reconstruct a single chromosome.

Alternatively, a small subpopulation of chromosomal fragments significantly larger than 23 kbp could be present in cells from which a single copy of the entire chromosome could be rebuilt. To increase our level of resolution as to the presence of a small cohort of larger fragments, we performed PFGE autoradiography using as a probe total *D. radiodurans* genomic DNA. Autoradiography produced results identical to those represented by the ethidium bromide-stained gel (Fig. 7B). As judged by the increased fragment size, substantial repair is discerned at very early times following irradiation. By 12 h, there is a significant migrational overlap of these fragments with those in the fully recovered 29-h sample and the unirradiated control (Fig. 7B). Importantly, in the postirradiation time zero sample, there is no detectable subpopulation of large fragments (i.e., of the control DNA sample size range) visualized by autoradiography compared with ethidium bromide staining. Both at time zero and in subsequent samples, there is not a bimodal distribution of DNA fragment sizes. The apparent absence of fragments larger than 23 kbp in the time zero sample excludes the possibility that a small group of large DNA fragments was acting as a special focus of repair. Instead, during the time course of repair, there are incremental increases in the physical length of all fragments, suggesting that a stochastic process produced this generalized increase of DNA sizes (Fig. 7B).

DSB-mediated repair proceeds by homologous chromosomal recombination. Recombination between alleles that are located at the same position on two different chromosomes is

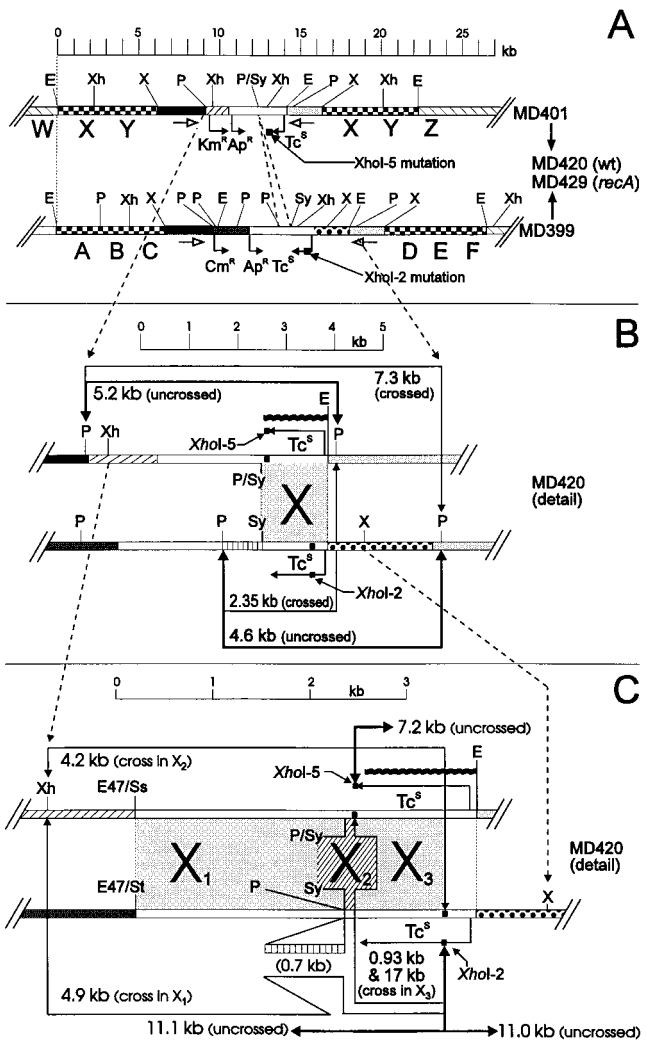

FIG. 5. Regional chromosomal maps, functions, probes, and restriction fragments indicative of recombination. (A) Maps of the chromosomal regions used to detect recombination. Strains MD420 (*recA*$^+$) and MD429 (*recA*) (Table 1) were constructed by cotransformation of high-molecular-weight DNA from strains MD401 and MD399 into either wild-type R1 or rec30 (*recA*). MD420 and MD429 contain both of two insertional cassettes: (i) a Cm^r *cat* gene cassette at locus 11, derived from MD399, and (ii) a Km^r-conferring *aphA* gene cassette at locus 14, derived from MD401. MD401 contains a tandem insertion of the diagnostic region. The tandem insertion is indicated by WXY to the left and XYZ to the right. The tandemly duplicated segment is XY, indicated by checkerboard segments to either side of the diagnostic region. MD401 can confer Km^r because the deinococcal promoter sequence derived from *D. radiodurans* SARK plasmid pUE11 (solid segment) is joined to a portion of the *E. coli* plasmid pMK20 (diagonally hatched segment) that contains the *aphA* gene (Km^r). MD399 expresses Cm^r as a result of the presence of the *cat* gene in a segment derived from pKK232-8 (dark gray segment) and the adjacent pUE11 promoter sequences (solid segment). The spotted segment shown in MD399 represents a physical polymorphism consisting of a rat cDNA fragment from pGABI that encodes a rat biliary glycoprotein. Open-headed arrows indicate the deinococcal promoter sequences. Filled arrows indicate drug resistance determinants as labeled. The 695-bp *Pvu*II-*Sty*I segment that is deleted in the pBR322 portion of MD401 is indicated by dotted lines extending from the *Pvu*II-*Sty*I fusion to the individual *Pvu*II and *Sty*I sites on MD399. This deletion does not affect function but, as in the rat DNA, introduces another physical polymorphism that can be diagnosed by restriction digestion. When the MD401 and MD399 gene cassettes are located within the same cell, recombination between the Tc^s alleles can result in Tc^r in *D. radiodurans* because of the upstream promoter sequences of another SARK plasmid, pUE10 (light gray segment). The *tet* gene is located in the sequence derived from pBR322 (white segment). Restriction site abbreviations: Sy, *Sty*I; others as in the legend to Fig. 1. (B) Detail of these same regions in MD420, showing the expected *Pvu*II restriction fragments made visible by using the *tet* gene fragment as a probe (wavy black line). This probe is the 1,268-bp *Eco*RI-*Xho*I fragment from pRDK39 (4, 5), which consists of the entire pBR322 *tet* gene. A *Pvu*II restriction digestion yields fragments diagnostic of the presence or absence of recombination occurring within the gray region labeled X. (C) Detail of these same regions in MD420 showing the expected *Xho*I restriction fragments, made visible by using the *tet* gene fragment as a probe. The 695-bp *Pvu*II-*Sty*I segment is shown as an expanded triangle from the MD399 portion of this MD420 detail. The recombinant (crossed) and parental (uncrossed) diagnostic *Xho*I restriction fragment sizes are shown together with their predicted origins in the three regions labeled X_1, X_2, and X_3.

referred to as allelic recombination, while recombination between alleles located at different positions is referred to as ectopic recombination (17). Since allelic recombination could not be observed (Fig. 3), we directed our studies to ectopic recombination. To implement this approach, we constructed deinococcal strains MD420 and MD429 (Fig. 5), which had genetic and physical polymorphisms present in the same cell but located at two different chromosomal loci. In samples isolated from time points during the course of recovery in the absence of any drug selection after irradiation, a *Xho*I digestion of genomic DNA followed by Southern blotting and hybridization with the *tet* probe shows the appearance of the predicted recombination products following irradiation (Fig. 5C and 8A). This view is predominantly qualitative rather than quantitative, since the occurrence of chromosomal crossover versus gene conversion cannot be distinguished by using the *Xho*I genomic digestion (Fig. 5C). All predicted recombinant bands are apparent in the *Xho*I digestion, each indicative of a crossover and/or gene conversion event in one of three regions marked X_1, X_2, and X_3 in Fig. 5C. There are a 4.9-kbp autoradiographic band, indicative of an event in X_1; a 4.2-kbp band, indicative of an event in X_2; and a 0.9-kbp band, indicative of an event in X_3 (Fig. 5C and 8A). It is noteworthy that this system resolves the 4.2-kbp band, demonstrating recombination in a region as small as 101 bp (between the *Xho*I-5 mutation and the *Sty*I site) (Fig. 5C and 8A). This diagnostic 4.2-kbp band can be seen at about 9 h postirradiation (Fig. 8A). This recombination is *recA* dependent since the *recA* strain MD429 does not show evidence of these new *Xho*I bands following irradiation (Fig. 8B).

To test the possibility that these recombination results were an artifact caused by transformation of DNA released from dead cells into living cells, the following control experiment was done. Two separate *recA*$^+$ cell populations that contained only one of the two inserts in each chromosome (MD399 and MD401; Table 1) were mixed just before a 1.75-Mrad exposure. After irradiation, the cells were allowed to recover together without selective drugs, as usual. The kinetics of cellular recovery and growth were normal for both irradiated and control cultures, and no recombination leading to any Tc^r clones occurred.

Two methods for evaluating recombination frequencies in *D. radiodurans*. While the *Xho*I digestion and *tet* probing provided qualitative evidence for recombination in the *D. radiodurans* chromosome, quantitative results were obtained by using *Pvu*II (rather than *Xho*I) for restriction cleavage and probing with the *tet* gene. The use of large heterologous flanking sequences to either side of the diagnostic *Pvu*II sites (Fig. 5B) allows us to visualize crossovers exclusively since gene conversion cannot be detected. Quantitative data on crossovers were obtained by measuring the signal in recombinant bands (Fig. 8C) which occur as the result of crossovers within the 1.37-kbp region marked X in Fig. 5B. A densitometric scan of the amount of signal in each peak of representative lanes of Fig. 8C is shown in Fig. 9. The parental bands indicative of

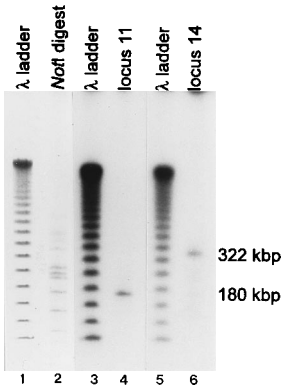

FIG. 6. PFGE of a strain MD420 chromosomal *Not*I digestion. Preparation of chromosomal DNA from strain MD420 in agarose plugs was followed by a 16-h digestion at 37°C with the restriction enzyme *Not*I. *Not*I chromosomal fragments were separated electrophoretically over a period of 16 h at 150 V with ramped pulse times from 5 to 120 s (lane 2). The PFGE product was subjected to Southern blotting followed by probing with a radiolabeled locus 11-specific *cat* probe (1,278-bp *Pvu*II fragment of pKK232-8) (lane 4) and a locus 14-specific *aphA* probe from *E. coli* vector pS27 (derivative of pMK20) (lane 6); both of the probes also contained λ DNA. Lanes 1, 3, and 5 show size markers consisting of λ DNA ladders. The sizes of the hybridizing *Not*I fragments are indicated.

chromosomes that have not undergone recombination within this window are 5.2 and 4.6 kbp, and both are equally abundant at all time points following irradiation. Both of the predicted recombinant bands of 7.3 and 2.35 kbp are first discernible at about 3 h following irradiation of MD420 (*recA*$^+$) and do not appear to increase in intensity after 9 h (Fig. 9). No recombination was detected by scanning the *recA* strain MD429 (not shown).

A second method of quantitatively evaluating recombination frequencies in the chromosomes is to use the genetic polymorphism in the *tet* gene. The intervening distance between the *Xho*I-5 and the *Xho*I-2 mutations is 929 bp, allowing detection of recombination in this region by selection for Tc^r. The frequency of Tc^r recombinants was 0.02 following irradiation (Fig. 2B), while Tc^r isolates were only very rarely found without irradiation (Fig. 2B). The interpretation of the preceding data is addressed in Discussion.

Structures of Tc^r recombination products. The recombinational events that led to Tc^r were determined by *Pvu*II digestions of minipreps of postirradiation Tc^r *D. radiodurans* colonies followed by Southern analysis (Fig. 10). The most abundant mechanism of recombination (15 of 36) was a crossover in *tet* (resulting in Tc^r) together with a second crossover at a site upstream of *aphA* and *cat*. The simplest explanation for the second most abundant mechanism (13 of 36) appears to be a gene conversion event between the *Xho*I-2 and *Xho*I-5 sites in which branch migration allowed for gene conversion of the *Xho*I-5 allele to the wild type. An additional mechanism would be the occurrence of two closely spaced crossovers. The third means of achieving Tc^r (8 of 36) is essentially like that for the first group described above: a crossover between the *Xho*I-5 and *Xho*I-2 mutations accompanied by a second crossover downstream in pBR322-derived sequences.

DISCUSSION

Members of the family *Deinococcaceae* are extremely resistant to radiation-induced cell killing and DSBs (25), in stark contrast to almost all other known organisms, which cannot survive and mend more than just a few such DSBs. Previous studies, aimed at determining how such nonspecific DSBs are processed, were restricted to organisms relatively sensitive to irradiation, such as *E. coli* (32) and *S. cerevisiae* (30), and were thus limited to the analysis of individual recombinants arising from a few DSB events. This constraint no longer exists now that *D. radiodurans* populations recovering from 500 DSBs per cell can be studied in mass culture.

A genetic system for studying *recA*-dependent interplasmidic homologous recombination in *D. radiodurans* has been described recently (5). These studies took advantage of the fact that extreme plasmid damage can be inflicted in vivo at exposures commensurate with a high degree of cellular survival. Detailed analysis of the timing and nature of the recombinational events in this study was made possible by using allelic and physical polymorphisms present in two slightly different plasmids. These same polymorphisms, shown in Fig. 5, were used in this study, this time being used as reporters of interchromosomal homologous recombination.

The possibility that interchromosomal recombination is the mechanism by which *D. radiodurans* chromosomes are rebuilt from hundreds of fragments renders important the consideration of its chromosome multiplicity, which ranges from 4 to 10 (13). The redundancy of chromosomal fragments, created by irradiation-induced DSBs, would thus provide the substrate for interchromosomal recombination.

We report the findings of two distinct approaches developed to investigate irradiation induced interchromosomal recombination in *D. radiodurans*: (i) an allelic study designed to assess recombination between two different mutant alleles of the *tet* gene at the same site on two different (but otherwise identical) chromosomes and (ii) an ectopic study using the same *tet*

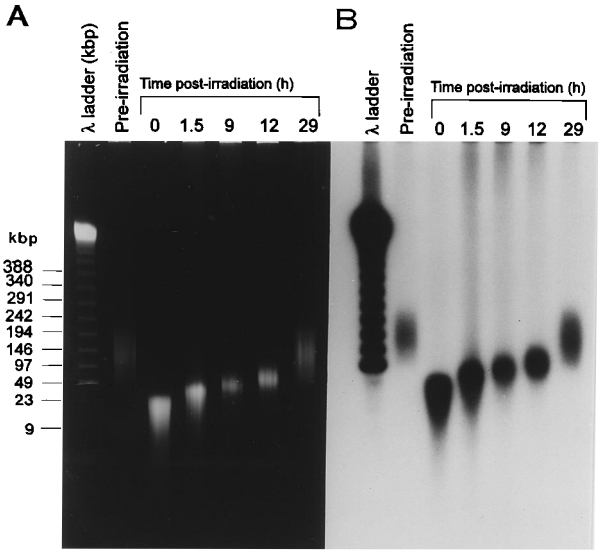

FIG. 7. (A) PFGE of chromosomal DNA prepared from an MD420 (Table 1) culture at various times postirradiation. MD420 (*recA*$^+$) was grown overnight to early plateau phase and γ irradiated on ice to 1.75 Mrad. Total genomic DNA was purified by the miniprep technique immediately thereafter, or the cells were diluted 1/50 in fresh TGY medium and incubated at 32°C for the times indicated, and total DNA was prepared by the same technique. Each lane contains DNA from 3×10^6 cells as determined with a hemocytometer as previously described (4). The DNA is visualized by ethidium bromide staining. The markers are composed of a λ DNA ladder, including a trace amount λ DNA cut with *Hin*dIII, to reveal the 23- and 9-kbp positions in panel B. (B) Autoradiogram of a PFGE Southern blot. After PFGE, the gel depicted in panel A was acid depurinated and alkali blotted to a nylon membrane. Total *D. radiodurans* DNA and some λ DNA was radiolabeled by the random priming technique (10^6 cpm/μg of DNA) and hybridized to the blot.

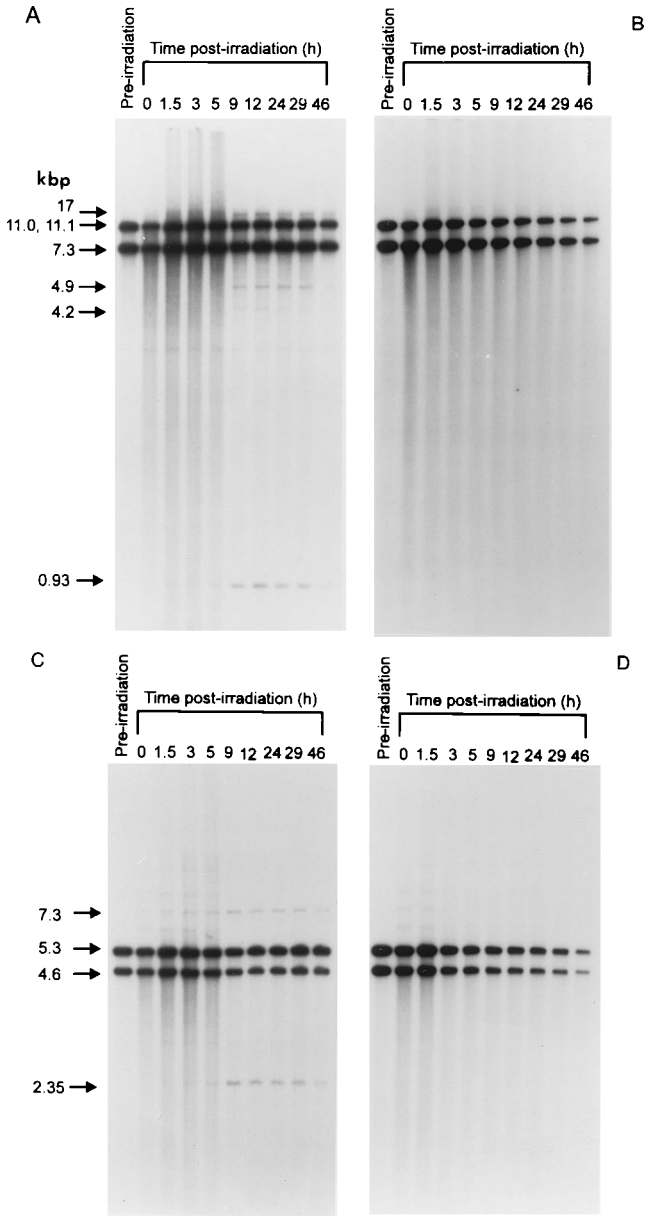

FIG. 8. Production of recombinant bands following irradiation of *D. radiodurans* MD420 (*recA*$^+$; A and C) and MD429 (*recA*; B and D). Cells were grown, irradiated, diluted, and allowed to recover without drug selection as described in the legend to Fig. 2. DNA was prepared from samples taken at the indicated times by the miniprep technique and cleaved with *Xho*I (A and B) or *Pvu*II (C and D). Each lane contains the DNA from 3×10^6 cells as determined by hemocytometer counting. Electrophoresis was in a 0.9% (for *Xho*I) or 0.7% (for *Pvu*II) agarose gel for 18 h at 55 V before blotting and probing of the blot with the full-length *tet* gene.

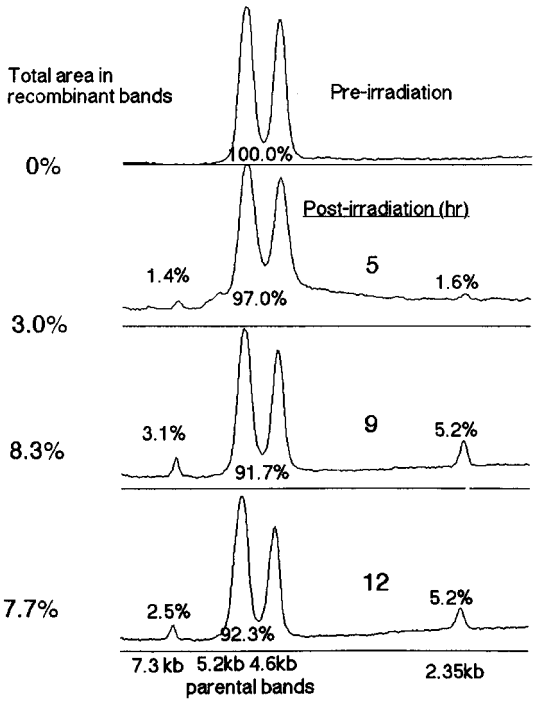

FIG. 9. Densitometric scanning of the gel lanes shown in Fig. 8C by the use of the gel scan module NIH 1.5.4. The scanned lanes include the postirradiation times indicated "Post-irradiation (hr)." The recombinant *Pvu*II bands at the top and bottom of each gel lane are represented by the far left and right peaks and are labeled with the amount of signal within each of these bands. The parental bands are centrally located and much larger; they are labeled with the total amount of signal in both of the bands combined. The sum of the signal in the two recombinant bands is indicated on the left.

heteroalleles located at two distant sites on the same chromosome.

Allelic recombination. We constructed chromosomes that have three important properties: (i) they contain different mutant alleles of the *tet* gene (Fig. 1), (ii) they contain physical polymorphisms that permit detection of crossing over by the appearance of novel restriction fragments resulting from exchanges between homologues in the regions of heterology, and (iii) these *tet* alleles and physical polymorphisms are linked to two different drug-resistant determinants, *aphA* (Km^r) and *cat* (Cm^r) (Fig. 5).

Cells containing either *cat* or *aphA* (and their associated polymorphisms) at the same site on two separate chromosomes failed to grow on solid substrate in the presence of double (kanamycin and chloramphenicol) selection and in liquid could grow only slowly under conditions of double selection (Fig. 2A). Without double selection, the chromosome type that was not selected was lost, and the cells then propagated at a wild-type rate. DNAs from these strains (MD373 and MD389; Table 1) recovering from irradiation in the presence of both chloramphenicol and kanamycin do not show any recombinant bands in Southern blots as detected by the *tet* probe (Fig. 3). The sluggish growth in liquid containing both chloramphenicol and kanamycin (Fig. 2A) is similar to previous observations on *D. radiodurans* in experiments designed to examine interplasmidic recombination (5). *D. radiodurans* is apparently extremely intolerant of heterologies located at the same chromosomal or plasmidic locations. While many explanations may be advanced, we suggest that during normal growth of this organism, there may be early partitioning of identical chromosomes into daughter cells. In *D. radiodurans*, cell replication is usually accomplished by alternate and synchronized divisions in two planes, and the daughter cell compartments can be in communication for some time after multiple rounds of nuclear segregation (26). In the case cited above, in which very modest growth is observed in liquid (with chloramphenicol and kanamycin dual selection), the two necessary genes (*aphA* and *cat*) each may be sequestered in different daughter cells capable of sharing drug resistance proteins. If early segregation of iden-

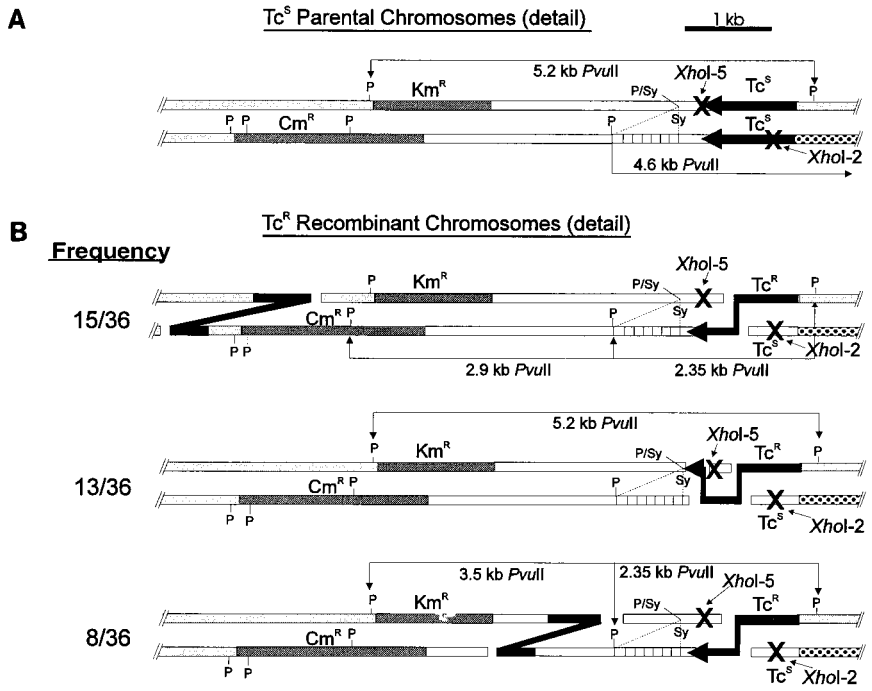

FIG. 10. Restriction enzyme analysis of Tc^r isolates obtained by plating MD420 on tetracycline selective agar medium. These isolates were taken from the 29-h postirradiation time point. (A) Detail of the two distant chromosomal insertions present in MD420, including those regions tested for recombination. DNA was prepared from individual Tc^r recombinant colonies by the miniprep technique. DNA was cleaved with *Pvu*II and subjected to Southern blot analysis. (B) Structures of the recombinant segments which were deduced by using the simplest mechanism by which the recombinational events could occur and be consistent with the diagnostic restriction fragments. Shading and restriction site abbreviations are as in the legend to Fig. 1; Sy, *Sty*I. The heavy black lines with arrowheads indicate the *tet* gene and the direction of transcription. Adjacent thick black segments correspond to a second crossover downstream of *tet*. In the case of the parental segments, which contain either the *Xho*I-2 or *Xho*I-5 mutation in the *tet* gene, this gene is labeled Tc^s, while in the recombinant structures, the *tet* genes are labeled Tc^r.

tical chromosomes into daughter cells does occur, then there would be no opportunity for irradiation-induced chromosomal fragments, containing different *tet* heteroalleles, to interact in the initial setting.

Ectopic recombination. Since interchromosomal recombination could not be evaluated by our allelic system of study, we turned to an ectopic recombination approach whereby the two *tet* heteroalleles and associated polymorphic markers were located on the same chromosome at two distant sites. Under such conditions, both the Cm^r and Km^r markers were retained within the same cell and both the *recA*$^+$ and *recA* strains (MD420 and MD429; Table 1) could be cultured in liquid or on solid media containing both antibiotics without difficulty. Thus, unlike the allelic approach, this system provided the setting for studying interchromosomal recombination by assessing recombination between the *tet* heteroalleles contained on different chromosomal fragments following irradiation.

The extensive fragmentation caused by 130 irradiation-induced DSBs per chromosome (Fig. 7) would be expected to eliminate chromosomal continuity, and the approximately 500 fragments generated per cell (4 chromosomal copies $\times$ 130) would be expected to be able to interact with each other. Because the two *tet* alleles are located at distant positions on the chromosome, none of the irradiation-generated chromosomal fragments ($\sim$23 kbp) should contain both of the *tet* heteroalleles.

We found that after an exposure to 1.75 Mrad, MD420 (*recA*$^+$; Table 1) underwent substantial homologous recombination within the windows of observation. These windows consisted largely of the *tet* gene and some flanking sequences in the pBR322 portion of these chromosomal insertions (Fig. 5B and C and 8A and C). There was no evidence for illegitimate recombination in MD420, and there was no evidence for any recombination in the *recA* strain MD429 (Fig. 8).

Frequency and reciprocity of recombination between chromosomes. By two different means, we have made estimates of the number of crossovers and/or gene conversions necessary to restore the integrity of a *D. radiodurans* chromosome following an exposure to 1.75 Mrad. These two estimates are as follows.

(i) Restriction length fragment polymorphisms (RFLPs). The frequency of crossovers in the 1.37-kbp region in *recA*$^+$ cells can be extrapolated to the whole chromosome. If it is assumed that all crossovers are nonreciprocal, then the total recombinant signal is 0.08 (from Fig. 9; the approximate sum of signal at 9 and 12 h in diagnostic recombinant bands); 0.08 $\times$ (3×10^6 bp per chromosome/1.37 kbp per window) equals 175 nonreciprocal crossovers per chromosome (700 crossovers per nucleoid). If all of the crossovers are reciprocal, then each crossover contributes equally to both the 2.35- and 7.3-kbp recombinant bands (the bands, therefore, should not be summed), and the number of reciprocal crossovers would be no more than half of the crossovers occurring by nonreciprocal mechanisms.

Some recombination is likely to be nonreciprocal since at the 9- and 12-h points (Fig. 9), the 7.3- and 2.35-kbp products are not present in an equal molar ratio. The 7.3-kbp product (which amounts to about 2.8% of signal at the 9- and 12-h time points) is underrepresented by a factor of about 2 compared with the 2.35-kbp product (which amounts to 5.2% of signal). Thus, if all of the 7.3-kbp product is taken to represent reciprocal recombination, the remaining 2.35-kbp product must arise by nonreciprocal recombination. If reciprocal recombi-

nation is maximized, then there would be (i) about 60 reciprocal crossovers per chromosome generating both the 7.3- and 2.35-kbp fragments and (ii) 53 nonreciprocal crossovers generating only the 2.35-kb fragment. Thus, the total number of crossovers per chromosome would be 113.

(ii) Genetic recombination of *tet* heteroalleles. The *tet* gene allelic polymorphisms, *Xho*I-2 and *Xho*I-5, which are 929 bp apart, are used to assay for the frequency of irradiation-induced Tc^r recombinants. Reconciliation of the *Pvu*II RFLP data and the Tc^r data requires a correction factor. The *Pvu*II RFLP densitometric determinations were made on total chromosomal DNA assayed from all cells at various time points during the preliminary lag phase (Fig. 2A). Thus, these DNA samples represent both those cells destined to survive and those destined to die, while the genetic recombination data gathered from determining the Tc^r colony counts solely measure Tc^r cells fated to survive. By this approach, the assay does not reflect Tc^r cells fated to die as a result of defects outside the *Pvu*II recombination window (Fig. 5B). To reconcile these two manifestations of recombination, it is necessary to multiply the Tc^r cell colony counts by 100%/37%, thereby taking into account all recombinational events in the *Xho*I window X_3 (Fig. 5C), including those cells fated to die (63%). The relevant calculation is as follows: 0.02 (Fig. 2B) $\times$ (100%/37%) $\times$ (3×10^6 bp/chromosome)/(929 bp/window [X_3]) equals 175 crossovers and/or gene conversion events per chromosome.

Thus, a range of estimates for the number of recombinational events occurring per chromosome following a dose of 1.75 Mrad is at the lowest 113 (maximizing reciprocal recombination) and at the highest 175 (calculated by assuming only nonreciprocal crossovers or calculated from Tc^r data which do not exclude gene conversion).

The timing of recombination following irradiation. Chromosomal recombination starts within the first several hours following irradiation of wild-type cells. Completed recombination events (resulting from exchanges between polymorphic markers) manifest themselves by the appearance of novel restriction fragments (Fig. 8A and C). The fact that such recombinant bands can be seen faintly as early as 3 h postirradiation (Fig. 8C) indicates that the DSB-mediated repair pathway is initiated before 3 h. Given that exponential growth starts sometime between 10 and 12 h (Fig. 2A), this separates kinetically the onset of recombination and the onset of growth by about 7 h, suggesting that substantial recombination precedes DNA replication. Furthermore, small increases in the intensity of recombinant bands relative to the parental bands (Fig. 8C and 9) can be seen between 3 and 9 h (but not at later times), suggesting that in *D. radiodurans*, like in *S. cerevisiae* (7), chromosomal recombination is slow, taking numerous hours from the time of a DSB to the completion of a crossover. *S. cerevisiae* sustains and can mend >100 region-specific DSBs per genome in meiosis (37), during which there is a structural alignment of the chromosomes held together by the synaptonemal complex. We suggest that a possible chromosomal alignment in *D. radiodurans* may be important in understanding its radioresistance, as described previously (22).

ACKNOWLEDGMENT

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