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Interplasmidic Recombination following Irradiation of the Radioresistant Bacterium *Deinococcus radiodurans*

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***Deinococcus radiodurans* R1 and other members of the eubacterial family *Deinococcaceae* are extremely resistant to ionizing radiation and many other agents that damage DNA. For example, after irradiation, *D. radiodurans* can repair >100 DNA double-strand breaks per chromosome without lethality or mutagenesis, while most other organisms can survive no more than 2 or 3 double-strand breaks. The unusual resistance of *D. radiodurans* is *recA* dependent, but the repair pathway(s) is not understood. Recently, we described how a plasmid present in *D. radiodurans* (plasmid copy number, ~6 per cell; chromosome copy number, ~4 per cell) during high-dose irradiation undergoes extreme damage like the chromosome and is retained by the cell without selection and fully repaired with the same efficiency as the chromosome. In the current work, we have investigated the repair of two similar plasmids within the same cell. These two plasmids were designed to provide both restriction fragment polymorphisms and a drug selection indicator of recombination. This study presents a novel system of analysis of in vivo damage and recombinational repair, exploiting the unique ability of *D. radiodurans* to survive extraordinarily high levels of DNA damage. We report that homologous recombination among plasmids following irradiation is extensive. For example, 2% of Tc^s plasmids become Tc^r as a result of productive recombination within a 929-bp region of the plasmids after repair. Our results suggest that each plasmid may participate in as many as 6.7 recombinational events during repair, a value that extrapolates to >700 events per chromosome undergoing repair simultaneously. These results indicate that the study of plasmid recombination within *D. radiodurans* may serve as an accurate model system for simultaneously occurring repair in the chromosome.**

Deinococcus (formerly *Micrococcus*) *radiodurans* R1 was discovered by Anderson and coworkers in Oregon in 1956 in X-ray-sterilized canned meat that was found to have undergone spoilage (1). Culture yielded a red-pigmented, gram-positive, nonsporulating, and nonpathogenic coccus that was extremely resistant to ionizing radiation and most other physical and chemical agents that cause DNA damage. Subsequently, four other similar radioresistant species have been isolated: *Deinococcus radiopugnans* from haddock tissue in Massachusetts (6), *Deinococcus radiophilus* from Bombay duck in India (16) as well as from weathered granite in Antarctica (4), and *Deinococcus proteolyticus* and *Deinobacter grandis* from irradiated feces of a llama and elephant, respectively (13, 26), both from a zoo in Ueno, Japan. The latter species is rod shaped but in all other respects is characteristically deinobacterial in phenotype and genotype. These five species have been grouped as members of the family *Deinococcaceae*, one of the 10 known families of eubacteria (25). Evolutionarily, the deinobacteria are extremely distant from any well-characterized bacterium, being just as far from *Escherichia coli* as from *Bacillus subtilis* (2, 25, 32, 33).

The most studied of the deinobacterial species is *D. radiodurans* R1, not only because it was the first to be discovered, but more importantly because it is the only deinobacterial species that is competent at transformation of chromosomal DNA (23, 31) and plasmid transfer (18, 28), rendering this species amenable to the techniques of bacterial genetics. The property of DNA damage resistance appears to be unrelated to competence at transformation since the other deinobacterial species, which are just as radioresistant as *D. radiodurans*, are to date nontransformable.

The resistance of *D. radiodurans* has been shown to be due to exceedingly efficient DNA repair (for reviews, see references 20 and 21). For example, following a 1-megarad exposure, it has been shown that *D. radiodurans* sustains >100 double-strand breaks per chromosome, which it repairs without lethality, mutagenesis, or rearrangements, whereas most other organisms cannot survive and mend 2 or 3 DNA damage-induced double-strand breaks per chromosome (5, 11, 12, 14, 21).

Recently, we reported that the repair capacity of *D. radiodurans* extends to include plasmids resident at the time of irradiation (5). Such studies have not been possible with other organisms. This is because damaging a plasmid in vivo requires supralethal damage of the chromosome since the plasmid is a very small target. This constraint, however, does not apply to *D. radiodurans* since extreme plasmid damage can be inflicted at in vivo exposures commensurate with a high degree of cellular survival. We previously constructed a 27.5-kbp *E. coli-D. radiodurans* shuttle vector (pMD66) (see Fig. 1A) that carries multiple drug resistance determinants that can be expressed in both organisms (5). The survival or regeneration of this plasmid was determined in vivo following exposure of *D. radiodurans* R1 that contained pMD66 to 1.75 megarads of ionizing radiation. The regeneration of both chromosomal and plasmid DNA in R1/pMD66 was compared with regeneration in a highly radiosensitive strain, rec30 (22), that has been shown recently to be defective in the deinococcal *recA* gene (9). Following exposure of R1/pMD66 and rec30/pMD66 to 1.75 megarads of radiation, both *D. radiodurans* plasmid and

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chromosomal DNA undergo extensive fragmentation as determined by Southern blot analysis and pulsed-field gel electrophoresis (5). Postirradiation, the cells were allowed to recover for various durations up to 46 h in aerated liquid culture without drug selection. In R1/pMD66, there was full regeneration of both plasmid and chromosomal DNA within 24 h. However, there was almost no regeneration of chromosomal DNA in rec30/pMD66 and only very limited regeneration of supercoiled plasmid (5). The supercoiled plasmid that was regenerated in rec30/pMD66 appeared to be defective since it failed to transform *E. coli*. This is in contrast to the plasmid regenerated in R1/pMD66, which transformed *E. coli* with high efficiency. At sublethal and lethal exposures of *D. radiodurans*, it was found that all survivors retained pMD66 without any drug selection for the plasmid, both in R1 and the radiosensitive *recA* strain rec30, at all exposures tested up to 3 megarads.

The previous studies show that *D. radiodurans* retains the plasmid during repair, that it repairs plasmid DNA with efficiency similar to that of its chromosomal DNA repair, and that deinococcal RecA is required for this efficient repair process in both the chromosome and the plasmid. Consequently, the study of DNA repair in plasmids that are present within the cell during irradiation might be a good reporter system for similar events occurring simultaneously in the chromosome. While the previous investigations show that the deinococcal RecA is important in plasmid repair, it has not been demonstrated that the postirradiation recovery of the plasmid is associated with crossover or gene conversion events. Therefore, in the current work, we have advanced these studies by developing a system in which two different plasmids, both similar to pMD66, are present together within each bacterium during irradiation. The two plasmids employed are alike except for small regions designed to provide several indicators of plasmidic recombination. Studies reported here using these two plasmids indicate that there is extensive interplasmidic recombination occurring during DNA repair.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *D. radiodurans* R1 is wild type (1), and the *D. radiodurans* R1 derivative rec30 was obtained via chemical mutagenesis using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (22). rec30 is defective in the deinococcal *recA* gene (9). All deinococcal derived DNA fragments used in the shuttle plasmids in *D. radiodurans* R1 are from *D. radiodurans* SARK plasmids pUE10 and pUE11, both of which have little homology with R1 chromosomal sequences and do not recombine with the R1 chromosome (5, 28). *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 grown in Luria-Bertani broth or on Luria-Bertani broth plates solidified with 1.5% agar. Selective drug concentrations for *D. radiodurans* were 3 μg of chloramphenicol (Cm) per ml, 8 μg of kanamycin (Km) per ml, or 2.5 μg of tetracycline (Tc) per ml. Selective drug concentrations for *E. coli* were 50 μg of ampicillin (Ap) per ml or 30 μg of tetracycline per ml.

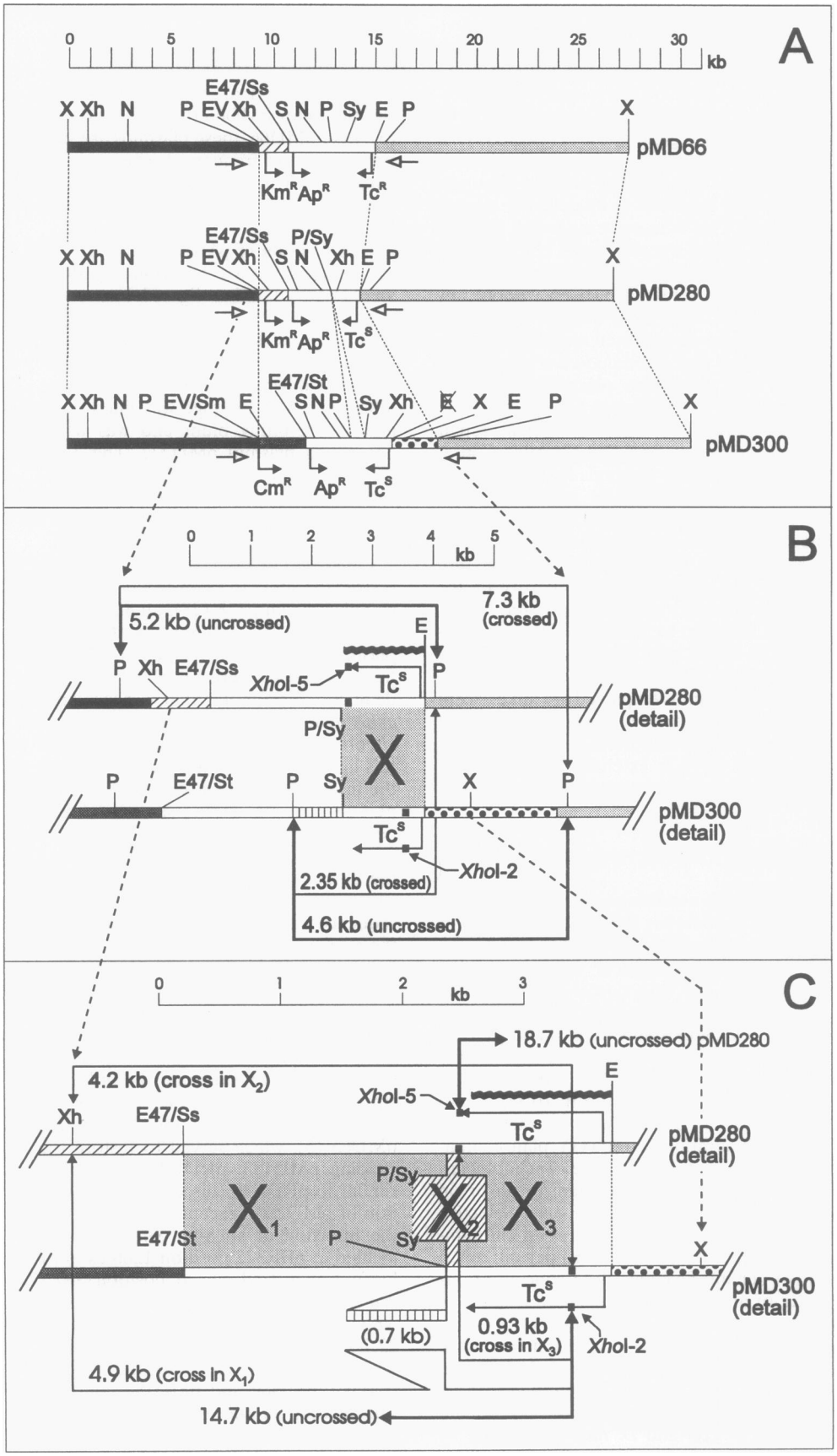
Transformation. Plasmid transfer of *E. coli* employed the $CaCl_2$ technique. Plasmid transfer of *D. radiodurans* also employed $CaCl_2$-treated cells, as detailed previously (19).

DNA isolation and manipulation. Isolation of plasmid and chromosomal DNA from *E. coli* and *D. radiodurans*, use of enzymatic reagents, gel electrophoresis, blotting, hybridization, washing of blots, and autoradiography were as described previously (5, 15, 29).

Plasmid transformation restriction in *D. radiodurans*. pMD280 and pMD300 (Fig. 1A), which were constructed for this investigation, are very similar to the *E. coli-D. radiodurans* shuttle vector pMD66 that was used in prior studies (Fig. 1A) (5). Like pMD66, these plasmids are subject to restriction (low transformation frequency) when used to transform *D. radiodurans* (5). Thus, these same plasmids when purified from *D. radiodurans* have a high transforming frequency for *D. radiodurans* recipients.

Construction of pMD280. pMD280 was constructed as follows. pRDK39 is a Tc^s version of pBR322 derived by inserting an 8-bp *Xho*I linker ligated into a *Taq*I site at nucleotide position 1268 of pBR322 (7). This mutation is referred to in the present work as the *Xho*I-5 mutation. pRDK39 was cleaved at its unique *Pvu*II and *Sty*I sites, thereby releasing a 695-bp fragment downstream from the *tet* gene. The 4-bp overhang left by the *Sty*I cleavage was filled in with Klenow fragment, and the resulting blunt end was ligated to the *Pvu*II site, generating plasmid pMD272. The 3.2-kbp *Eco*RI-*Sca*I fragment from this plasmid (pMD272) was substituted for the similar fragment in pMD66 (Fig. 1A), thereby transferring to pMD66 both the 695-bp deletion and the *Xho*I-5 mutation, resulting in plasmid pMD280 (Fig. 1A).

Construction of pMD300. Because of the lack of useful restriction sites, the construction of pMD300 (Fig. 1A) was complex. From pS18 (28), an 8.5-kbp *Fsp*I-*Eco*47III fragment was excised. This fragment contains a portion of the *E. coli* plasmid pMK20 that includes the *aphA* gene (Km^r) and the flanking SARK-derived pUE11 sequence that includes a deinococcal promoter that is necessary for expression of the *aphA* gene in *D. radiodurans*. The fragment was ligated to the *Hin*cII site in the multiple cloning site of pUC18. All ends in this ligation were blunt. A plasmid where the *Eco*47III end of the insert was most closely adjacent to the *Hin*dIII site in the pUC18 multiple cloning site was chosen, and this clone was designated pMD154. The 5.1-kbp *Sma*I-linearized pKK232-8, which contains a promoterless *cat* gene (3), was inserted into the unique *Eco*RV site and blunt-end ligated, and a clone in which the *cat* gene is promoted by the pUE11 deinococcal sequence was chosen, generating pMD177, thereby interposing the *cat* gene between the pUE11 promoting sequences and the *aphA* gene. From pMD177, the 9.3-kbp *Xba*I-*Eco*47III fragment was then purified. This fragment contains the pUE11 sequence that promotes the *cat* gene, eliminates the pMK20-derived *aphA* gene (Km^s), and also eliminates all *E. coli* plasmid sequences and pUE10-derived sequences. This step was necessary to eliminate the *aphA* (Km^r) sequence downstream of *cat* (Cm^r). The 9.3-kbp fragment from pMD177 thus derived was joined to the 4.3-kbp *Xba*I-*Stu*I fragment of pMD65 (5), which served to restore the pBR322 sequences, yielding pMD183. pRDK37 is a Tc^s derivative of pBR322 similar to pRDK39 (discussed above with respect to construction of pMD280), except that the *tet* mutation, resulting from the insertion of an 8-bp *Xho*I linker at a *Taq*I site, is not located at pBR322 position 1268 as in pRDK37 but instead is located at a *Taq*I site at position 339 (7). This mutation is referred to in the current work as the *Xho*I-2 mutation. The 3.8-kbp *Eco*RI-*Sca*I fragment of pRDK37 was ligated to the 22.7-kbp *Eco*RI-*Sca*I fragment of pMD66, thereby transferring the *Xho*I-2 mutation into the pMD66 *tet* gene, yielding pMD89. pMD89 and pMD183 contain two *Nde*I sites each. The ligation of the 10.5-kbp *Nde*I fragment from pMD183 (which contains the pUE11-derived promoter and the *cat* gene) with the 17.3-kbp *Nde*I fragment from pMD89 (which


A
0 5 10 15 20 25 30 kb
E47/Ss
X Xh N P EV Xh S N P Sy E P X
pMD66
Km^R Ap^R Tc^R
E47/Ss P/Sy
X Xh N P EV Xh S N Xh E P X
pMD280
Km^R Ap^R Tc^S
E47/St
X Xh N P EV/Sm E S N P Sy Xh X E P X
pMD300
Cm^R Ap^R Tc^S
B
0 1 2 3 4 5 kb
5.2 kb (uncrossed)
7.3 kb (crossed)
E
P Xh E47/Ss XhoI-5 Tc^S P
pMD280 (detail)
P/Sy
P E47/St P Sy X X P
pMD300 (detail)
Tc^S
XhoI-2
2.35 kb (crossed)
4.6 kb (uncrossed)
C
0 1 2 kb 3
18.7 kb (uncrossed) pMD280
4.2 kb (cross in X2)
E
XhoI-5
Xh E47/Ss
Tc^S
pMD280 (detail)
P/Sy
X1 X2 X3
E47/St P Sy X
pMD300 (detail)
Tc^S
(0.7 kb)
0.93 kb (cross in X3)
XhoI-2
4.9 kb (cross in X1)
14.7 kb (uncrossed)

contains the pUE10 sequences necessary for replication in *D. radiodurans* as well as the *Xho*I-2 *tet* mutation) yielded pMD 189. pMD189 is identical to pMD300 except that pMD300 contains a segment of rat cDNA that is absent in pMD189 (Fig. 1A).

The segment of rat cDNA was introduced into pMD189 to create an additional nonhomologous physical polymorphism relative to pMD280. This was accomplished as follows. A 2.2-kbp *Eco*RI fragment of rat DNA from pGABI (a cDNA from a rat biliary glycoprotein gene; gift of G. Dveksler) was ligated with the 6.2-kbp *Eco*RI fragment from pMD189 that included the *Xho*I-2 *tet* mutation, pBR322 replication sequences, the *bla* gene (Ap^r), and the downstream half of the *cat* gene, yielding the 8.4-kbp pMD289. A partial digestion of pMD289 with *Eco*RI, fill-in of 5′ overhangs with Klenow fragment, and blunt-end ligation yielded plasmids in which one of the *Eco*RI restriction cleavage sites was destroyed and the other remained intact. A clone in which only the *Eco*RI site at the junction of the pMD289 rat sequences and the *cat* gene were intact was chosen, yielding the 8.4-kbp pMD294. The 17.3-kbp *Nde*I-*Nde*I fragment of pMD66 was ligated to the 10.5-kbp *Nde*I-*Nde*I fragment of pMD183 (referred to earlier in the description of this construction), to yield the 27.3-kbp pMD186. The 20.6-kbp *Eco*RI fragment of pMD186 was then ligated with the full length of *Eco*RI-linearized 8.4-kbp pMD 294, which includes the *Xho*I-2 mutation, to form pMD300.

Measurements of time course of repair and recombination. Early-plateau-phase cells from overnight cultures (approximately 10^8 cells per ml) were irradiated on ice without change of broth with various total exposures at a rate of 1.3 megarad/h of ^{60}Co, diluted 1/50 in fresh TGY medium without selective drugs, and incubated at 32°C with samples taken at the times indicated. Viable cell counts were determined by plating for colony formation. Visible cell counts were determined with a Neubauer hemocytometer as described previously (5). Transforming activity of plasmid DNA was determined by purification of genomic DNA by miniprep, and 1 μg of the genomic DNA was used to transform *E. coli* DH10B to Ap^r, as determined by plating on selective Luria-Bertani agar.

RESULTS

Characteristics of pMD300 and pMD280. *D. radiodurans-E. coli* shuttle plasmids pMD300 and pMD280 (Fig. 1A) were designed to assess interplasmidic recombination when both plasmids are present in *D. radiodurans* R1. These plasmids are the same in those portions derived from the *D. radiodurans* SARK natural plasmids pUE10 and pUE11. However, pMD300 and pMD280 differ within their *E. coli*-derived segments in three ways.

(i) Different drug resistance determinants. pMD300 contains and expresses the *cat* gene (Cm^r) but not *aphA* (Km^s), while for pMD280, the reverse is true. This facilitates the retention of both plasmids in *D. radiodurans* during growth in liquid culture by use of double drug selection with both chloramphenicol and kanamycin.

(ii) Physical polymorphisms. pMD300 and pMD280 are physically polymorphic in the region of the *tet* gene, containing flanking segments that differ both upstream of *tet* (the 2.2-kbp insert of rat cDNA in pMD300) and downstream of *tet* (the 695-bp *Sty*I-*Puv*II deletion of pBR322 DNA in pMD280) (Fig. 1A). These polymorphisms allow for detection of the recom-

FIG. 1. Plasmid maps, functions, probes, and restriction fragments indicative of recombination. (A) Full-length maps of pMD66, pMD280, and pMD300. In *D. radiodurans*, pMD66 and pMD280 can confer Km^r because of the deinococcal promoting sequences in the fragment derived from the *D. radiodurans* SARK natural plasmid pUE11 (solid segment) that is joined to a portion of the *E. coli* plasmid pMK20 (diagonally hatched segment) that contains the *aphA* gene (Km^r). In *D. radiodurans*, pMD66, pMD280, and pMD300 can confer Tc^r (if the *tet* gene is wild type as it is in pMD66) because of the promoting sequences in a segment derived from another SARK natural plasmid, pUE10 (light gray segment). The *tet* gene is located within the sequence derived from pBR322 (white segment). In *D. radiodurans*, pMD300 expresses Cm^r as a result of the presence of the *cat* gene in a segment from pKK232-8 (dark gray segment) and the adjacent pUE11 promoting sequences. Both pMD280 and pMD300 replicate as plasmids in *D. radiodurans* R1 and SARK because of a deinococcal plasmid origin of replication located within the pUE10-derived portion of these plasmids (light gray segment). In *E. coli*, pMD66, pMD280, and pMD300 can confer Tc^r (if the *tet* gene is wild type) as a result of pBR322 (white segment) promoting sequences for *tet*. In *E. coli*, pMD66 and pMD280 confer Km^r because of the *E. coli* pMK20-derived promoting sequences immediately upstream of *aphA*. For any gene, such as *aphA*, to be expressed in both *E. coli* and *D. radiodurans*, two sets of promoting sequences are necessary, one promoter for *D. radiodurans* and one for *E. coli*, because *D. radiodurans* and *E. coli* recognize each other's promoters very poorly (5, 30). In *E. coli*, pMD66, pMD280, and pMD300 confer Ap^r because of the *bla* gene in pBR322. pMD300 does not express Cm^r in *E. coli* in low copy number because of the lack of an *E. coli* promoting sequence for this gene. pMD66, pMD280, and pMD300 replicate in *E. coli* as a result of the pBR322 replication origin. The spotted segment, which indicates the introduction of a physical polymorphism that can be seen by restriction digestion, is a rat cDNA fragment from pGABI that encodes a rat biliary glycoprotein. Open-headed arrows indicate deinococcal promoting sequences. Filled arrows indicate drug resistance determinants as labeled. The 695-bp *Puv*II-*Sty*I fragment that is deleted from the pBR322 portion of pMD280 is indicated by dotted lines between pMD280 and pMD300, connecting the *Puv*II-*Sty*I region of pMD300 to the *Puv*II-*Sty*I fusion site in pMD280. This deletion does not affect function, but like the segment of rat cDNA, the deletion was introduced to yield a physical polymorphism that can be traced by appropriate restriction digestions. Restriction site abbreviations: E, *Eco*RI; EV, *Eco*RV; EV/Sm, *Eco*RV-*Sma*I fusion; E47/Ss, *Eco*47III-*Ssp*I fusion; E47/St, *Eco*47III-*Stu*I fusion; Ⓔ (E crossed out with an X), destroyed *Eco*RI site; N, *Nde*I; P, *Puv*II; P/Sy, *Puv*II-*Sty*I fusion; S, *Sca*I; Sy, *Sty*I; X, *Xba*I; Xh, *Xho*I. (B) Detail of pBR322 regions and flanking regions of pMD280 and pMD300, showing expected *Puv*II restriction fragments, made visible by use of the *tet* gene probe. The *Puv*II restriction digestion yields fragments indicative of recombination or lack of recombination within the region of homology (gray field) labeled 'X'. The probe, indicated by a wavy black line, is the 1.2-kbp *Eco*RI-*Xho*I fragment from pRDK39 that contains the *tet* gene. Parental fragments visible by autoradiograpy are 5.2 kbp (from pMD280) and 4.6 kbp (from pMD300). Fragments indicative of recombination are 7.3 and 2.35 kbp. These two latter fragments can be generated only by a crossover and not a gene conversion. The locations of the *Xho*I-2 mutation in pMD300 and the *Xho*I-5 mutation in pMD280 are indicated. Both mutations consist of an 8-bp *Xho*I linker inserted into *Taq*I sites, as described in Materials and Methods. The segments' shading is as described for panel A, with the addition that the vertically hatched segment indicates the *Puv*I-*Sty*I 695-bp segment of pBR322 that is present in pMD300 but deleted in pMD280. Restriction site abbreviations are the same as those described for panel A. (C) Detail of pBR322 regions and flanking regions of pMD280 and pMD300, showing expected *Xho*I restriction fragments, made visible by use of the same *tet* gene probe described for panel B. The *Xho*I restriction digestion generates three different-length fragments predicted by recombination, depending upon where the crossover occurred: X_1, X_2, or X_3. Parental fragments are 14.7 kbp from pMD300 and 18.7 kbp from pMD280, respectively. Fragments indicative of recombination are 4.9 kbp if there is a crossover in the 2-kbp region X_1, 4.2 kbp if a crossover occurs in the 101-bp region X_2, and 0.93 kbp if a crossover occurs within the 929-bp region X_3. Segments, restriction sites, probe, and sites of mutation in the *tet* gene are as described for panel B.

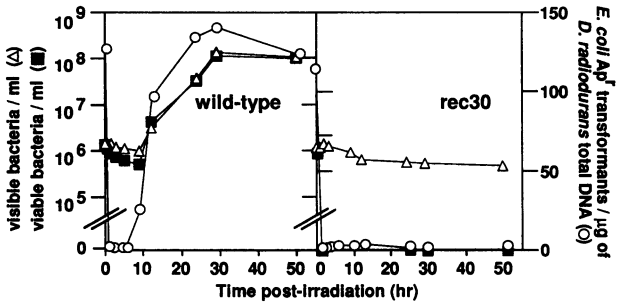


FIG. 2. Viable cells, visible cells, and plasmid transformation activity for R1/pMD280/pMD300 and rec30/pMD280/pMD300. Early-plateau-phase cells (~10^8/ml) were grown in the presence of chloramphenicol and kanamycin. The cells were then irradiated without change of broth on ice with 1.75 megarads, subsequently diluted 1/50 in fresh TGY medium without selective drugs, and allowed to incubate at 32°C with aeration for the times indicated. Viable cell counts were determined by plating for colony formation at the times indicated. Visible cell counts were determined with a hemocytometer as described previously in detail (5). Plasmid transforming activity was determined by purification of total DNA by the miniprep technique at the times indicated, 1 μg of which was used to transform *E. coli* DH10B to Ap^r, as determined by plating on ampicillin selective agar. In both the viable cell assay and the plasmid transformation assay, cells were grown for 3 days prior to counting.

bination region via restriction cleavage and Southern blot analysis (Fig. 1B and C).

(iii) Allelic polymorphisms in the *tet* gene. pMD280 is Tc^s as a result of the presence 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, while pMD300 is Tc^s as a result of the *Xho*I-2 mutation, consisting of the same linker inserted at pBR322 nucleotide position 339 in the *tet* gene. These mutations are 929 bp apart, allowing for detection of recombination in this region (or possibly gene conversion) by selecting for Tc^r.

Cellular survival. As described previously (5), cells were grown prior to irradiation to the plateau phase with appropriate selective drugs for maintenance of plasmids. Following irradiation, cellular survival was assessed by the formation of colonies on nonselective TGY agar plates in samples taken at various times during irradiation at various exposures. It was found that the simultaneous presence of two different plasmids in strain R1 or rec30 (pMD280 and pMD300) at the time of irradiation did not have any effect on cell survival (not shown), as demonstrated previously for the presence of a single plasmid (pMD66) in R1 and rec30 compared with the same strains that did not contain pMD66 (5).

Kinetics of cellular recovery. The kinetics of cellular recovery (Fig. 2) in the current experiments are very similar to those observed previously following the same radiation exposure of R1/pMD66 or rec30/pMD66 (5). Before irradiation, both chloramphenicol and kanamycin were used during the growth of R1/pMD280/pMD300 and rec30/pMD280/pMD300 to the plateau phase to ensure that both plasmids were present at the time of irradiation, as confirmed by diagnostic restriction digestions (not shown). Postirradiation recovery of R1/pMD280/pMD300 and rec30/pMD280/pMD300 was allowed to proceed in fresh TGY broth with vigorous aeration at 32°C in the absence of any drug. The radiation exposure employed was 1.75 Megarads, the dose required to produce 37% survival under our conditions for strain R1 (5). This dose produces little effect on subsequent growth, since the initial population (100%) is regenerated in just 1.4 cell divisions. Following irradiation of R1/pMD280/pMD300, there was a lag phase in the wild type of about 10 h (Fig. 2). A profound growth lag in *D. radiodurans* following any form of DNA damage (nonlethal or partially lethal) has been well described; during this lag, a limited degree of chromosomal DNA degradation that is intrinsic to the repair process(es) occurs (5; for reviews, see references 20 and 21). Over the next 14 h (from 10 to 24 h postirradiation), there were seven divisions, which is nearly normal exponential growth for *D. radiodurans*, and then an additional eighth division over the next 4 h, thereby achieving stationary phase (Fig. 2). Following irradiation of the radiosensitive *recA* strain rec30/pMD280/pMD300, there was no recovery, as determined by plating for viable cells and by the complete absence of growth in liquid culture (Fig. 2).

In R1/pMD280/pMD300, plasmid repair, as shown by regeneration of supercoiled plasmid DNA visualized on Southern blots, paralleled repair of chromosomal DNA (not shown) as demonstrated previously in R1/pMD66 (5), and the regenerated supercoiled plasmid was as efficient as unirradiated control genomic DNA when used for plasmid transformation of *E. coli* DH10B to Ap^r (Fig. 2). This result is also similar to previous observations on regeneration of supercoil and repair of R1/pMD66 (5). Following irradiation of rec30/pMD280/pMD300, small amounts of supercoiled plasmid were regenerated during the time period 9 to 29 h following irradiation (not shown), similar to that previously shown for rec30/pMD66 (5). As in the case of rec30/pMD66, the regenerated plasmid in rec30/pMD280/pMD300 appeared to be defective since it was unable to transform *E. coli* DH10B to Ap^r (Fig. 2), unlike plasmid purified from postirradiation R1/pMD280/pMD300 (5).

Physical polymorphisms of deinococcal plasmid DNA following irradiation. Southern blot analysis of plasmid DNA permits assessment of plasmidic recombination within specific windows made possible by the introduction of physical polymorphisms, as noted above (Fig. 1B and C). At various times following 1.75 megarads of exposure, total DNA was purified from nonselective liquid cultures of recovering cells, cleaved with *Pvu*II, and analyzed by Southern blot hybridization using the radiolabeled *tet* gene as a probe. This procedure permits observation of recombinant bands if a crossover occurs within the 1.37-kbp region marked X in Fig. 1B. The parental bands, indicative of plasmids that have not undergone recombination within this window, are 5.2 and 4.6 kbp, and both are abundant, as demonstrated by the autoradiogram (Fig. 3A). However, both of the predicted recombinant bands of 7.3 and 2.35 kbp (Fig. 1B) are also evident, faintly visible as early as 4 h following irradiation and easily detected by 7 h following irradiation and at later times in wild-type R1 (Fig. 3A). Because of the nature of the flanking polymorphisms and the *tet* probe used to observe the polymorphisms, the recombinant bands visible when a *Pvu*II digestion was used can occur only as the result of a crossover and not a gene conversion. Densitometric analysis of the 7-, 12-, 24-, and 29-h lanes (Fig. 4) shows recombinant bands at 7 h postirradiation, before the onset of cellular growth (as determined both by the CFU assay and the visible cell count assay) (Fig. 2). Subsequent to 7 h postirradiation, the total amount of signal in the recombinant bands averaged about 0.08 of total signal within all bands, with the remaining signal being in the two nonrecombinant bands (Fig. 4). In contrast, in the *recA* strain rec30, there was no evidence of recombinant bands (Fig. 3B).

Recombination was also detectable with these two constructions when a *Xho*I digestion of genomic DNA and the same *tet* fragment as radiolabeled probe were used. All three predicted recombinant bands, each indicative of an event in one of three

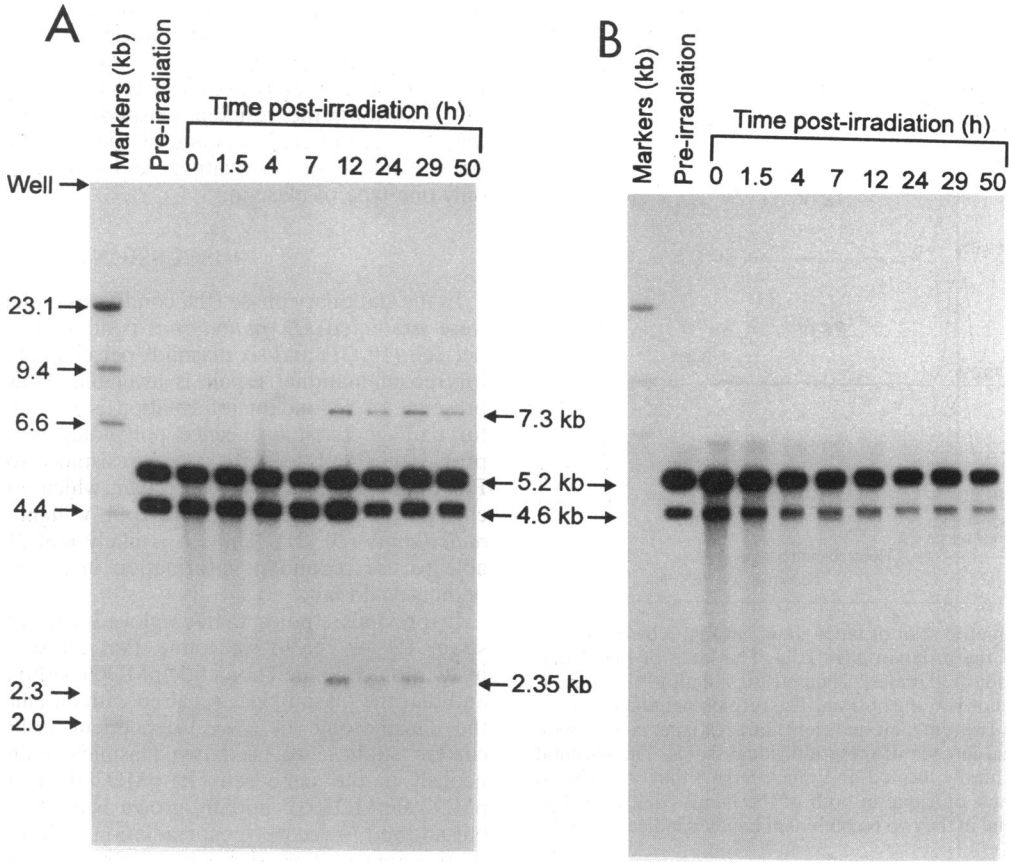


FIG. 3. Production of recombinant bands following irradiation of *D. radiodurans* wild-type strain R1 (A) and *recA* strain rec30 (B). Cells were grown, irradiated, diluted, and allowed to recover without drug selection as described in the legend to Fig. 2. DNA was prepared from samples taken at the indicated times by the miniprep technique and cleaved with *Pvu*II. Each lane contains the DNA from 3×10^6 cells, as determined by hemocytometer count. Electrophoresis was in a 0.7% agarose gel for 18 h at 55 V prior to blotting and probing of the blot with a 1.2-kb fragment of the *tet* gene that had been radiolabeled with [^{32}P]dCTP by the random priming method. The markers are λ phage DNA cleaved with *Hin*dIII.

regions (X_1, X_2, and X_3 [Fig. 1C]), are present in the autoradiogram of genomic DNA from wild-type R1 (Fig. 5A). In particular, a 4.2-kbp fragment that occurs as the result of recombination within a 101-bp region between the *Xho*I-5 mutation and the *Sty*I site is detectable at later times. This very small region is indicated as X_2 in Fig. 1C. In *recA* strain rec30, there was no evidence of recombination between plasmids or of gene conversion when the *Xho*I digestion and the *tet* gene probe were used (Fig. 5, right), similar to the finding in rec30 when a *Pvu*II digestion was used (Fig. 3, right). Following irradiation, Southern blot evidence of recombination in wild-type R1 occurred only in R1 cells that contained both plasmids. This was tested by a control experiment in which two separate R1 cell populations that contained only one variety of each of the two plasmids were mixed prior to irradiation, with the finding that in irradiated and control cultures, no recombination or gene conversion occurred (not shown). This rules out the formal possibility that recombination was occurring secondary to transformation of plasmid DNA among cells.

Structures of Tc^r-conferring plasmids. Gel electrophoresis of deinococcal total DNA, both before and after irradiation, showed that all supercoiled plasmid migrated as a monomer (not shown), as observed previously (5). Subsequent transformation of *E. coli* DH10B using 29-h postirradiated *D. radiodurans* genomic DNA produced numerous Ap^r colony isolates (Fig. 2). Of these, 809 isolates were replica plated onto tetracycline-containing plates, giving 16 recombinant Tc^r clones, equal to 2.0% of the total patches screened. Each of these colony isolates were either Cm^r or Km^r, in addition to being Tc^r, but never both as predicted for a heterodimer. Furthermore, gel electrophoresis of supercoiled Tc^r recombinant plasmids showed them all to be monomeric. This frequency of irradiation-induced Tc^r-conferring plasmids was 1 log greater than the sporadic occurrence of Tc^r-conferring plasmids in samples treated identically except for the lack of irradiation treatment. Thus, more unirradiated than irradiated samples had to be screened to acquire the 11 sporadic Tc^r recombinants (Fig. 6). The structure of the 16 Tc^r recombinant plasmids derived from irradiated *D. radiodurans* was compared with the structure of 11 sporadically occurring Tc^r-conferring plasmids by using diagnostic restriction digestions and Occam's razor (Fig. 6). In both cases, the most frequent event within this window of analysis was a single crossover in the 929-bp region between the *Xho*I-2 and *Xho*I-5 mutations in *tet* (11 of 16 [64%] irradiated and 7 of 11 [69%] control Tc^r recombinants), all of which were Cm^r. The plasmids derived from this event could not have arisen by gene conversion because the recombinants lacked both of the flanking polymorphisms (the 695-kb *Pvu*II-*Sty*I deletion of pMD280 and the 2.2-kb rat DNA insertion from pMD300). In the postirradiation group of Tc^r plasmids, we found an unexpected number of plasmids consistent with the occurrence of a crossover in the 101-bp region

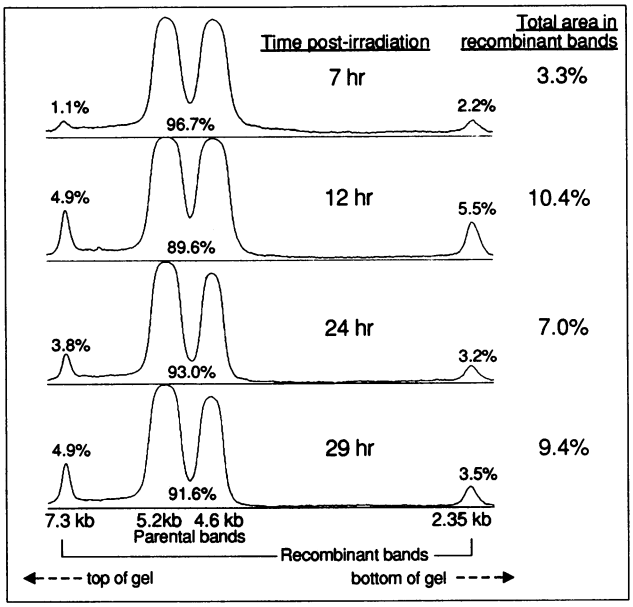


FIG. 4. Densitometric scan of lanes shown in Fig. 3 by use of the gel scan module of the program NIH 1.5.4. The lanes of Fig. 3 that were scanned are indicated under "Time post-irradiation." The orientation of the gel is shown at the bottom. The recombinant bands are at the top and bottom (extreme left and right) of each lane, labeled with the amount of signal in each of these individual bands. The parental bands are more centrally located and much larger; they are labeled with the total amount of signal in both of the bands combined. The total amount of signal in the two recombinant bands is indicated on the right.

between the *Xho*I-5 mutation and the *Pvu*II-*Sty*I deletion (4 of 16 plasmids [25%]). This mapping assignment agrees with the prediction that such plasmids are Km^r, not Cm^r, which is the case. No such sporadic recombinants of this type were detected in the current sampling. To date, it is not possible to determine whether the plasmids in this group occurred by gene conversion or two crossovers within this window of observation. In contrast, in the 339-bp region between the *Xho*I-2 mutation and the upstream *Eco*RI site, mapping of the recombinant Tc^r plasmids indicates that there were few radiation-induced events in this region that lead to a Tc^r plasmid (1 of 16 [6%]), while 4 of 11 (36%) of the sporadic Tc^r plasmids suggest the occurrence of gene conversion or two crossovers in this region.

All *D. radiodurans* survivors following irradiation retained only one variety of plasmid. Because of radiation-induced damage to plasmid DNA, it is possible that the plasmids might not be repaired or retained by each *D. radiodurans* bacterium following irradiation and subsequent recovery in liquid culture. Thus, the liquid culture results shown in Fig. 3 and 5 might be due to retention and repair by only a subset of bacteria in which the plasmids sustained only minor damage. In previous studies that employed only one reporter plasmid (R1/pMD66 and rec30/pMD66), we found that all survivors following irradiation contained bona fide pMD66 without drug selection (5).

However, in the current study that employed two types of plasmids per cell at the time of irradiation, we found that R1 did not retain both plasmids while recovering in liquid culture without selective pressure. Instead, numerous minipreps showed that all survivors contained only one type of plasmid, bona fide pMD280 (Km^r) or pMD300 (Cm^r), or a recombinant of the types shown in Fig. 6. In no case by the miniprep technique did we find an absence of plasmids following recovery, and in no case did we find more than one plasmid present, the most commonly encountered being pMD280. Thus, following irradiation of *D. radiodurans* R1 (allowed to recover without selection), the resident plasmids either did or did not undergo recombination (some of which is evident within our small windows of observation) and then eventually retained only one type of plasmid.

DISCUSSION

In the stationary phase (the conditions under which the cells were irradiated), *D. radiodurans* contains four chromosomes per cell (10, 31) and six plasmids per cell (5). Thus, substrate for recombinational repair is available. However, the mere presence of redundant information is insufficient to account for damage resistance, since polyploidy is common among prokaryotes and does not confer resistance to DNA damage. For example, *Azotobacter vinelandii*, which contains up to 80 chromosomes (27), is sensitive to UV light (17), unlike *D. radiodurans* (20, 21). Thus, it is likely that *D. radiodurans* is able to use redundant information in a manner that other organisms do not.

In previous studies that employed only pMD66 alone in strain R1 or rec30, we found that all survivors following irradiation contained bona fide pMD66 without drug selection and that the plasmid was repaired with an efficiency similar to the chromosome in a *recA*-dependent fashion (5). In the current studies, we used two plasmids maintained simultaneously in the same cell (R1/pMD280/pMD300 and rec30/pMD280/pMD300), initially grown with dual drug selection but allowed to recover postirradiation without drug selection. By this approach, we determined that a high level of homologous recombination was occurring among plasmids following irradiation. In addition, we found that under nonselective conditions only a single variety of plasmid in R1 or rec30 was retained. This may be related to the fact that both pMD280 and pMD300 use the same replicon, from SARK plasmid pUE10. Previous studies of strain R1 using two different plasmids with different autonomous replication sequences showed that they can be maintained in the same host for long durations without selection (28, 30). Thus, *D. radiodurans* R1 apparently prefers its plasmids homozygous, in which case plasmids of a given replicon may survey each other for homozygosity, perhaps by Holliday junctions, as we suggested previously (5).

We found that pMD280 and pMD300 in the wild type underwent substantial homologous recombination within a window of observation consisting largely of the *tet* gene and some flanking sequences in the pBR322 portion of these plasmids (Fig. 1B and C and 3 to 5). While many hypotheses may be advanced, we suggest the following. The window of 1.37 kbp observed in the *Pvu*II digestion in these experiments shows a crossover frequency of 0.08 (Fig. 4). We would expect nonproductive crosses within this region to occur at the same frequency as that of crossovers. Thus, the theoretical number of crosses occurring within this window should be doubled, i.e., to 0.16. The number of crossovers and nonproductive crosses per monomeric plasmid would then be predicted to be at least (28.5 kbp/1.37 kbp) $\times$ (0.16) $\times$ 2 (for a monomer), which equals 6.7. The number of base pairs in the *D. radiodurans* chromosome is about 3×10^6 (8). If the same number of recombinational events occurring per kilobase pair in the plasmid are also occurring simultaneously in the chromosome, the predicted number of crosses per chromosome yielded during repair would be >700, as determined by: [(3×10^6 bp

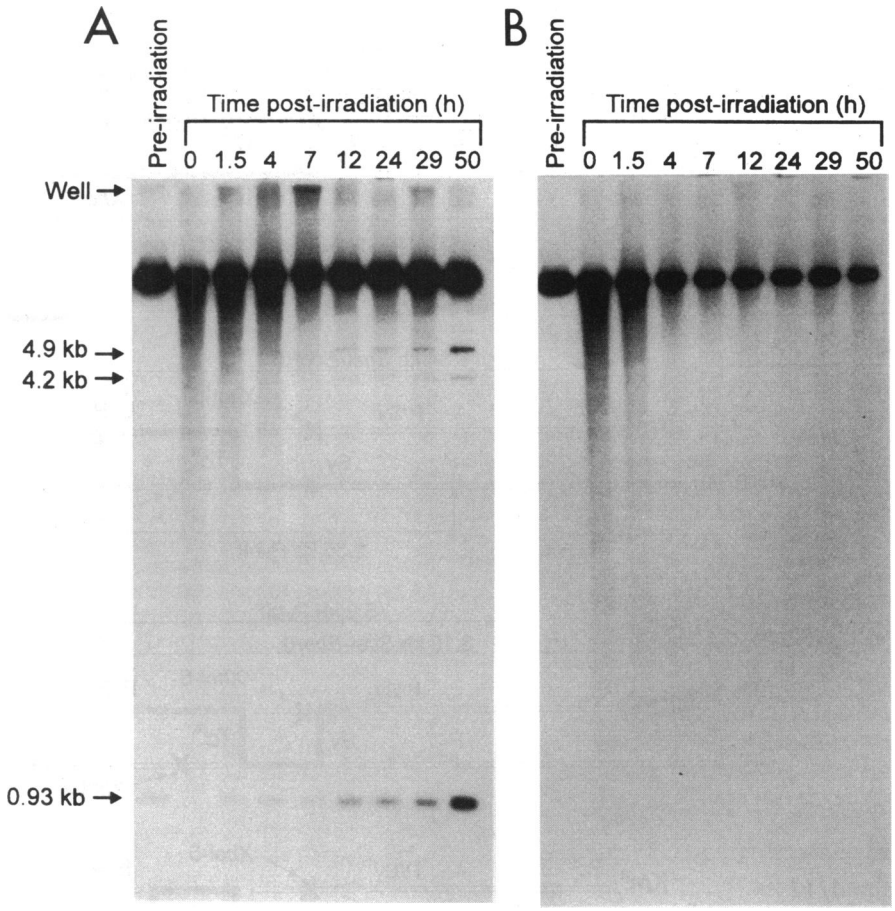


FIG. 5. Production of recombinant bands following irradiation of *D. radiodurans* wild-type strain R1 (A) and *recA* strain rec30 (B). Cells were treated as described in the legend to Fig. 3, except that enzymatic cleavage employed *Xho*I instead of *Pvu*II. The electrophoresis was in a 0.9% agarose gel at 55 V for 18 h.

per chromosome)/(28.5 × 10^3 bp per plasmid)] × 6.7 productive and nonproductive crosses per plasmid.

The occurrence of Tc^r isolates was also studied to assess recombination in the region of the *tet* gene (Fig. 6). In both postirradiation and sporadically occurring Tc^r mutants, about 65% of events may be interpreted as having occurred by the simplest route, a single crossover between the mutant *tet* genes, while the remaining plasmids achieved Tc^r-conferring status by less obvious routes, attributable to either gene conversion or two crossovers (Fig. 6). About 8% of all plasmids undergo productive recombination within a window of 1,369 bp, consisting of the entire *tet* gene plus flanking sequences (Fig. 3 and 4). Among these recombinants, a fraction should be Tc^r, lacking both the *Xho*I-2 and *Xho*I-5 mutations, i.e., 50% of the instances where there is a crossover between these two sites on pMD300 and pMD280, respectively, a distance of 929 bp. Consequently, we would predict the frequency of Tc^r recombinants to be 2.7%, as determined by the following equation: [(929/1,369) × 0.08] × 50%, where 929 is the number of base pairs in the Tc^r phenotype window, 1,369 is the number of base pairs in the *Pvu*II restriction fragment polymorphism window, 0.08 is the frequency of crossovers within the *Pvu*II window, and 50% is the expected frequency of recombinants that contain neither mutant allele. This value of 2.7% is approximately the case, since we found that 2.0% of all Ap^r *E. coli* transformants were also Tc^r. As expected, plating of postirradiation R1/pMD280/pMD300 on tetracycline-selective plates gave the same frequency of 2% Tc^r transformants per CFU (not shown).

We do not know of any other systems in which plasmids can recover from being heavily irradiated in vivo. We are thus limited to very indirect comparisons between our current results and those of others. One comparison might be with the occurrence in other organisms of host cell reactivation of γ-irradiated plasmids when the recipient unirradiated cells contain homologous sequences. One such study using γ-irradiated plasmids damaged in vitro, and then transformation of *E. coli*, demonstrated a dramatic dose-dependent decrease in plasmid survival that was not improved by the presence of homology in the recipient and was *recA* independent (24). However, plasmid irradiation did result in *recA*-dependent recombination of the plasmid DNA with homologous chromosomal sequences to form recombinant colonies. Whereas unirradiated plasmids did not recombine, plasmids irradiated to produce mostly open circular and linear forms (i.e., similar to the extent of damage induced in plasmids in the current study) formed recombinant colonies at a yield of 10^{-4} (24). This value implies that recombination in *E. coli* following irradiation may be as much as 2 to 3 logs less efficient than that in *D. radiodurans*, where we found that DNA damage resulted in 2% (2 × 10^{-2}) Tc^r recombinants by crossover within a small region between pMD280 and pMD300. This observation is also

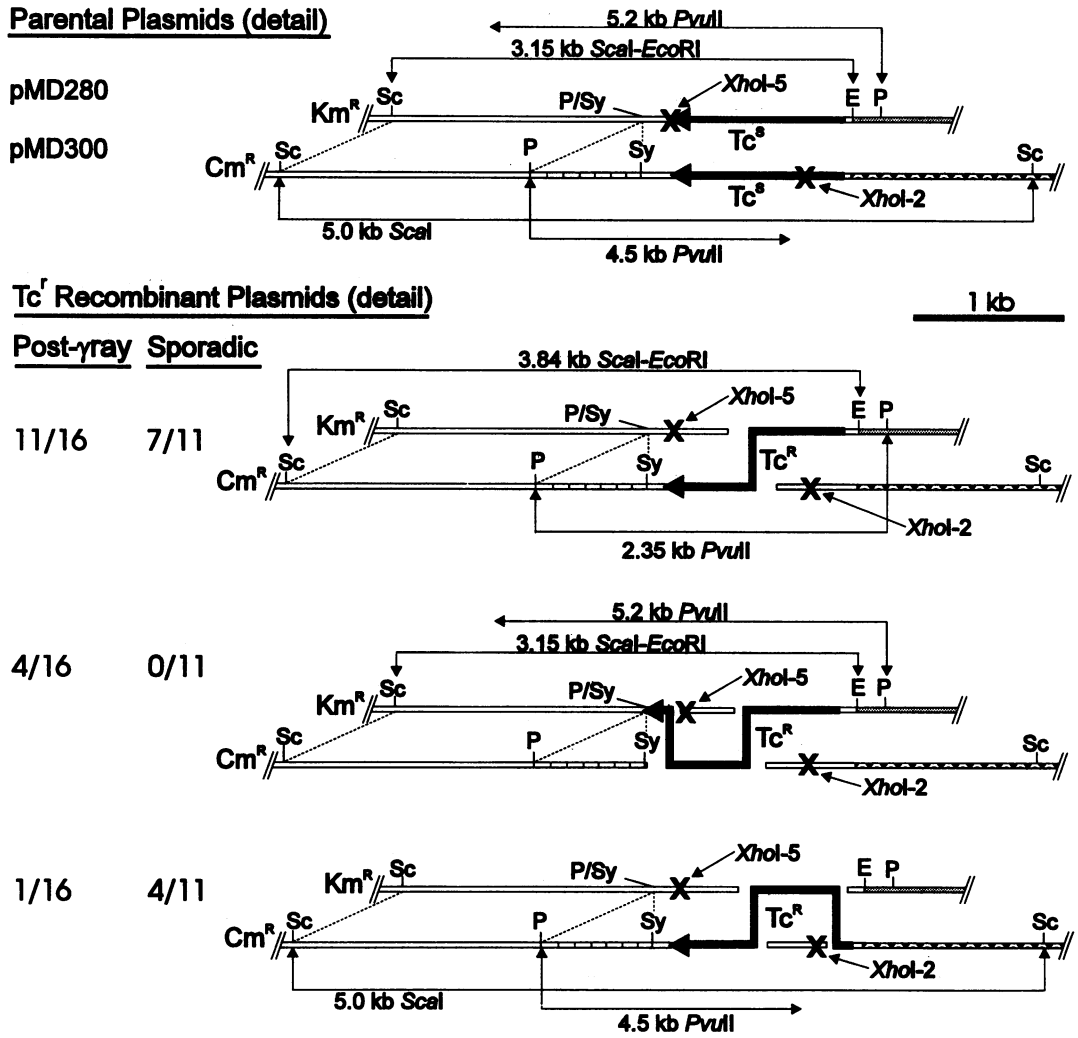


FIG. 6. Restriction enzyme analysis of Tc^r plasmids arising postirradiation or sporadically. The top of the figure shows a detail of the two parental plasmids, pMD280 and pMD300, including those regions tested for recombination. The lower portion of the figure shows the restriction cleavage sites and the simplest mechanism by which recombinational events could occur and be consistent with the diagnostic restriction fragments. Segment shading and restriction site abbreviations are described in the legend to Fig. 1. In addition, the heavy black line indicates the *tet* gene, with the arrowhead showing the direction of transcription. In the case of the parental plasmids, which contain either the *Xho*I-2 or *Xho*I-5 mutation in the *tet* gene, this gene is labeled Tc^s, while below the recombinant (or gene converted), the *tet* genes are labeled Tc^r. The 4.5-kb *Pvu*II fragment is the same fragment labelled 4.6 kb in Fig. 1 and 3.

consistent with the fact that *D. radiodurans* can repair its chromosomal DNA with 2-log-greater efficiency than *E. coli* (5). Thus, it appears that recombination between plasmids occurs with the same frequency in *D. radiodurans* as it does among its chromosomes, strongly supporting the concept that study of plasmid repair damaged in vivo is a legitimate model for concurrent repair occurring in the *D. radiodurans* chromosome.

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