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# Duplication Insertion of Drug Resistance Determinants in the Radioresistant Bacterium *Deinococcus radiodurans*

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***Escherichia coli* drug resistance plasmids were introduced into *Deinococcus radiodurans* by cloning *D. radiodurans* DNA into the plasmids prior to transformation. The plasmids were integrated into the chromosome of the transformants and flanked by a direct repeat of the cloned *D. radiodurans* segment. The plasmid and one copy of the flanking chromosomal segment constituted a unit ("amplification unit") which was found repeated in tandem at the site of chromosomal integration. Up to 50 copies of the amplification unit were present per chromosome, accounting for approximately 10% of the genomic DNA. Circular forms of the amplification unit were also present in *D. radiodurans* transformants. These circles were introduced into *E. coli*, where they replicated as plasmids. The drug resistance determinants which have been introduced into *D. radiodurans* in this fashion are *cat* (from Tn*9*) and *aphA* (from Tn*903*). Transformation of *D. radiodurans* to drug resistance was efficient when the donor DNA was from *D. radiodurans* or *E. coli*, but was greatly reduced when the donor DNA was linearized with restriction enzymes prior to transformation. In the course of the study, a plasmid, pS16, was discovered in *D. radiodurans* R1, establishing that all *Deinococcus* strains so far examined contain plasmids.**

Bacteria of the genus *Deinococcus* share an extreme degree of resistance to ionizing and UV radiation (20). Our major interest in this genus is the genetic and molecular mechanisms responsible for its resistance to ionizing radiation. Currently, genetic techniques applicable to *Deinococcus* species are limited. *Deinococcus radiodurans*, the most-studied species of this genus, has previously been shown by Moseley and co-workers to be naturally transformable (21, 28). Their work has used high-efficiency transformation by homologous DNA containing spontaneous or chemically induced mutations. For example, DNA from the rifampin-resistant (Rif$^r$) *D. radiodurans* strain R1 KRASE transforms up to 6% of competent *D. radiodurans* R1 recipients to Rif$^r$ (28). The restoration of normal phenotype in *D. radiodurans* mutants via transformation by wild-type *D. radiodurans* sequences has been used as an assay to clone several *D. radiodurans* genes (1). Although both double- and single-stranded DNA is taken up by competent *D. radiodurans*, double-stranded DNA is more effective for transformation (21). Despite several attempts, no drug resistance determinants, transposons, or plasmids have been introduced into any *Deinococcus* strain (15, 20).

We sought to determine whether we could exploit transformation in *D. radiodurans* to achieve insertion of heterologous DNA by using a strategy effective in transformable species of *Bacillus*, *Streptococcus*, and yeast (8, 11, 27). In these organisms, heterologous sequences may be integrated into the host chromosome by in vitro ligation of host DNA sequences to the heterologous sequence prior to transformation. Transformants contain the heterologous sequence flanked by a direct repeat of the host sequence. Using this approach, we report the first genomic integration and expression of heterologous drug resistance determinants in any *Deinococcus* species. In addition, we detail the fate of these transformed sequences, including the occurrence of direct repeats flanking the heterologous sequence upon genomic integration, the amplification of the heterologous sequence with linked flanking sequences to very high copy numbers, and the generation of covalently closed circular (CCC) DNA within *D. radiodurans*, apparently due to intrachromosomal recombination within the amplified sequences. A model is presented to account for the occurrence of these events, and similar events in other transformable species are compared.

## MATERIALS AND METHODS

**Materials.** Tryptone, yeast extract, and Bacto-Agar were from Difco Laboratories, Detroit, Mich. Restriction enzymes, T4 DNA ligase, S1 nuclease, lambda phage DNA digested with *Hin*dIII, and φX174 phage DNA digested with *Hae*III were from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; New England BioLabs, Inc., Beverly, Mass.; or Boehringer Mannheim Biochemicals, Indianapolis, Ind. Antibiotics and most other reagents were from Sigma Chemical Co., St. Louis, Mo. Gene Screen-Plus and [α-$^{32}$P]dCTP were from New England Nuclear Corp., Boston, Mass.

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used are described in Table 1. Several strains may be thought of as having insertions of a plasmid which contains both chromosomal and heterologous sequences and are so designated in accordance with convention (22). For example, *D. radiodurans* R1 derivative LM1 has an insertion of pS10 and so is described as R1ΩpS10. *D. radiodurans* strains were grown at 32°C in TGY broth in a rotating shaker or on TGY plates containing 1.5% agar (6). *Escherichia coli* strains were grown at 37°C in LB broth or on LB plates containing 1.5% agar (16).

**Transformation.** Exponentially growing *D. radiodurans* cultures were stored at −70°C in TGY plus 10% glycerol and 30 mM $CaCl_2$. Samples were thawed and immediately transformed by the method of Tigari and Moseley (28). A typical

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TABLE 1. Bacterial strains and plasmids[a]

| Strain or plasmid | Description[b] | Source (reference)[c] |
|---|---|---|
| *D. radiodurans* | | |
| R1 | Wild type (pS16) | Moseley (2) |
| R1 KRASE | R1 Rif$^r$(pS16) | Moseley (28) |
| Sark | Wild type (pUE10, pUE11) | Moseley (15; Murray and Robinow)[d] |
| LM1 | R1ΩpS10 | R1 × pMK20::R1 KRASE *Eco*RI |
| LM2 | R1ΩpS11 | R1 × pMK20::R1 KRASE *Eco*RI |
| LM20 | R1ΩpS14 | R1 × pMK20::R1 KRASE *Eco*RI |
| LM102 | R1ΩpEL2 | R1 × pEL1 *Bam*HI::R1 *Bcl*I |
| LM103 | R1ΩpEL3 | R1 × pEL1 *Bam*HI::R1 *Bcl*I |
| LM104 | R1ΩpEL4 | R1 × pEL1 *Bam*HI::R1 *Bcl*I |
| *E. coli* | | |
| DH5α | F$^-$ *recA1* | Bethesda Research Laboratories |
| Plasmids | | |
| pMK20 | *aphA* | Helinski (13) |
| pACYC184 | *cat* | (4) |
| RK2 | 60 kb | Guerry (13) |
| pS10 | pMK20::R1 KRASE | DH5α × LM1 |
| pS11 | pMK20::R1 KRASE | DH5α × LM2 |
| pS12 | pS10 *Dra*I-*Nar*I | pS10 *Dra*I-*Nar*I resection[e] |
| pS14 | pMK20::R1 | DH5α × LM20 |
| pS16 | 60 kb | Endogenous to R1 |
| pEL1 | *cat* | pACYC184 derivative[f] |
| pEL2 | pEL1::R1 | DH5α × LM102 |
| pEL3 | pEL1::R1 | DH5α × LM103 |
| pEL4 | pEL1::R1 | DH5α × LM104 |

[a] Transformations are noted as "recipient × donor." Donor preparations containing ligase-treated mixtures of pEL1 (cleaved with *Bam*HI) and R1 DNA (cleaved with *Bcl*I) are shown as "pEL1 *Bam*HI::R1 *Bcl*I."

[b] Wild-type R1 and all of its derivatives contain pS16. *aphA* and *cat* designations are per convention (3, 22).

[c] Plasmids or strains for which a person or reference is not noted were constructed during this study.

[d] R. G. E. Murray and C. F. Robinow, Abstr. VIIth Int. Cong. Microbiol., p. 427, 1958.

[e] pS12 was created by cleaving pS10 with *Dra*I and *Nar*I, followed by S1 nuclease and T4 DNA ligase treatments. pMK20 has no *Nar*I sites, but pS10 has over 5 *Nar*I sites.

[f] pSA3′ was obtained from Ferretti as an *Sph*I chimera of pGB301′ (pIP501 derivative similar to pGB301) and pACYC184, linked at the *Sph*I site (5). pEL1 is a *Hin*cII self-ligation of pSA3′.

transformation was 100 μl of cells ($5 \times 10^7$ cells per ml) exposed to 1 μg of DNA. Transformants were selected by plating on TGY supplemented with the appropriate drug (5 μg of rifampin, 8 μg of kanamycin sulfate, or 3 μg of chloramphenicol per ml). Strain R1 spontaneous mutants resistant to 8 μg of kanamycin per ml, but not 20 μg/ml, were observed at a frequency of about $4 \times 10^{-7}$ CFU. *E. coli* strains were transformed by the $CaCl_2$ method (19), and transformants were selected by plating on LB containing 25 μg of chloramphenicol or 30 μg of kanamycin per ml (16).

**DNA isolation and manipulation.** *D. radiodurans* genomic DNA was isolated by a modification of prior techniques (15, 28). Briefly, crude lysates were extracted with chloroform and then precipitated with isopropanol. *E. coli* plasmids were isolated on a small scale by boiling and on a large scale by precipitating sodium dodecyl sulfate lysates with NaCl, followed by CsCl-ethidium bromide centrifugation (16). *D. radiodurans* CCC DNA was isolated from 500-ml cultures by isopycnic banding of cell lysates in 38 ml of CsCl-ethidium bromide, followed by rebanding in 12 ml. Yields were typically 3 to 5 mg of open and linear DNA and 1 to 5 μg of CCC DNA. Restriction endonuclease, S1 digests, and ligations were performed according to the recommendations of the manufacturer. Ligations were at 40 μg of DNA per ml at 4°C for 18 h. *E. coli* plasmid/*D. radiodurans* DNA ratios ranged from 4:1 to 1:4 (microgram/microgram).

**Electrophoresis and hybridization.** Digests were electrophoresed on horizontal gels (1% agarose in Tris-acetate buffer) at 1 V/cm (16). Unless specified otherwise, lanes contained about 500 ng of DNA. Southern blotting (25) was performed with 0.4 N NaOH for denaturation and transfer to a nylon Gene Screen-Plus membrane according to the recommendation of the manufacturer (New England Nuclear). $^{32}$P-labeled probe was prepared by using Bethesda Research Laboratories nick translation kits followed by gel filtration on a 5-ml column of G-75 (Pharmacia, Inc., Piscataway, N.J.). Hybridization was for 18 h with about $10^6$ cpm/ml in 50% formamide at 37°C (16). Blots were washed at high stringency (60°C; 15 mM NaCl, 1.5 mM sodium citrate). Probe was stripped from the blots by washing with 0.2 N NaOH, followed by washing with water.

## RESULTS

**Introduction of *E. coli* plasmids pMK20 (Km$^r$) and pEL1 (Cm$^r$) to *D. radiodurans*.** pMK20 is a 4.1-kilobase (kb) ColE1-based plasmid that confers resistance to kanamycin and neomycin by virtue of the gene encoding aminoglycoside phosphotransferase I (*aphA*) from Tn*903* (Fig. 1). Most of the inverted repeats of Tn*903* were deleted in the construction of pMK20 (13, 23). pEL1 is a 3.5-kb derivative of *E. coli* plasmid pACYC184 (4) that confers resistance to chloramphenicol by virtue of the *cat* gene (Table 1; see Fig. 5). In both cases *D. radiodurans* was not transformed to drug resistance with pMK20 or pEL1 alone (<10 transformants per μg of DNA). However, if the restriction-cleaved *E. coli* plasmid was mixed with restriction-cleaved *D. radiodurans* DNA with compatible ends and treated with T4 DNA ligase

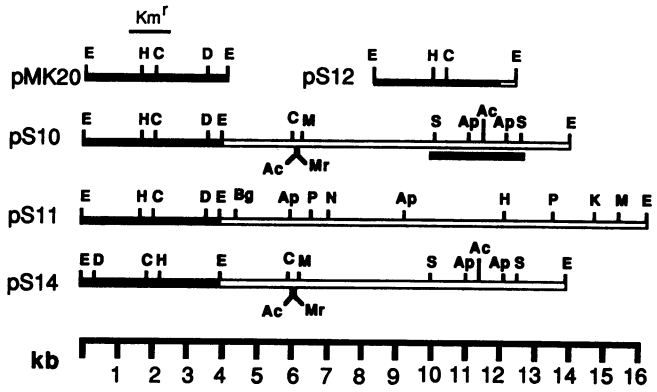


FIG. 1. Restriction maps of pMK20 and derivatives. The location of the pS10 *Sst*I-B fragment which was used as a probe is marked with a heavy line. pMK20 sequences (striped boxes) contain the *aphA* gene, which confers kanamycin resistance (Km$^r$). Restriction sites: Ap, *Apa*I; Ac, *Acc*I; Bg, *Bgl*II; C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; Mr, *Mra*I; P, *Pvu*II; S, *Sst*I. There are several *Nar*I and *Bgl*I sites in pS10 and pS11, but none in pMK20.

prior to transformation, drug-resistant transformants occurred at a frequency of 10 to 1,000 transformants per μg. All transformants tested contained DNA which strongly hybridized to pMK20 or pEL1 (not shown). The transformation efficiency appeared to be dependent on the efficiency of the T4 DNA ligase reaction, as assessed by gel electrophoresis before and following ligation (not shown).

**Characterization of pMK20 transformant strains LM1, LM2, and LM20.** *D. radiodurans* R1 was transformed to kanamycin resistance (Km$^r$) with a ligation mixture composed of *Eco*RI-cleaved genomic DNA from *D. radiodurans* R1 KRASE and *Eco*RI-linearized pMK20. Several single-colony isolates were characterized, including the three which are described in detail.

**Genomic DNA from *D. radiodurans* Km$^r$ transformants transformed *E. coli* to Km$^r$.** *E. coli* DH5α was transformed to Km$^r$ with genomic DNA from *D. radiodurans* LM1. Plasmid preparations from six *E. coli* Km$^r$ transformants were cleaved with *Eco*RI and *Hin*dIII and found to be indistinguishable from one another (data not shown). A similar result, though a different plasmid, was obtained with LM2 donor DNA. The plasmid arising from LM1 (pS10) and that from LM2 (pS11) were mapped (Fig. 1). Both plasmids were *Eco*RI cointegrates of pMK20 and a large *Eco*RI fragment. These large *Eco*RI fragments came from the *D. radiodurans* chromosome (see below).

**LM1 and LM2 genomic DNA contained multiple copies of pS10 and pS11.** Results for strains LM1 and LM2 (Fig. 2) are addressed in this section. Results for strain LM20 (Fig. 2) are covered in the next section. Genomic DNA from *D. radiodurans* wild-type strain R1 and transformants LM1 and LM2 were cleaved with *Eco*RI or *Hin*dIII and electrophoresed. The ethidium bromide stain of the digests of wild-type R1 DNA showed a complex banding pattern typical of restriction enzyme digests of bacterial genomic DNA, but the LM1 and LM2 digests were slightly different from each other and R1. *Eco*RI digests of genomic DNA from LM1 and LM2 contained a 4.1-kb fragment which was barely visible by ethidium bromide fluorescence, comigrated with *Eco*RI-linearized pMK20, and hybridized to the pMK20 probe (Fig. 2A and 2B, lane 5 and *Eco*RI lanes 6 and 7). This indicated that strains LM1 and LM2 each contained the pMK20 sequence and that multiple pMK20 copies per chromosome were present, since the bands as viewed by ethidium bromide fluorescence were bright in comparison to other chromosomal fragments in the same area of the gel.

The *Eco*RI digests of LM1 and LM2 contained additional fluorescent bands, suggesting amplification of chromosomal sequences. LM1 DNA contained a visible 9.8-kb *Eco*RI band that did not hybridize to pMK20 (Fig. 2A and 2B, *Eco*RI lane 6) but did hybridize to a 1-kb probe (pS10 *Sst*I-B of Fig. 1) from the 9.8-kb *Eco*RI fragment of pS10 (Fig. 2C, *Eco*RI lane 6). A *Hin*dIII digest of LM1 DNA contained a visible 14-kb fragment that hybridized to both probes (Fig. 2, *Hin*dIII lane 6) and comigrated with *Hin*dIII-linearized pS10 (not shown). The lengths of these *Eco*RI and *Hin*dIII frag-

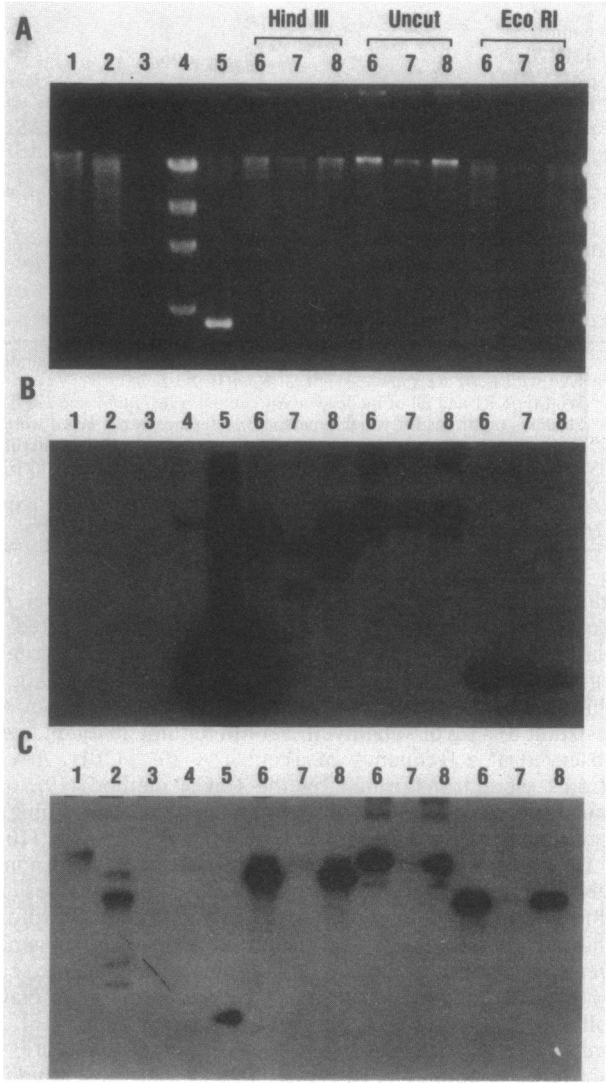


FIG. 2. Agarose gel electrophoresis of *D. radiodurans* genomic DNA. (A) Ethidium bromide stain. (B) Autoradiogram of a Southern blot probed with pMK20. (C) Autoradiogram of the same Southern blot after removal of pMK20 probe and hybridization with pS10 *Sst*I-B. Note that removal of pMK20 was incomplete in lane 5. Lanes contain the following DNA: 1, R1 genomic cleaved with *Hin*dIII; 2, R1 genomic cleaved with *Eco*RI; 3, ϕX174 *Hae*III digest; 4, lambda *Hin*dIII digest (23.1, 9.4, 6.6, and 4.4 kb); 5, pMK20 cleaved with *Eco*RI (4.1 kb); 6, LM1 genomic; 7, LM2 genomic; 8, LM20 genomic. Lanes labeled 6, 7, or 8 contain undigested DNA ("uncut") or DNA digested with the indicated enzyme.

ments from LM1 correspond to those indicated by the map of pS10 (Fig. 1, two *Eco*RI sites and one *Hin*dIII site), suggesting that the sequence of pS10 is the same as the amplified sequence in LM1.

The 9.8-kb *Eco*RI fragment in LM1 that hybridizes with pS10 *Sst*I-B is also found in wild-type R1 (Fig. 2C, lane 2). The intensity of this band is much less in strain R1 than in LM1 (Fig. 2C, cf. lane 2 and *Eco*RI lane 6), again indicating that there are multiple copies of pS10 in LM1, a finding that supports the amplification suggested by the ethidium bromide stain. The *Hin*dIII digest of R1 also contains a band that hybridizes to pS10 *Sst*I-B, but it is of a different size than that in *Hin*dIII-digested LM1 (Fig. 2C, cf. lane 1 and *Hin*dIII lane 6). This difference in size is expected since the *D. radiodurans* fragment in pS10 contains no *Hin*dIII sites (Fig. 1).

Some hybridization between pMK20 and lambda *Hin*dIII fragments A and D was observed, perhaps because of weak homology between them. Four bands of hybridization were occasionally observed in *Eco*RI digests of LM1 or R1 probed with pS10 SStI-B (Fig. 2C, lane 2; see Fig. 4). The identity of these bands is unknown, but they may represent chromosomal fragments with partial homology to the probe.

LM1 DNA and pS10 were digested with *Mlu*I, *Apa*I, *Nar*I, and *Bgl*I, electrophoresed, blotted, and probed with pMK20 and pS10 *Sst*I-B. In each case the hybridization signals comigrated (data not shown). These experiments indicate that LM1 contained multiple copies of the pS10 sequence, which is composed of pMK20 plus a linked 9.8-kb *Eco*RI fragment.

Strain LM2 DNA contained a visible 12-kb *Eco*RI band that did not hybridize to pMK20 (Fig. 2A and B, *Eco*RI lane 7) and comigrated with the 12-kb *Eco*RI fragment of pS11 (not shown). *Hin*dIII digests of LM2 DNA contained two visible bands (7 and 9 kb) that hybridized to pMK20 but not to pS10 *Sst*I-B (Fig. 2, *Hin*dIII lane 7) and comigrated with the *Hin*dIII fragments of pS11 (not shown). LM2 DNA and pS11 were digested with *Nar*I, *Bgl*I, *Cla*I, and *Xba*I, electrophoresed, blotted, and probed with pMK20. In each case the hybridization signals comigrated (data not shown). These results indicate that LM2 contained multiple copies of pS11.

**A *D. radiodurans* transformant was the inverse of LM1.** A transformant (LM20) was isolated from a transformation that occurred in a tube separate from that from which LM1 and LM2 were isolated, and yet it appeared to be identical to LM1 (Fig. 2, cf. all lanes 6 and 8). Further analysis demonstrated that LM20 was different in that pMK20 was inverted with respect to the *D. radiodurans* *Eco*RI fragment that flanked it. CCC DNA from LM20 was used to transform *E. coli* DH5α, and six transformants were analyzed. All contained pS14, which was the same as pS10 except that the pMK20 sequences were inverted (Fig. 1).

**pS10 and pS11 were present in *D. radiodurans* transformants LM1 and LM2 as both tandem repeats in the chromosome and CCC DNA.** pMK20 hybridized to uncleaved LM1 and LM2 DNA at several places. Most of the hybridization occurred at the chromosomal DNA band where large linear DNA migrates (Fig. 2B, Uncut lanes 6 and 7). Thus, most of the pMK20 sequences in LM1 and LM2 were in large linear fragments similar in size to chromosomal fragments. Since these fragments were much larger than pS10 or pS11, and yet were cut to pS10- or pS11-sized fragments with *Hin*dIII (for pS10; Fig. 2B and C, *Hin*dIII lane 6) or *Cla*I (for pS11; not shown), it follows that they were arranged in tandem, since any other arrangement would have yielded multiple fragments of varying sizes (see model in Fig. 9). Similar observations were made for LM20 (and pS14), which in Fig. 2 is indistinguishable from LM1.

In uncleaved DNA, some pMK20 sequences were detected by hybridization as discrete bands between the wells and the chromosomal bands of LM1, LM2, and LM20, indicating that some pMK20 sequences were in the form of circles (Fig. 2B, Uncut lanes 6, 7, and 8).

To further investigate the nature of the CCC DNA forms seen in the uncleaved DNA lanes of Fig. 2B, CCC DNA was prepared from R1, LM1, and LM2 by isopycnic CsCl-ethidium bromide centrifugation (Fig. 3). Mackay et al. (15) have described plasmids in every *Deinococcus* strain examined except *D. radiodurans* R1. We found that a single plasmid (60 kb; pS16) was present in wild-type R1 and its derivatives. pS16 was isolated from R1 in low amounts (≤0.1 plasmid recovered per chromosome), and its size was estimated by the addition of restriction fragment sizes (not shown). CCC DNA from LM1 and LM2 contained pS16 and pS10 or pS11, respectively, in what appeared to be monomer and multimeric forms (Fig. 3, lanes 5 to 13). Note that the CCC DNA preparations are not entirely free of chromosomal DNA. Digests of LM1 CCC DNA contained *Eco*RI and *Hin*dIII fragments indistinguishable from those of pS10 by ethidium bromide staining and by hybridization with pMK20 and pS10 *Sst*I-B (Fig. 3, *Eco*RI lanes 1 and 2, *Hin*dIII lanes 1 and 2). Similarly, digests of LM2 CCC DNA contained *Eco*RI and *Hin*dIII fragments indistinguishable from those of pS11 as seen by ethidium bromide staining and hybridization with pMK20 (Fig. 3, *Eco*RI lanes 3 and 4, *Hin*dIII lanes 3 and 4). These results indicate that the CCC DNAs within *D. radiodurans* LM1 and LM2 are the same as pS10 and pS11.

**Transformation of R1 with LM1 and LM2 DNA.** *D. radiodurans* wild-type R1 was transformed with pS10 and pS11 (isolated from *E. coli* DH5α) as well as by genomic and CCC DNA from LM1 and LM2 (Table 2). DNA from all of these sources transformed *D. radiodurans* to $Km^r$ efficiently. A pS10 derivative, pS12, was constructed in which all but about 0.5 kb of the *D. radiodurans* chromosomal DNA was deleted (Fig. 1; Table 1). pS12 did not transform R1 to $Km^r$ at a detectable level (Table 2). Transformation of R1 to kanamycin resistance with LM2 genomic DNA or pS11 was reduced to levels below detection (<two transformants per μg of DNA) when the donor DNA was cleaved with *Eco*RI, *Cla*I, *Hin*dIII, *Mlu*I, *Bgl*II, or *Apa*I prior to transformation. Similar results were seen for LM1 and pS10, using *Cla*I, *Hin*dIII, *Acc*I, and *Sst*I.

pS11 consistently transformed at a higher frequency than pS10 (Table 2). Since R1 produces restriction endonuclease *Mra*I (29), these results suggest that the *Mra*I site in pS10 was affecting the frequency of transformation. The higher transforming activity of LM1 or LM2 DNA (genomic or CCC) when compared with pS10 and pS11 may reflect unknown host modifications or may be due to the multimeric nature of the LM1 or LM2 donor. The latter interpretation is supported by the observation that linearization of the donor, even in the region of homology with the recipient, abolished transformation.

**Copy number of pS10 in LM1.** As noted above (Fig. 2A and C), pS10 and pS11 sequences in LM1 and LM2 were present in multiple copies per chromosome. The degree of amplification in LM1 was quantitated by serially diluting *Eco*RI digests of LM1 DNA, electrophoresing the dilutions along with *Eco*RI digests of R1 and LM2 DNA, blotting the gel, and probing with pS10 *Sst*I-B (Fig. 4). The results showed equivalent hybridization with 25 ng of LM1 DNA

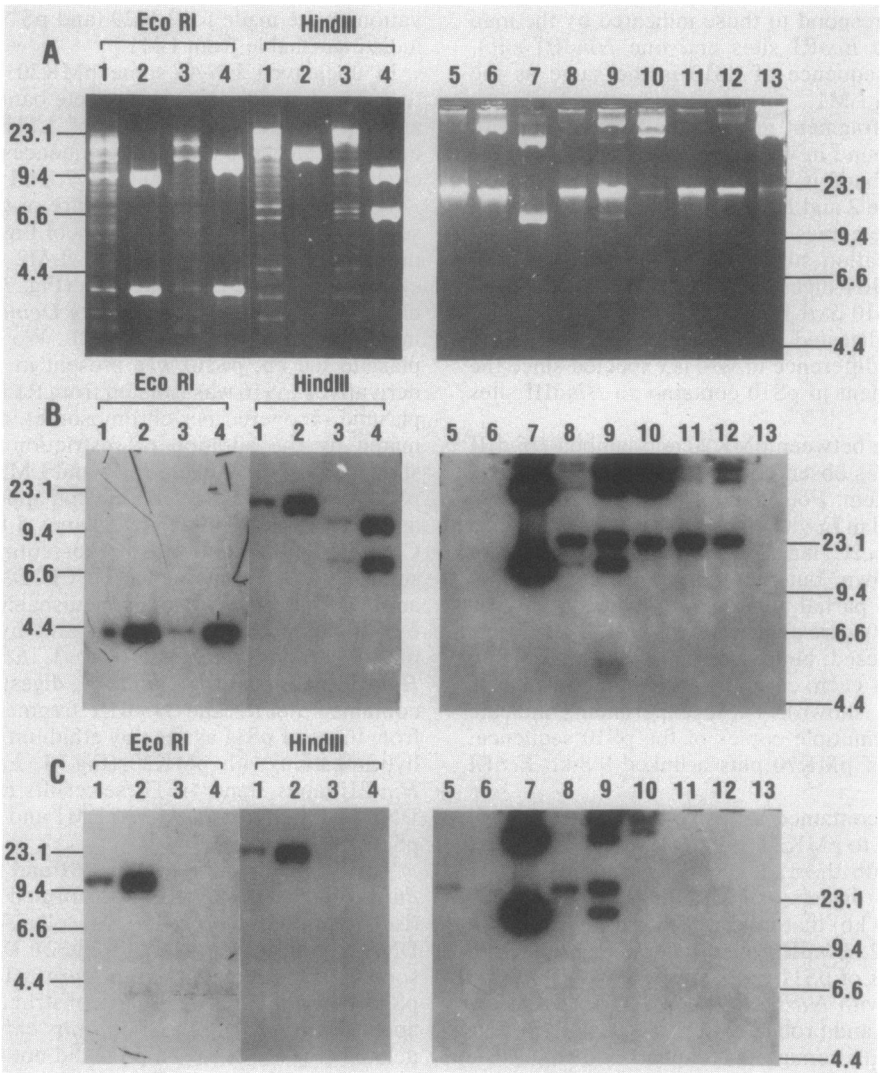


FIG. 3. CCC DNA from *D. radiodurans* LM1 and LM2 compared with pS10 and pS11 from *E. coli*. (A) Ethidium bromide stain. (B) Southern blot probed with pMK20. (C) Southern blot probed with pS10 *Sst*I-B. Lanes contain DNA digested with the indicated enzyme (left) or uncleaved (right): 1, LM1 CCC; 2, pS10; 3, LM2 CCC; 4, pS11; 5, R1 genomic; 6, R1 CCC; 7, pS10; 8, LM1 genomic; 9, LM1 CCC; 10, pS11; 11, LM2 genomic; 12, LM2 CCC; 13, RK2.

and 1,000 ng of R1 DNA, which indicated a 50-fold amplification. LM2 contained the fragment in the same unamplified copy number as R1. LM1 liquid cultures were grown for 10 generations in concentrations of kanamycin varying twofold from 100 to near 1 μg/ml and in the absence of drug. *Eco*RI digests of genomic DNA from each culture were electrophoresed, blotted, and probed with pMK20. High levels of gene amplification were detected in all cultures. There was no correlation between the amount of kanamycin added to the culture and the number of copies of pMK20 per cell (data not shown). The high level of amplification on LM1 was therefore stable over 10 generations without selection. When LM1 was grown in drug-free liquid culture for 20 generations, 40% of the CFU were kanamycin sensitive and so had evidently lost pMK20.

**Characterization of pEL1 transformant strains LM102, LM103, and LM104.** *D. radiodurans* R1 was transformed to chloramphenicol resistance ($Cm^r$) by a ligation mixture of *Bam*HI-cleaved pEL1 and *Bcl*I-cleaved *D. radiodurans* DNA from strain R1. Since the desired junction was *Bam*HI/*Bcl*I, no attempt was made to inactivate either

TABLE 2. Transformation of *D. radiodurans*

| Donor DNA[a] | Transformants per μg[b] |
|---|---|
| LM1 genomic | $8 \times 10^3$ $Km^r$ |
| LM1 CCC | $2 \times 10^5$ $Km^r$ |
| pS10 | $3 \times 10^2$ $Km^r$ |
| LM2 genomic | $3 \times 10^3$ $Km^r$ |
| LM2 CCC | $5 \times 10^4$ $Km^r$ |
| pS11 | $1 \times 10^4$ $Km^r$ |
| pS12 | <10 $Km^r$ |
| pEL2 | $4 \times 10^3$ $Cm^r$ |
| pEL3 | $4 \times 10^3$ $Cm^r$ |
| R1 KRASE (genomic) | $6 \times 10^5$ $Rif^r$ |
| | <10 $Km^r$ |
| | <1 $Cm^r$ |
| No DNA | <10/ml $Km^r$ |
| | <1/ml $Cm^r$ |

[a] Genomic, Unfractionated DNA from *D. radiodurans*; CCC, CCC DNA fraction from *D. radiodurans* strains, isolated on CsCl isopycnic gradient; pS10 to pS12, pEL2, and pEL3: CCC DNA fraction from *E. coli* transformants, isolated on CsCl isopycnic gradients.

[b] For LM1 CCC and LM2 CCC, 50 ng of donor DNA was used; for all others, 1 μg of donor DNA was used.

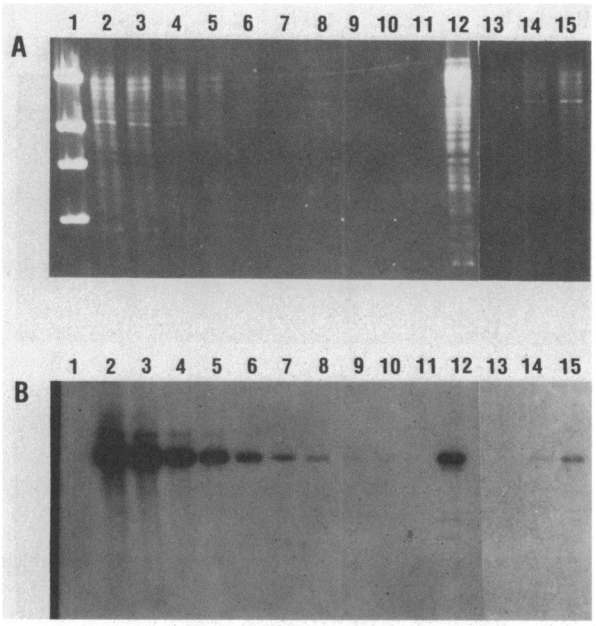


FIG. 4. Copy number of pS10 in LM1. (A) Ethidium bromide stain of *Eco*RI digests of genomic DNA of LM1, R1, and LM2 after agarose gel electrophoresis. (B) Southern blot probed with pS10 *Sst*I-B. Markers: lane 1, lambda *Hin*dIII digests (23.1, 9.4, 6.6, and 4.4 kb); 2, 200 ng of LM1; 3, 100 ng of LM1; 4, 50 ng of LM1; 5, 25 ng of LM1; 6, 12 ng of LM1; 7, 6 ng of LM1; 8, 3 ng of LM1; 9, 1.5 ng of LM1; 10, 0.8 ng of LM1; 11, 0.4 ng of LM1; 12, 1,000 ng of R1; 13, 25 ng of LM2; 14, 50 ng of LM2; 15, 100 ng of LM2.

*Bam*HI or *Bcl*I prior to ligation. This occasionally resulted in the deletion of the pEL1 *Bcl*I-*Bam*HI B fragment due to infrequent cutting of pEL1 with *Bcl*I (Fig. 5). Genomic DNA from 31 independent Cm$^r$ transformants was digested with *Eco*RI, electrophoresed, blotted, and probed with pEL1 (Fig. 6). *Eco*RI cleavage of genomic DNA should result in one major hybridizable fragment if there is no *Eco*RI site within the duplicated chromosomal segment, the only *Eco*RI site being that in pEL1. The size of such a fragment is equivalent to the length of the sequence which has undergone amplification (see model in Fig. 9). *Eco*RI digests of transformant DNA should contain two fragments that hybridize to pEL1 if there are any *Eco*RI sites in the duplicated chromosomal segment. Twenty-two transformants contained two major bands of hybridization, seven contained one major band of hybridization, and two contained multiple bands (Fig. 6). In most cases the locations of the bands that hybridized to pEL1 correlated with the locations of bright bands visible by ethidium bromide staining of the agarose gel (not shown), indicating the presence of amplification. Since the fragments were of various sizes, it appeared that the insertions were at different sites in the R1 chromosome. The transformants with multiple bands were not analyzed further.

**DNA from *D. radiodurans* Cm$^r$ transformants transformed *E. coli* to Cm$^r$.** DNA from LM102, LM103, and LM104 transformed *E. coli* DH5α to Cm$^r$. These *E. coli* transformants were found to contain pEL2, pEL3, and pEL4, respectively, which were mapped (Fig. 5). *E. coli* DH5α was transformed to Cm$^r$ at low frequency (1 to 20 transformants per μg) with genomic DNA from *D. radiodurans* transformant LM104, but at a high frequency ($10^4$ transformants per 50 ng) with the CCC DNA fraction from LM104. Similar results were found for the pMK20 derivative LM20.

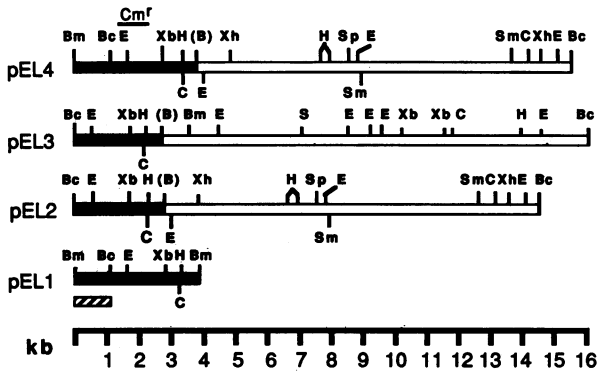


FIG. 5. Restriction maps of pEL1 and derivatives. pEL1 sequences (black boxes) contain the *cat* gene which confers chloramphenicol resistance (Cm$^r$). Other sequences are from *D. radiodurans*. The *Bcl*I site in pEL1 is infrequently cleaved when pEL1 is harvested from *E. coli* because the *Bcl*I recognition site is methylated (16). There are two *Pvu*II sites in pEL1 within 150 bases of each other and no *Xho*I sites. Note that the pEL1 *Bcl*I-*Bam*HI B fragment, designated by a striped box under pEL1, is missing in pEL2. Bc, *Bcl*I; Bm, *Bam*HI; (B), *Bam*HI/*Bcl*I fusion; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Xb, *Xba*I; Xh, *Xho*I.

**LM102 and LM103 contain multiple tandem insertions of pEL1 linked to a chromosomal segment.** R1, LM102 and LM103 genomic DNA, and pEL2 and pEL3 were digested with several restriction enzymes, electrophoresed, blotted, and probed with pEL1 (Fig. 7). For each restriction digest, LM103 DNA showed amplified bands on the ethidium bromide stain at locations corresponding to the restriction fragments of pEL3 (Fig. 7A, *Eco*RI lanes 2 and 3, *Ava*I lanes 2 and 3). Similarly, each restriction digest of LM102 DNA

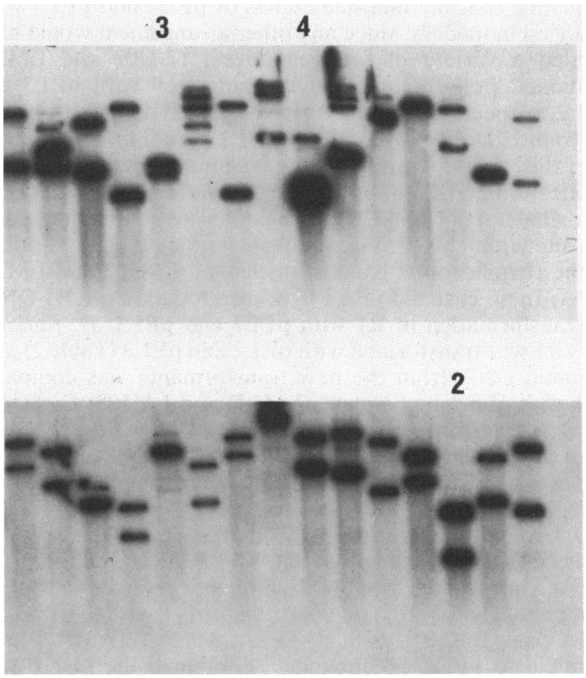


FIG. 6. Agarose gel electrophoresis of *Eco*RI digests of genomic DNA from several R1 × pEL1 transformants (see text). Southern blot probed with pEL1. Only three isolates are indicated: 2, LM102; 3, LM103; 4, LM104.

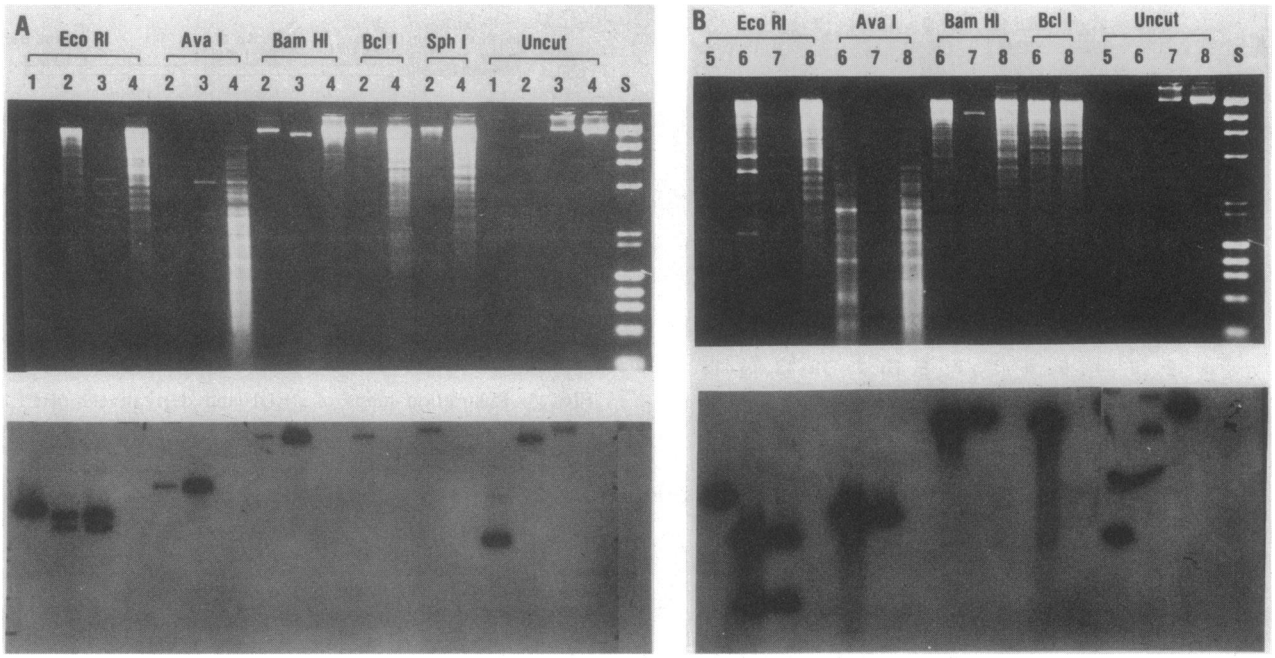


FIG. 7. Comparison of (A) pEL3 with genomic DNA from LM103 and (B) pEL2 with genomic DNA from LM102. (Top) Ethidium bromide stain of the indicated restriction digests after agarose gel electrophoresis. (Bottom) Southern blot probed with pEL1. Lanes: 1, pEL1; 2, LM103; 3, pEL3; 4, R1; 5, pEL1; 6, LM102; 7, pEL2; 8, R1; S, lambda *Hin*dIII and φX174 *Hae*III digests (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 1.4, 1.1, 0.87, 0.60, and 0.31 kb). Note that in panel A strain R1 (lanes 4) contain fourfold as much DNA as in strain LM103 (lanes 2).

showed amplified bands by ethidium bromide staining at locations corresponding to the restriction fragments of pEL2 (Fig. 7B, *Eco*RI lanes 6 and 7, *Ava*I lanes 6 and 7, *Bam*HI lanes 6 and 7). pEL1 hybridized to the same bands in LM103 DNA as in pEL3 and to the same bands in LM102 DNA as in pEL2 (Fig. 7A and B, *Eco*RI, *Ava*I, and *Bam*HI digests), indicating that the multiple copies of pEL2 and pEL3 were arranged in tandem, since any other arrangement would have yielded a variety of fragment sizes. LM103 and LM102 contained *Bcl*I fragments that comigrated with pEL3 and pEL2, respectively, and hybridized to pEL1 (Fig. 7A and B, *Bcl*I lane 2). Since pEL2 and pEL3 contain only one *Bcl*I site, this finding confirms the presence of multiple tandem duplications. pEL1 hybridized to *Sph*I-cleaved LM103 DNA at a single major band, a finding consistent with the lack of this site within pEL3 (Fig. 7A, *Sph*I lane α). pEL1 hybridized to the chromosomal band of uncleaved LM102 and LM103 DNA. In no case did pEL1 hybridize to wild-type R1 DNA.

**Transformation of R1 with pEL2 and pEL3.** *D. radiodurans* R1 was transformed with pEL2 and pEL3 (Table 2), and genomic DNA from the new transformants was compared with that of original isolates LM102 and LM103. Digests of the new transformants were probed with pEL2 or pEL3 and were indistinguishable from those of LM102 or LM103, respectively (Fig. 8). Similar results were found for pS10 and pS11 (not shown).

***D. radiodurans* transformant that contains the same *D. radiodurans* sequence as LM102.** The *Eco*RI genomic digest of one $Cm^r$ isolate, LM104, hybridized strongly with pEL2 probe (not shown). *E. coli* was transformed to $Cm^r$ with CCC DNA from LM104, yielding pEL4, which was found to be identical to pEL2 except that it contained the pEL1 *Bcl*I-*Bam*HI B fragment that was missing from pEL2 (Fig. 5). As was the case with LM1 and LM20, LM102 and LM104 came from separate transformation tubes.

**Demonstration of chromosomal location of pEL3 in LM103.** The data presented above do not exclude rigorously the possibility that pEL3 and related plasmids may not be integrated into the chromosome of R1 transformants, but instead exist as tandems that are stably inherited even after 10 generations in the absence of selection. A chromosomal restriction fragment in strain R1 should be missing from strain LM103 if pEL3 were really in the chromosome. This is demonstrated in Fig. 8E, in which an *Xho*I fragment of R1 which hybridizes to pEL3 (Fig. 8E, lane 7) is missing from an *Xho*I digest of LM103 (Fig. 8E, lane 8). Instead the LM103 *Xho*I species which hybridizes to pEL3 is much larger, which was expected since pEL3 has no *Xho*I site. Two "junction fragments" between pEL3 and flanking chromosomal DNA should be evident in small amounts if the restriction enzyme cleaves within pEL1 but not within the *D. radiodurans* segment of pEL3. This is demonstrated in Fig. 8E, in which a *Pvu*II digest of R1 DNA contains a single fragment that hybridizes to pEL3 (Fig. 8E, lane 7), but a *Pvu*II digest of LM103 has three hybridizing species (Fig. 8E, lane 8). The three species represent linear pEL3 (the darkest band) and two junction fragments. Superposition of the original autoradiogram over a photograph (of the same size) of the ethidium bromide-stained gel confirmed that the R1 species in lane 7 is in fact larger than the largest junction fragment in lane 8.

## DISCUSSION

We found that two *E. coli* plasmids, pMK20 and pEL1, may be inserted into the *D. radiodurans* chromosome if they are ligated to DNA sequences from the recipient prior to transformation and that the plasmid-encoded *aphA* and *cat* genes were expressed by *D. radiodurans* (Fig. 2 and 6). After integration, the plasmid plus flanking *D. radiodurans* sequences (termed the amplification unit [12]) were amplified, yielding tandem arrays of 30 to 50 amplification units within

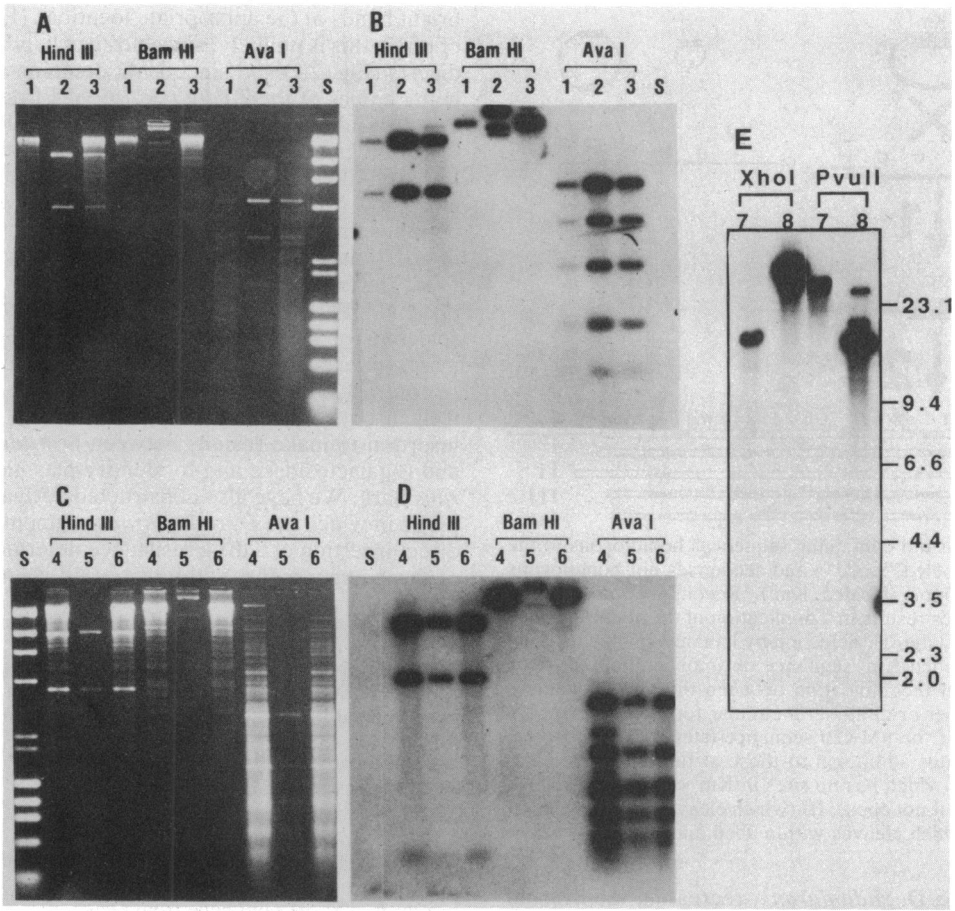


FIG. 8. Comparison of restriction digests of genomic DNA from LM103 with an R1 × pEL3 transformant and from LM102 with an R1 × pEL2 transformant. (A and C) Ethidium bromide stain of the indicated restriction digests after agarose gel electrophoresis. (B) Southern blot probed with pEL3. (D) Southern blot probed with pEL2. Lanes: 1, LM103; 2, pEL3; 3, R1 × pEL3 transformant; 4, LM102; 5, pEL2; 6, R1 × pEL2 transformant. (E) Linkage of pEL3 to chromosomal DNA in LM103. Autoradiogram of a Southern blot of a 0.5% agarose gel, probed with pEL3. Lanes: 7, R1 genomic DNA cleaved as indicated; 8, LM103 genomic DNA cleaved as indicated. Standards, in kilobases, are shown at the right.

the chromosome (Fig. 2, 4, 6, and 7). DNA from *D. radiodurans* transformants transformed *E. coli* to drug resistance. The plasmids from the *E. coli* transformants contained *D. radiodurans* sequences, together with pMK20 or pEL1, and were identical to the amplification unit within the chromosome of the parent *D. radiodurans* transformant (Fig. 2, 7, and 8). The CCC DNA within the *D. radiodurans* transformants was physically documented in the case of LM1, LM2, and LM20 by purification of the CCC DNA fraction from the transformants and direct comparison with pS10, pS11, and pS14 (Fig. 3; see text). The chromosomal location of an insertion was demonstrated in LM103 by hybridization to new restriction fragments created by the insertion of pEL3 into the chromosome. Finally, we found that plasmids pEL2 and pEL3 derived from the *E. coli* transformants (that is, identical to the amplification unit) transformed wild-type *D. radiodurans* to strains which were indistinguishable from the original parent *D. radiodurans* transformants (Fig. 8).

A model accounting for the above findings is shown in Fig. 9. The transforming construct contains the *E. coli* plasmid joined to a *D. radiodurans*-derived sequence. After uptake by *D. radiodurans*, a recipient-derived sequence contained within the chimeric donor plasmid permits homologous recombination with the host genome, resulting in integration of the *E. coli* plasmid sequence. This event results in a direct repeat of the recipient sequence flanking the nonhomologous *E. coli* plasmid (Fig. 9, step 1). This process in *D. radiodurans* resembles "duplication insertion," which has been described previously in *Bacillus subtilis* (8), *Streptococcus pneumoniae* (17, 24, 27), and *Saccharomyces cerevisiae* (11). We suggest that the requirement for the covalent linkage of the plasmid to host sequences prior to transformation is due to the inability of the *E. coli* plasmids to be replicated by *D. radiodurans*. In addition, the drug resistance marker may not be expressed unless linked to *D. radiodurans* sequences, or amplified, or both.

The *E. coli* plasmid sequence flanked by direct repeats resembles drug resistance determinants flanked by "recombination sequences" (26) and can amplify many times when selection is for expression of internal sequences (Fig. 9, step 2). This process can amount to >1% of total chromosomal DNA in *B. subtilis* (12). The amplified structure can generate CCC DNA identical to the original transforming plasmid (or a multimer of it) by intrachromosomal recombination between repeats (Fig. 9, step 3). The CCC DNA from the recipient transforms *E. coli* in which the plasmid can replicate (17, 24). Since the plasmid replicating in *E. coli* is identical to the amplification unit, containing both *E. coli* plasmid and *D. radiodurans* sequences, it may be used to

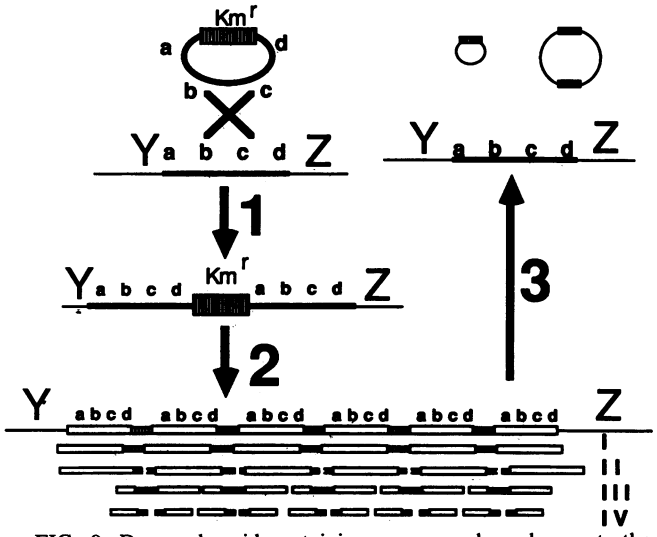


FIG. 9. Donor plasmid containing sequences homologous to the recipient genome (labeled "abcd") and sequences not homologous to the recipient genome (labeled Km$^r$). Recombination between homologous sequences results in a duplication of the abcd sequence on either side of Km$^r$ (step 1). Selection by kanamycin may result in amplification of the abcd-Km$^r$ sequence or amplification unit (step 2). Intrachromosomal recombination between the abcd sequences may produce monomer or multimeric circular forms of the amplification unit, or loss of the pMK20 sequence (step 3). Hypothetical restriction enzyme sites additional to those at the abcd-Km$^r$ junctions are shown as I (which has no sites in Km$^r$ or abcd), II (which cleaves within Km$^r$ but not abcd), III (which cleaves within abcd but not Km$^r$), and IV (which cleaves within abcd and Km$^r$).

transform wild-type *D. radiodurans*, recreating the original transformant. These observations have been made in different and more characterized systems, but have not all been made within a single species except now in the case of *D. radiodurans*. The data presented document the occurrence of duplication insertion in *D. radiodurans*, but we have also detected occasional transformants in which the amplification unit is the heterologous plasmid itself, without detectable flanking chromosomal sequences. Perhaps these variants came from donor plasmids which contained tandem copies of the heterologous plasmid as well as the *D. radiodurans* segment.

Amplification of the duplication structure in the presence of the selective agent produced up to 50 copies of the amplification unit per R1 chromosome. This amplified structure was stable for 10 generations, but loss of resistance could be detected after 20 generations. Fifty copies of pS10 per chromosome represents about 500 kb, or >10% of the chromosome, given that the R1 chromosome is about 3,000 kb (9, 28).

pS10 derived from strain LM1, while its inverted form, pS14, resulted from an independent transformant, LM20. Likewise, pEL4 and pEL2 were derived from independent transformants LM104 and LM102 (pEL4 contains the pEL1 *Bcl*I-*Bam*HI B fragment, while pEL2 does not) and contain the same *D. radiodurans* fragment. This nonrandom pattern is not due to escape from a restriction system, since the R1 DNA used in the initial transformation was already modified. Perhaps some *D. radiodurans* chromosomal fragments are repeated sequences and therefore more likely to be joined to pMK20 or pEL1 in the ligation mixtures. In the case of pEL2, this is possible, since the genomic digests of *Eco*RI- and *Ava*I-cleaved R1 DNA appear to contain relatively bright bands at the appropriate locations (Fig. 7). In the case of pS10, this is unlikely, since no such bands are visible in R1 digests (Fig. 2A) and since LM1 contains about 50 times as many copies of the 9.8-kb pS10 *Eco*RI fragment as R1 (Fig. 4). If R1 already contained five copies of the pS10 *Eco*RI 9.8-kb fragment per chromosome, then half of the genomic DNA of LM1 would have been this sequence. Instead, the efficiency of forming a healthy colony with an amplified chromosomal fragment may vary depending on the fragment in question.

We introduced the pUB110-based *cat* promotor probe plasmid pPL703 to strain R1 by duplication insertion by selecting for Km$^r$, but were unable to recover Cm$^r$ transformants with any of the restriction enzyme combinations used. In contrast, the *E. coli cat* promotor probe pKK223-8 works well in both R1 and Sark. We have also used duplication insertion to make fusions between *D. radiodurans* proteins and β-galactosidase and to identify plasmids in *D. radiodurans* Sark. We have also constructed derivatives of pS11 and pEL2 in which the *D. radiodurans* segment is interrupted by direct insertion of a drug resistance determinant. The results of these experiments will be reported elsewhere.

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