Gene Fusions with *lacZ* by Duplication Insertion in the Radioresistant Bacterium *Deinococcus radiodurans*

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Deinococcus radiodurans is the most-studied species of a eubacterial family characterized by extreme resistance to DNA damage. We have focused on developing molecular biological techniques to investigate the genetics of this organism. We report construction of *lacZ* gene fusions by a method involving both in vitro splicing and the natural transformation of *D. radiodurans*. Numerous fusion strains were identified by expression of β -galactosidase. Among these fusion strains, several were inducible by exposure to the DNA-damaging agent mitomycin C, and four of the inducible fusion constructs were cloned in *Escherichia coli*. Hybridization studies indicate that one of the damage-inducible genes contains a sequence reiterated throughout the *D. radiodurans* chromosome. Survival measurements show that two of the fusion strains have increased sensitivity to mitomycin C, suggesting that the fusions within these strains inactivate repair functions.

Species of the family *Deinococcaceae* share in common extreme resistance to both the lethal and mutagenic effects of ionizing and UV radiation and to most chemical agents that damage DNA (8, 17). Although repair of DNA damage by these organisms is known to be unusually efficient, the molecular mechanisms responsible remain largely unidentified (8). The family is not closely related to any bacterium whose genetics is well understood (1), and genetic techniques applicable to them are limited. The species most studied is *Deinococcus radiodurans*, since it is the only member of the family naturally transformable by homologous DNA, a property that has facilitated efforts at genetic characterization (8). However, characterization of this organism and the other *Deinococcaceae* has been limited by the lack of other applicable techniques, including conjugation, protoplast fusion, or transduction for introduction of genetic markers. In addition, there are no known transposons. Recently, we have identified heterologous drug resistance determinants capable of expression in *D. radiodurans* and succeeded in inserting these determinants into the *D. radiodurans* chromosome and into the resident cryptic plasmids by duplication insertion and direct insertion (13, 14). Extending these results, we have constructed several shuttle vectors capable of replicating in *D. radiodurans* and *Escherichia coli* (13, 15).

We now report the first translational gene fusions achieved in any member of the *Deinococcaceae*, using a truncated *lacZ* structural gene introduced by duplication insertion into the *D. radiodurans* chromosome. Expression of *lacZ* requires fusion with a *D. radiodurans* promoter as well as transcriptional and translational start sites. Random fusions with numerous transcribed genes were detected by virtue of *lacZ*-encoded β -galactosidase expression. Several fusions were found to be induced in the presence of the DNA-damaging agent mitomycin C (MMC), a finding of potential relevance to DNA repair in this resistant organism. From protein gel electrophoresis experiments, it is known that there are at least three proteins in *D. radiodurans* induced after MMC exposure (4). This study is the first effort to identify the genes encoding these functions.

The DNA damage-inducible fusion constructs were cloned from their corresponding fusion strains by a novel technique that exploits posttransformation events that we previously described in this organism (14; Fig. 1). Briefly, chromosomal integration of heterologous DNA (in this case, an *E. coli* cloning vector that contains a *lacZ* gene deleted of its promoter and its first seven codons) is accomplished by in vitro ligation of random *D. radiodurans* DNA fragments into the *E. coli* vector. Upon transformation, recombination of the *D. radiodurans* fragment in the vector with its homologous chromosomal sequence yields integration of the heterologous DNA, flanked by a direct repeat of the host sequence, referred to as duplication insertion. The duplicated host sequence is identical to the *D. radiodurans* sequence ligated in vitro into the *E. coli* vector before transformation (14). The *E. coli* plasmid sequence plus one of the flanking genomic *D. radiodurans* sequences (termed the amplification unit [AU]; 5) can undergo amplification in *D. radiodurans* in the presence of continued drug selection, producing up to 50 tandem repeats of the AU per *D. radiodurans* chromosome (14). The amplified chromosomal structure generates covalently closed circular (CCC) AUs identical to the original transforming plasmid (or a multimer of it), presumably by intrachromosomal recombination between repeats (14). The CCC AUs cannot replicate in *D. radiodurans* and are in low abundance but can be propagated in *E. coli* because of the presence of the original cloning vector sequence within the circular AU (14). Total DNA or CCC DNA from an amplification-containing *D. radiodurans* strain can readily transform *E. coli*, yielding the AU as an *E. coli* plasmid. Consequently, in the experiments described here, any gene fusion of interest is readily cloned in *E. coli*.

Mapping and hybridization studies suggested that the *lacZ* fusions were in different areas of the chromosome. Interestingly, one of the AUs from a *lacZ* fusion strain identified a sequence that was reiterated throughout the genome. In addition, we report the first application of a shuttle vector constructed from a resident plasmid in *D. radiodurans* Sark to determine whether the AU from a strongly inducible clone had all of the necessary signals to autonomously respond to MMC.

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FIG. 1. Protocol for generating *lacZ* gene fusions. The boxed section contains a schematic of relevant intracellular events (see text). (1) pRF *E. coli* fusion vectors were cleaved at the solitary *Bgl*II site located at the upstream end of the truncated *lacZ* gene and ligated with a partial *Sau*3AI digest of *D. radiodurans* DNA. (2) The ligation mixture was used to transform wild-type *D. radiodurans* Sark. (Box) Duplication insertion results in integration of the pRF vector, including the *aphA* kanamycin resistance determinant, flanked by a direct repeat of *D. radiodurans* sequence (*bcd*). Gene amplification yields up to 50 tandem copies of the AU per chromosome and, by recombination, circular AUs. (3) *D. radiodurans* is plated on kanamycin to select for *aphA*-containing transformants. (4) Individual *Km*^r transformants are tested for β -galactosidase expression in the absence and presence of MMC to identify fusion strains as described in Materials and Methods. (5) Genomic DNA, prepared from *D. radiodurans* fusion strains, is used to transform *E. coli* to *Km*^r. Circular AUs, unable to replicate in *D. radiodurans*, propagate as pRF derivatives in *E. coli*, permitting growth in large quantities and enabling characterization. The individual fusion construct plasmids transform wild-type *D. radiodurans*, reproducing the original fusion strain.

MATERIALS AND METHODS

Materials. Sources for most materials were described previously (14). Methylumbelliferyl- β -D-galactoside (MUG), methylumbelliferone (MU), 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-Gal), and MMC were from Sigma Chemical Co., St. Louis, Mo.

Bacterial strains and plasmids. Bacterial strains and plasmids are described in Table 1 and in Fig. 4 and 7. *D. radiodurans* Sark naturally contains two cryptic plasmids, pUE10 and pUE11 (Table 1). Strains carrying added plasmids are designated as strain/plasmid, for example, Sark/pS19. pS19, an *aphA*-containing derivative of pUE11, replaced pUE11 in Sark/pS19 (13). pEL16, a *cat*-containing derivative of pUE10, replaced pUE10 in Sark/pEL16.

Growth and selection. *D. radiodurans* was grown at 32°C in liquid TGY broth and plated on TGY agar as described elsewhere (14). *Escherichia coli* was grown in LB broth or LB agar at 37°C. For *D. radiodurans* Sark, selective drug

concentrations in agar were 5 μ g/ml for kanamycin sulfate and 3 μ g/ml for chloramphenicol; in broth, kanamycin at 3 μ g/ml was used. For *D. radiodurans* R1, kanamycin was added to 8 μ g/ml in agar, while other selective concentrations were the same as for strain Sark. For *E. coli*, selective broth and agar contained chloramphenicol (25 μ g/ml), kanamycin (50 μ g/ml), and/or ampicillin (50 μ g/ml).

DNA manipulations and characterization. Large-scale DNA isolation from *D. radiodurans* and large- and small-scale DNA isolation from *E. coli* were done as described previously (14). In addition, DNA was isolated from 5 ml of *D. radiodurans* cultures by treatment of cells with lysozyme, sodium dodecyl sulfate, and proteinase K, followed by isopropanol precipitation and overnight centrifugation in CsCl-ethidium bromide in a 100.3 rotor on a TL-100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). Southern blotting, hybridization, washing of blots, autoradiography, and removal of probe from blots were done as described previously (14) except that probe was purified by gel filtration on a Sephadex G-50 NICK-Column (Pharmacia, Uppsala, Sweden). Most hybridizations were done overnight at 37°C with 10⁶ cpm/ml in 50% formamide, and final washing of blots was at high stringency (65°C; 15 mM NaCl, 1.5 mM sodium citrate) for 1 h. Ligations of *D. radiodurans* DNA with pRF vectors were at DNA ratios of 1 μ g:2 μ g or 1 μ g:3 μ g, at 4°C for 18 h with T4 DNA ligase. Electrophoresis was done on horizontal 0.8% agarose gels for restriction-cleaved chromosomal DNA and on 1.0% gels for plasmid restriction mapping in Tris-acetate-EDTA buffer overnight at 1 V/cm.

Transformation. Transformation of *D. radiodurans* was done as previously reported (14). Briefly, 0.1 ml of competent bacteria (approximately 5×10^6 cells) was mixed with ≥ 1 μ g of DNA and incubated at 32°C for 90 min. The cells were diluted 10-fold in TGY, incubated for 3 to 5 h, and plated on selective medium. Primary selection of transformants was usually done by plating cells in 2.0 ml of kanamycin-free TGY top agar (0.7%) onto TGY bottom agar supplemented with kanamycin. *Km*^r transformants were observed at 2 to 5 days after plating. *E. coli* transformations were done by the CaCl₂ method.

Screening for β -galactosidase expression. Wild-type *D. radiodurans* does not express β -galactosidase activity (1). *Km*^r *D. radiodurans* transformants were screened for the expression of β -galactosidase and its increased production in the presence of MMC. Individual transformants were grown overnight in TGY broth supplemented with kanamycin (3 μ g/ml) in 96-well plates on a rotating shaker at 32°C. Samples of 5 μ l were used to inoculate wells in fresh 96-well plates containing 100 μ l of TGY per well with MUG at 50 μ g/ml plus MMC at 0, 0.0125, 0.025, or 0.05 μ g/ml. MUG is cleaved by β -galactosidase to fluorescent MU (19). Plates were incubated overnight at 32°C on a rotating shaker and examined on a transilluminator (Fotodyne, Inc., New Berlin, Wis.) for fluorescence, indicating a productive *lacZ* fusion. At the same time, isolates that produced higher levels of β -galactosidase when grown in the presence of MMC were identified. After overnight growth, each well contained approximately 10⁶ bacteria, monitored by optical density at 600 nm on a Titertek Multiskan MC (Flow Laboratories, Inc., McLean, Va.). To quantify the amount of fluorescent MU generated by cleavage of MUG by *D. radiodurans* isolates, a standard series composed of twofold dilutions of MU in TGY was added to the 96-well assay plates. The fluorescence intensity produced by MU in each experimental well was compared with that of the standard dilution series. In control experiments, it was determined that large amounts

TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid	Description ^b	Source or reference ^c
Strains		
<i>D. radiodurans</i>		
Sark	Wild type (pUE10, pUE11)	1; Murray and Robinow ^d
L105	SarkΩpEL5	Sark × pRF1 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
L107	SarkΩpEL14	Sark × pRF1 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
L108	SarkΩpEL20	Sark × pRF1 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
L109	SarkΩpEL21	Sark × pRF1 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
L110	SarkΩpRF3 ^e	Sark × pRF3 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
L114	SarkΩpEL17	Sark × pRF1 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
L119	SarkΩpRF1 ^e	Sark × pRF1 <i>Bgl</i> II::Sark <i>Sau</i> 3AI
<i>E. coli</i> DH5α	<i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁻) F ⁻ φ80d <i>lacZ</i> ΔM15	Bethesda Research Laboratories
Plasmids		
pRF1, pRF2, pRF3 ^f	<i>lac</i> 'Z <i>aph</i> A	10
pS19	pUE11::pMK20 <i>Eco</i> RV-A <i>aph</i> A	13
pEL5	pRF1::Sark	DH5α × L105
pEL17	pRF1::Sark	DH5α × L114
pEL14	pRF1::Sark	DH5α × L107
pEL20	pRF1::Sark	DH5α × L108
pEL16	pEL5 <i>Sal</i> I::pS31 <i>Sal</i> I, 31 kb	In vitro ligation
pEL21	pRF1::Sark	DH5α × L109
pS31	pUE10-derived shuttle vector; Cm ^r Ap ^r ; 20 kb	15

^a Transformations are noted as recipient × donor. Donor preparations containing ligase-treated mixtures of pRF plasmids (cleaved with *Bgl*II) and Sark DNA (incomplete digest with *Sau*3AI) are shown as pRF *Bgl*II::Sark *Sau*3AI.

^b Wild-type *D. radiodurans* Sark naturally contains pUE10 and pUE11. Several strains may be thought of as having insertions of a plasmid that contains both chromosomal and heterologous sequences and are so designated in accordance with convention (9). For instance, *D. radiodurans* Sark derivative L105 has an insertion of pEL5 and so is described as SarkΩpEL5. Cm^r, Chloramphenicol resistant; Ap^r, ampicillin resistant; *aph*A, expresses kanamycin resistance.

^c Strains or plasmids for which a person or reference is not noted were constructed during this study.

^d R. G. E. Murray and C. F. Robinow, Abstr. VII Interim Congr. Microbiol., p. 427, 1958.

^e The Sark DNA-pRF fusion was not molecularly cloned from strain L110 and L119. In L110, the AU consisted solely of pRF3. See text for details.

^f Contains a *lacZ* gene deleted of its promoter and first seven codons (*lac*'Z) (2, 3). Unique *Bgl*II sites are present adjacent to the eighth codon. pRF1, pRF2, and pRF3 differ by one or two base pairs between the *Bgl*II site and the eighth codon, providing all three reading frames.

of *D. radiodurans* added to the standard dilution series of MU had no observable effect on fluorescence intensity. In preliminary experiments, β-galactosidase activity was screened with the chromogenic indicator substrate X-Gal; however, the red pigmentation of *D. radiodurans* obscured the blue color imparted by the cleaved substrate, making identification and quantitation difficult.

Survival after MMC exposure. Sark fusion strains and Sark/pS19 (pS19 confers Km^r) were grown to exponential phase in TGY with kanamycin (3 μg/ml). Cultures were centrifuged, and the pellets were suspended in 0.9% NaCl at an optical density (600 nm) of 1.0. MMC was added to a concentration of 10 μg/ml, and samples were withdrawn at specific intervals, diluted, and plated on TGY supplemented with kanamycin at 5 μg/ml.

RESULTS

Production of *lacZ* gene fusions by duplication insertion. The protocol for generating *lacZ* fusion strains and cloning the *lacZ* fusion constructs for these strains as well as a schematic diagram of relevant intracellular events are shown in Fig. 1. Total DNA from *D. radiodurans* Sark was partially digested with *Sau*3AI and ligated in vitro with the three pRF reading frame vectors at the unique *Bgl*II site adjacent to the eighth codon of the *lac*'Z gene. Transformation of *D. radiodurans* Sark yielded a total 1,225 Km^r isolates: 699, 332, and 194 from ligations with pRF1, pRF2, and pRF3, respectively. A total of 810 transformants were tested for expression of β-galactosidase. Of these, 165 (82 of 486 SarkΩpRF1, 5 of 35 SarkΩpRF2, and 78 of 289 SarkΩpRF3) expressed β-galactosidase, as determined by fluorescence assay using

MUG as described in Materials and Methods. Several strains showed increased β-galactosidase expression when exposed to MMC, with highly reproducible inductions from 3- to 30-fold in the presence of 0.05 μg of MMC per ml (Fig. 2). Transformation of *D. radiodurans* R1 by using the pRF reading frame vectors ligated to strain R1 chromosomal DNA produced no Km^r transformants, in contrast to the results for strain Sark. There are two *Mra*I (isoschizomer of *Sst*II) sites in the pRF vectors (Fig. 3). Apparently, the *Mra*I restriction system in *D. radiodurans* R1 (18) precluded transformation of these constructs at detectable levels.

Gene fusions in transformant strains are amplified. Genomic DNA from MMC-inducible fusion strains was cleaved

FIG. 2. Induction by MMC of increased β-galactosidase in five fusion strains. β-Galactosidase was measured by the production of MU at various concentrations of MMC as described in Materials and Methods. The strains assayed were L105 (■), L110 (△), L109 (○), L107 (□), and L108 (▲).

FIG. 3. Fusion constructs from *D. radiodurans* fusion strains with MMC-inducible phenotype. Plasmids were isolated from *E. coli* transformed with DNA from *D. radiodurans* fusion strains: pEL5 from strain L105, pEL14 from strain L107, pEL20 from strain L108, and pEL21 from strain L109. $\square$, *D. radiodurans* DNA. The complete plasmid, including pRF and the *D. radiodurans* fragment, is shown only for pEL5. Arrows indicate direction of transcription. Restriction sites: Ba, *Bam*HI; B, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; N, *Nru*I; P, *Pvu*II; Ps, *Pst*I; RV, *Eco*RV; S, *Sal*I; Sa, *Sau*3AI; Sm, *Sma*I; Ss, *Sst*II; St, *Stu*I.

with *Eco*RI and resolved by agarose gel electrophoresis. A faint single new band was visible in the ethidium bromide-stained gel in each fusion strain, representing the amplified duplication insertions, as expected, since the pRF vectors contain only a single *Eco*RI restriction site and the *D. radiodurans* portions of these AUs do not contain *Eco*RI sites (Fig. 3). The AUs are clearly seen in a Southern blot of this gel probed with pRF (Fig. 4). In accordance with prior findings (14), the size of each new band in the fusion strain was the same length as the circular AUs recovered by propagation in *E. coli* (cf. Fig. 3 and 4). The lighter bands detectable in some lanes of Fig. 4 are probably junction fragments, their lengths being the distance from the most upstream and downstream *Eco*RI sites within the amplified region of the chromosome to the nearest *Eco*RI sites in flanking nonamplified chromosomal DNA (14). The junction

FIG. 4. Southern blot of *Eco*RI-cleaved total DNA from *D. radiodurans* fusion strains. The Southern blot of the indicated fusion strains was hybridized with pRF1, highlighting the amplified bands. Wild-type Sark has no detectable homology with pRF.

FIG. 5. Southern blot of *D. radiodurans* Sark wild-type DNA cleaved with *Eco*RI or *Stu*I (first and second lanes, respectively, of each panel). Blots were probed with 32 P-labeled pRF1 (A), pEL5 (B), pEL14 (C), pEL20 (D), and pEL21 (E).

fragments are faint or inapparent because they are in single copy per chromosome, in contrast to the many copies of the AU (14).

Unlike the other fusion strains, L110 showed an amplified band that was the same size as the linearized pRF vector itself (Fig. 4). As expected, the circular AU recovered by propagation in *E. coli* was the pRF vector without any *D. radiodurans*-derived insert. This observation is compatible with an original in vitro ligation which included two head-to-tail tandem copies of pRF3, joined at the *Bgl*II cleavage site, plus a *D. radiodurans* genomic *Sau*3AI fragment. In this case, the tandem duplication of pRF underwent amplification, rather than the duplicated flanking *D. radiodurans* chromosomal sequences. We previously observed a similar phenomenon when the *cat*-containing Tn9 was introduced into *D. radiodurans* R1 by duplication insertion. Although no transposition of Tn9 was observed, under chloramphenicol selection there was extensive chromosomal amplification of Tn9. However, the amplification occurred via the two IS1 direct repeats at each end of Tn9, without amplification of the *D. radiodurans* DNA flanking repeats generated by the original duplication insertion. Similar to the present case, circular amplification units consisted of monomeric and multimeric forms of Tn9, without *D. radiodurans* sequences (M. Smith and K. Minton, unpublished observations).

Characterization of the fusion constructs isolated from fusion strains. *D. radiodurans* DNA was used to transform *E. coli* to *Km*^r, and the resulting plasmid DNA was mapped (Fig. 3). The *D. radiodurans* inserts were 1.5 kilobases (kb) in pEL5, 350 bases in pEL14, 5.5 kb in pEL20, and 420 bases in pEL21, and each contained different restriction sites. A Southern blot of wild-type *D. radiodurans* Sark chromosomal DNA, cut with either *Eco*RI or *Stu*I and probed with each of the above-named plasmids, showed hybridization to different chromosomal fragments (Fig. 5). Since pEL5 (Fig. 5B) has a *Stu*I site in the *D. radiodurans* DNA insert, there was hybridization to two chromosomal bands with this plasmid. Plasmid pEL14 (Fig. 5C) strongly hybridized to a single band in both the *Eco*RI and *Stu*I digests. However, there was some minor hybridization to two bands of about

FIG. 6. Survival of fusion strains after MMC exposure. Shown are data for the surviving fraction after exposure to MMC (10 μ g/ml) for the indicated times, measured by dilution plate count. The strains assayed were Sark/pS19 (○), L105 (■), L107 (▲), L108 (+), L109 (●), L110 (▽), L114 (△), and L119 (□).

5.0 kb in the *Eco*RI digest. Plasmid pEL20 (Fig. 5D), which contained a 5.5-kb insert, showed cross-hybridization with several bands in the chromosomal digests. pEL21, which contains a 420-base insert, hybridized extensively with the genomic DNA (Fig. 5E), suggesting the presence of a reiterated sequence. Since no means of mapping the chromosome of any member of the eubacterial family *Deinococcaceae* has been developed, it was not possible to assign these genes to specific chromosomal loci.

E. coli DH5 α contains a *lacZ* deletion and does not express β -galactosidase (Table 1). DH5 α /pEL21 clearly expressed β -galactosidase, as determined by MUG and chromogenic X-Gal assays. DH5 α /pEL5 and DH5 α /pEL20 expressed β -galactosidase at barely detectable levels, whereas DH5 α /pEL14 did not express β -galactosidase. Expression of β -galactosidase by these plasmids in *E. coli* was not induced by MMC (data not shown).

Wild-type *D. radiodurans* was transformed to MMC-inducible phenotype with plasmids isolated from *E. coli*. Plasmids pEL5, pEL14, pEL20, and pEL21 were used to transform wild-type *D. radiodurans* strain Sark to Km^r. Transformation of wild-type *D. radiodurans* Sark produced Km^r isolates identical to the original fusion strains with respect to noninduced and MMC-induced β -galactosidase expression. Furthermore, isolates of circular AUs by transformation of *E. coli* with DNA from these second-round transformants yield the same plasmids: pEL5, pEL14, pEL20, and pEL21. These results are similar to those previously described with respect to insertion of drug resistance determinants (14).

MMC survival. If the MMC-inducible genes are necessary for resistance to damage caused by MMC, duplication insertion of heterologous DNA within these genes may be deleterious (Fig. 6). After MMC exposure, the MMC-inducible fusion strains L105, L107, and L109 did not significantly differ in survival from wild-type Sark and two other strains that demonstrated constitutive noninducible expression of β -galactosidase, L114 and L119. Two MMC-inducible strains, L108 and L110, were less resistant to MMC: both showed survival 2 orders of magnitude less than that the other strains after MMC exposure of 20 min (Fig. 6). The normal MMC resistance of the inducible fusion strains L105, L107, and L109 does not preclude a role for the corresponding genes in MMC resistance. The insertions within these genes do not necessarily cause mutations, since the *D. radiodurans* flanking DNA is duplicated, or the fusion protein in these strains may retain its normal activity.

The gene fusion in pEL5 does not express β -galactosidase if

FIG. 7. Restriction map of pEL16. Portions of pEL16 from pEL5 and the shuttle vector pS31 are indicated. Arrows indicate direction of transcription of *cat*, *aphA*, and *lacZ* genes. pEL16 contains six *Cla*I sites, three of which (*) do not cut in pEL16 harvested from *dam*⁺ *E. coli* because of methylation. However, all *Cla*I sites are cut when pEL16 is harvested from *D. radiodurans*, in which no methylated bases have been observed (12, 16). Restriction sites are as in Fig. 3.

maintained as plasmid in *D. radiodurans*. *D. radiodurans* containing pEL5 integrated into the chromosome expresses β -galactosidase at a low constitutive level and in the presence of MMC at a high induced level (Fig. 2). The shuttle vector pS31 is composed of the promoter selection vector pKK232-8, which contains a full-length *amp* gene and a promoterless *cat* gene, ligated to a portion of pUE10 (15). pS31 retains the pUE10 origin of replication and pUE10 sequences that promote expression of the *cat* gene. pS31 replicates in *D. radiodurans* Sark and R1, where it confers chloramphenicol resistance, and in *E. coli*, where it confers ampicillin resistance (Table 1; 15). pS31 was ligated in vitro with pEL5 at their unique *Sal*I sites, generating pEL16 (Fig. 7), which contained the full length of both pS31 and pEL5. pEL16 replicated as plasmid in both *D. radiodurans* Sark and *E. coli* but could not be introduced into *D. radiodurans* R1, presumably because of the two *Mra*I sites in the pEL5 portion of pEL16, as previously discussed.

When pEL5 was maintained as plasmid (as part of pEL16) in *D. radiodurans* by either chloramphenicol or kanamycin selection, β -galactosidase was not expressed, either constitutively or in the presence of MMC, indicating that all requisite sequences for expression are not within the 1.5-kb insert in pEL5. Southern blot analysis of total DNA from Sark/pEL16 showed only the expected fragments for the plasmid, without evidence of plasmid integration into the chromosome (not shown). Four isolates that did express β -galactosidase were all inducible by MMC. These four isolates were characterized by Southern blotting and hybridization studies. In all four, it was found that various fragments of pEL16 had integrated into the Sark chromosome, always including the pEL5 β -galactosidase fusion portion of pEL16. In these isolates there were major deletions of the pS31 portion of the integrated plasmid (data not shown). Since the pS31 portion of the shuttle vector includes the origin of replication for Sark plasmid pUE10, we speculate that chromosomal integration may be restricted by endogenous mechanism(s). An alternative explanation is that the large size of pEL16 was incompatible with full-length integration.

DISCUSSION

Members of the family *Deinococcaceae* are the most radiation-resistant organisms discovered to date (8). Despite this property, there has been little investigation of this family

(8). The primary hindrance has been lack of microbial genetic techniques applicable to these organisms. Our *lac*'Z fusion strategy exploited prior work from our laboratory demonstrating the introduction of drug resistance determinants into *D. radiodurans* by duplication insertion (14). In the study described here, this capability was extended to yield *lac*'Z gene fusions by ligating *D. radiodurans* DNA in vitro to the reporter *lac*'Z gene in an *E. coli* plasmid that also contained the *aphA* kanamycin resistance determinant. After duplication insertion and amplification in *D. radiodurans*, the Km^r transformants that produced β -galactosidase were studied further, and fusion strains that have increased β -galactosidase expression when exposed to MMC were identified. An asset of this duplication insertion strategy is the presence of CCC AUs in *D. radiodurans* that allow rapid cloning of the fusion construct in *E. coli*. Plasmid DNA isolated from *E. coli* included the *D. radiodurans* sequence of interest, as it formed part of the AU. Transformation of wild-type *D. radiodurans* with these plasmids reproduced the original transformation event, yielding *D. radiodurans* of identical phenotype and containing the identical CCC AU as the original transformant. This technique for creating and cloning fusion constructs does not require transposable elements or shuttle vectors.

Hybridization of the cloned *D. radiodurans* DNA against wild-type chromosomal *D. radiodurans* digests showed varied patterns, including one sequence that appeared to be repeated many times throughout the chromosome (pEL21). Two of the five fusion strains demonstrated reduced resistance to the lethal effects of MMC exposure, suggesting that the fusions in these isolates may have impaired the function of these loci. It is also possible that the fusion protein is itself dominant to the wild-type product and deleterious to survival.

About 20% of all Km^r transformants expressed β -galactosidase, a high frequency for a translational gene fusion, considering six possible reading frames and the presumed presence of at least some untranscribed sequences in *D. radiodurans*. This high frequency may be partially explained by the likelihood that Km^r transformants are only those in which the fusion vector has inserted downstream of *D. radiodurans* promoter sequences. We have shown that the *aphA* gene is not well expressed from its own promoter in *D. radiodurans* and apparently requires upstream *D. radiodurans* sequences for expression (13). Therefore, Km^r expression may be biased toward duplication insertions in which the *lac*'Z gene is transcribed, facilitating downstream readthrough of the *aphA* determinant.

Most fusion strains expressing β -galactosidase were derived from the reading frame fusion vectors pRF1 and pRF3 (Table 1). Ligations with pRF2 resulted in only rare Km^r transformants; the reason for this low efficiency was not investigated. The most successful transformation experiment using pRF3 generated several Km^r isolates that expressed β -galactosidase in an MMC-inducible fashion. However, they were similar to strain L110 in that the AU consisted solely of pRF3 (data not shown), suggesting that the initial transformation was with a random ligation construction containing two tandem pRF3 sequences. The fusion constructs in these strains were not readily clonable in *E. coli* since the *D. radiodurans* CCC AU was the pRF3 vector itself. Other strategies will need to be used to obtain the fusion constructs from these strains.

To determine whether requisite signals for expression were present on pEL5 (the fusion construct from the most highly inducible fusion strain), it was ligated with an auton-

omously replicating *D. radiodurans*-*E. coli* shuttle vector. When the resulting plasmid, pEL16, was maintained as an autonomously replicating element, β -galactosidase expression did not occur in *D. radiodurans* (or *E. coli*). Only rare isolates in which pEL5 fusion-containing fragments of pEL16 had integrated into the chromosome showed the characteristic phenotype of low-level constitutive β -galactosidase expression induced to a high level of expression after MMC exposure. These observations suggest that the MMC-inducible gene was only partially cloned in pEL5, possibly because it is a long gene or part of an operon. Another possibility is that requisite *cis*-acting sequences for control of gene expression (promoter or otherwise) are upstream of the 1.5-kb chromosomal fragment cloned within pEL5.

DNA damage-inducible gene expression plays an important role in the tolerance and repair of DNA damage by a variety of other organisms, including *E. coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* (6, 7, 11). If DNA repair mechanisms used by these relatively DNA damage-sensitive organisms are relevant to our understanding of DNA repair in the *Deinococcaceae*, it is likely that DNA damage-induced genes, such as those detected in this study, will play a significant role.

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