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Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments

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We have developed a radiation resistant bacterium for the treatment of mixed radioactive wastes containing ionic mercury. The high cost of remediating radioactive waste sites from nuclear weapons production has stimulated the development of bioremediation strategies using *Deinococcus radiodurans*, the most radiation resistant organism known. As a frequent constituent of these sites is the highly toxic ionic mercury (Hg) (II), we have generated several *D. radiodurans* strains expressing the cloned Hg (II) resistance gene (*merA)* from *Escherichia coli* strain BL308. We designed four different expression vectors for this purpose, and compared the relative advantages of each. The strains were shown to grow in the presence of both radiation and ionic mercury at concentrations well above those found in radioactive waste sites, and to effectively reduce Hg (II) to the less toxic volatile elemental mercury. We also demonstrated that different gene clusters could be used to engineer *D. radiodurans* for treatment of mixed radioactive wastes by developing a strain to detoxify both mercury and toluene. These expression systems could provide models to guide future *D. radiodurans* engineering efforts aimed at integrating several remediation functions into a single host.

Key words: mercuric reductase, bioremediation

Most of the wastes generated as a result of global nuclear weapons production between 1945 and 1986 were discharged to the ground and are now contaminating the subsurface at thousands of sites. In the United States, about one third of the reported 3,000 waste sites are radioactive, with radiation levels as high as 10 mCi/L, in or close to the contaminating sources[1]. It is estimated that these leaking buried wastes (3×10^6 m^3) have contaminated 7.5×10^7 m^3 of surface and subsurface soils and about 2×10^{12} dm^3 of groundwater[2]. These highly toxic wastes contain inorganic and organic contaminants that include radionuclides such as ^{235}Uranium $[\gamma, \alpha]^E$, heavy metals such as mercury, and solvents such as toluene[1].

The clean-up cost of these waste sites, by physicochemical methods, was estimated recently to be about $265 billion[3,4]. These sites, therefore, represent targets for less expensive bioremediation technologies using specialized microorganisms that can remediate both metallic and organic contaminants. The development of microbiological methods for the treatment of highly radioactive waste will largely be determined by the ability of microorganisms catalyzing the desired function(s) to survive and function under radiation stress.

Numerous microorganisms (including *Shewanella*, *Geobacter,* and *Pseudomonas* spp.) have been studied in detail for their ability to detoxify or immobilize a variety of metallic pollutants[5–12]. Generally, microorganisms are sensitive to the damaging effects of ionizing radiation, and most of the bacteria[10–13] and plants[14,15] currently being studied as candidates for bioremediation are no exception. Therefore, radiation resistant organisms that can remediate toxic metals, the most common contaminants at Department of Energy (DOE) sites, are needed to address this problem.

The most radiation resistant organism yet discovered is the bacterium *Deinococcus radiodurans*[16–18], and its genome (strain R1) has recently been sequenced and annotated[19]; it consists of 4–10 identical copies[20] of a chromosome (2.65 Mbp), two megaplasmids (412 and 177 kbp), and a plasmid (46 kbp). *D. radiodurans* is a nonpathogenic, solvent tolerant[21], soil bacterium that can grow continuously in the presence of 60 Gy/h (a dose rate that exceeds those in most radioactive waste sites[1]) with no effect on either its growth rate or its ability to express foreign genes[21].

The ability of a microorganism to resist the toxic effect of metals is frequently associated with its ability to transform those metals to less soluble and less toxic chemical states. Cloning metal resistance genes into *D. radiodurans*, therefore, would serve two important objectives: (1) it would confer resistance to the most common metallic waste constituents; and (2) it would transform those metals to less toxic and less soluble chemical forms. For example, the bacterial mercuric reductase gene, *merA*, encodes mercuric ion reductase (MerA), that reduces highly toxic, thiol-reactive mercuric ion, Hg (II), to much less toxic and nearly inert monoatomic volatile Hg (0)[22, 23]. Of the mercury contaminants in DOE wastes, free Hg (II) ions predominate[1,2]; there are hundreds of radioactive DOE waste sites contaminated with Hg (II)[1].

To demonstrate the applicability of the strategy to confer both metal resistance and metal remediating capabilities on a radiation resistant bacterium, we cloned the highly characterized *merA* locus from the *E. coli* strain BL308 (*E. coli* K12 containing pDB7)[24] into *D. radiodurans*. To illustrate the feasibility of strategies for combining different gene-encoded functions into a single host, we combined an organic toxin degrading function[21] into a mercury trans-

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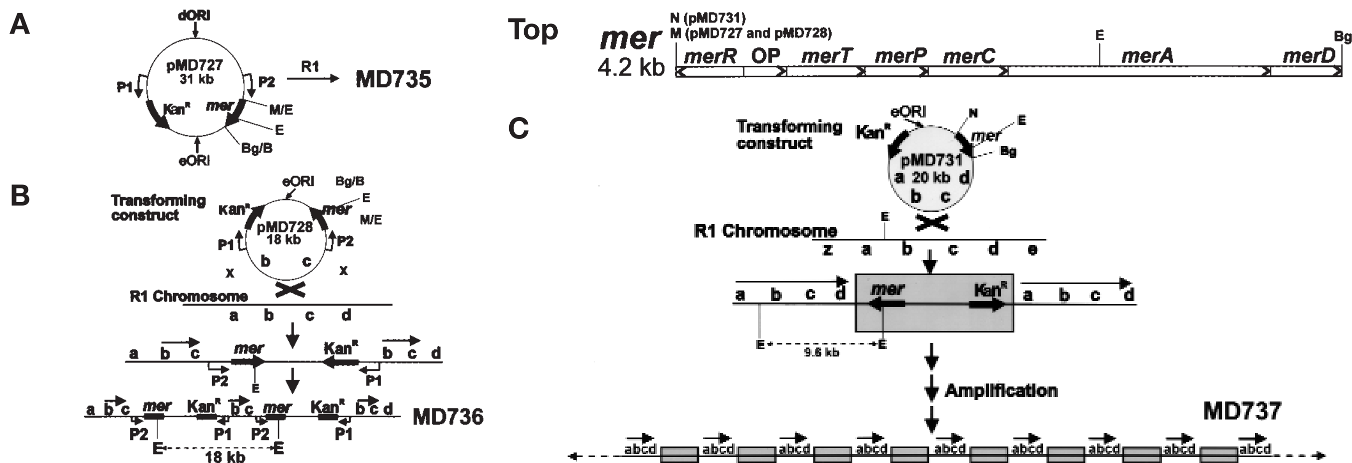

Figure 1. Plasmid and chromosomal maps. (A) 4.2-kb *mer* operon of pBD7[24] encodes six proteins: MerR, activation/repression of the *mer* operon; MerT, mercuric ion transport protein; MerP, periplasmic mercuric ion binding protein; MerC, transmembrane protein; MerA, mercuric reductase; and MerD, putative secondary regulatory protein. OP, operator/ promoter sequence; M, *Mfe*I; N, *Nco*I; E, *EcoR*I; Bg, *Bgl*II. (A) pMD727 was transformed into *D. radiodurans* strain R1 by selection with kanamycin (Kan), giving MD735. dORI, deinococcal origin of replication[18]; eORI, *E. coli* origin of replication[18]. P1 and P2 are two different constitutive deinococcal promoters[21,25]. Kan^R, kanamycin resistance gene *aphA*; *mer*, mercury operon. Bg/B, *Bgl*II/*BamH*I fusion; M/E, *Mfe*I/*EcoR*I fusion. (B) pMD728 was transformed into strain R1 with *Km* selection, giving MD736. Two rounds of recombinative duplication are illustrated, yielding two vector copies on a chromosome. bc, duplicated chromosomal target sequence; X, *Xba*1; all other abbreviations and symbols, as in A. (C) pMD731 was transformed into strain R1 with *Km* selection, giving MD737. Several rounds of recombinative duplication are illustrated, yielding many insertions per chromosome. abcd, duplicated chromosomal target sequence; all other abbreviations and symbols, as in A and B above.

forming *D. radiodurans* strain, generating a strain that expresses both functions.

Results

Construction and characterization of Hg (II) resistant *D. radiodurans* strains. The cloned *mer* operon encodes six proteins that confer mercury resistance functions on *E. coli*[22,23] (Fig. 1, Top). This operon was cloned into strains on autonomous plasmids or recombined into the chromosome as previously described[18,21]. The entire *D. radiodurans* genomic DNA sequence[19] was searched for similarity to these *mer* operon sequences using the basic local alignment search tool (BLAST)[26]. No authentic *mer* operon sequences were identified in the *D. radiodurans* genome.

MD735: the *mer* operon was cloned into the *D. radiodurans* autonomously replicating plasmid pMD66[18] forming pMD727, and transformed into R1 (Fig. 1A). This construction placed the *mer* genes under the control of a constitutive *D. radiodurans* promoter (P2; Fig. 1A), and Southern blot analysis showed that the *mer* operon was present at about one copy per cell (Fig. 3A, B).

MD736: this strain has the *mer* operon integrated into the previously described chromosomal S11 locus[27] (position 1,677,743–1,689,109)[19] as a tandem duplication. The functional difference between the *mer*-containing tandem duplication vector and pMD727 is that the plasmid origin of replication segment (dORI; Fig. 1A) was replaced with a 4-kb internal segment of the *D. radiodurans* chromosomal S11 locus[26,28] (bc; Fig. 1B), that allows it to recom-

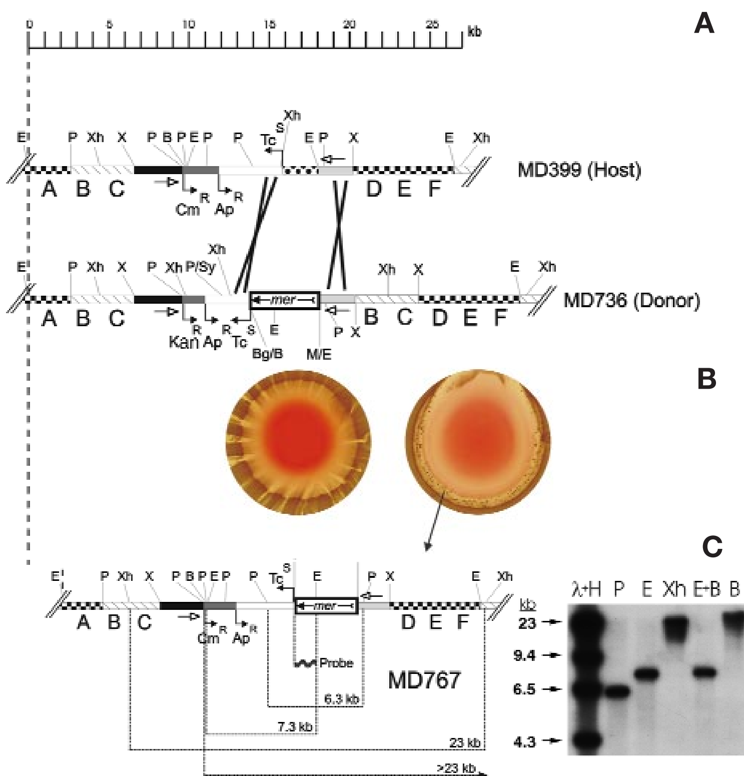

Figure 2. Construction and structure of a chromosomal direct insertion of the *mer* operon. (A) MD399[29] contains a direct insertion of a plasmid having regions of identity with the tandem duplication in MD736. ABC and DEF are unique contiguous chromosomal sequences in wild-type *D. radiodurans* strain R1. BC is the duplicated chromosomal flanking region in MD736. Open-headed arrows are constitutive deinococcal promoters[21, 25]. Black crosses between the MD399 and MD736 chromosomes link regions of homology and show where crossovers occurred. Cm^R, chloramphenicol resistance gene, *cat*; Kan^R, kanamycin resistance gene, *aphA*; Tc^S, mutated tetracyclin gene[25], *tet*; E, *EcoR*I; P, *Pvu*II; X, *Xba*I; Xh, *Xho*I; B, *BamH*I; P/Sy, *Pvu*II/*Sty*I fusion; mer, 4.2-kb *mer* operon. (B) MD736 genomic DNA was transformed into MD399. Transformed cells were spread on solid TGY medium spotted with Merbromin. MD767 was isolated from within the zone of wild-type growth inhibition. MD399 (Left, control); MD399 + MD736 DNA (Right). (C) Right, MD767 was subjected to a detailed mapping of the *mer* operon integration site using restriction enzymes, Southern blotting, and probing with various radiolabeled DNA fragments, including a probe made from the *EcoR*I-*Bgl*II fragment of the *mer* operon (black wavy line). Left, The chromosomal structure of the direct chromosomal insertion containing the *mer* operon in MD767. Abbreviations and symbols, as described in A.

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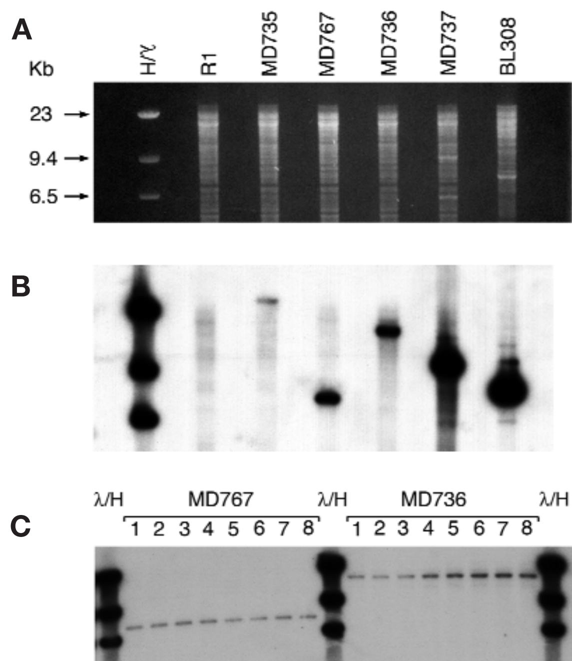

Figure 3. Determination of *mer* operon copy number and associated mercury resistance phenotype. (A) ~600 ng genomic DNA from each of the indicated strains was electrophoresed in an agarose gel. λ/H, lambda phage DNA cut with *Hind*III. (B) The gel was blotted and hybridized to a radiolabeled 1.5-kb *Eco*RI-*Bgl*II fragment of pMD726, containing part of *merA* and all of *merD*. The order of lanes is as in A. The number of disintegration counts in each of the hybridizing bands was determined and used to calculate the *mer* copy number per cell: MD735, ~1; MD767, ~10; MD736, ~10; MD737, ~150. (C) Genomic DNA was prepared from MD767 (direct insertion) and MD736 (tandem duplication) growing in increasing concentrations of Merbromin (0–35 μM in 5 μM steps, lanes 1–8, respectively). DNA was analyzed as described in A and B. The 23 kb, 9.4 kb, and 6.5 kb λ/H size markers are shown. (D) Growth curves for each of the strains described in A and B were determined by inoculating ~5 x 10^6 cells of each into growth medium containing 50 μM Merbromin (Left) or 50 μM $HgCl_2$ (Right).

bine into the targeted S11 chromosome sequence. In MD736, the *mer* operon is present at ~10 copies per cell (Fig. 3A, B).

MD737: whereas the tandem duplication strain (MD736) has ~10 *mer* copies per cell, amplification vectors such as pS11[27], integrate themselves into the chromosome at 150–200 vector copies per cell[27]. When integrated, the duplicated chromosomal flanking sequences of pS11 (11.4 kb) (abcd; Fig. 1C), can readily recombine with identical insertions on other chromosomes, leading to amplification. This occurs because the vector lacks a deinococcal constitutive promoter (P1; Fig. 1B) upstream of the antibiotic resistance marker (Km^R; Fig. 1C), and the only cells able to grow under selective conditions are those that have highly amplified antibiotic resistance determinants[27]. In MD737, the situation is analogous; the *mer* operon is present at ~150 copies per cell (Fig. 3A, B).

MD767: unlike the tandem duplication and amplification vectors, a vector integrated into a host cell's chromosome by direct insertion becomes a permanent fixture in the cell's genome as a result of the unique chromosomal DNA sequences flanking the integrated vector that will not recombine (ABC and DEF; Fig. 2A). Therefore, we used a recombinative transformation approach to construct a *D. radiodurans* strain containing a direct insertion of the *mer* operon (Fig. 3). Following selection for mercury resistance (Fig. 2B), individual clones were prescreened for resistance to chloramphenicol to select for crossovers in the desired chromosomal regions, yielding MD767 (Fig. 2C), which contains one *mer* operon per chromosome (Fig. 3A, B), ~10 copies per cell.

***mer* copy number.** The *merA* copy number in engineered strains, grown exponentially, was determined by Southern blotting and hybridization with a *mer*-specific probe (Fig. 3B). The number of radioactive disintegrations of each hybridizing band was compared to the hybridizing band of MD767 (Fig. 3B), that contains ~10 copies of the *mer* operon per cell. The direct insertion of strain MD767 is located on the chromosome, which exists at 8–10 copies per exponentially growing cell[20]. The approximate number of *merA* copies per cell in R1, MD735, MD767, MD736, and MD737 is 0, 1, 10, 10, and 150, respectively. An exponentially growing *D. radiodurans* cell contains about five times the DNA content of an *E. coli* stationary-phase cell[19]. Taking this into consideration, it is estimated from data shown in Figure 3A, B that *E. coli* BL308 has about 20–30 *mer* copies per cell.

Resistance to Hg (II). The engineered *D. radiodurans* strains, as well as the *E. coli* strain BL308[30], were inoculated into liquid medium containing Merbromin or $HgCl_2$ and their growth monitored over a period of 13 days (Fig. 3D). Wild-type *D. radiodurans* did not grow in the presence of 50 μM Hg (II) and was inhibited by 10–15 μM Merbromin or $HgCl_2$ (data not shown). The strains containing the cloned *mer* operon were variably affected by Hg (II), showing the following order of resistance: BL308 > (MD737, MD736) > MD735 > MD767 > R1 (Fig. 3D).

We tested the ability of MD736 to further increase its number of *mer* tandem duplications with Hg (II) selection. By increasing the mercury concentration over a range of 0–35 μM, the copy number of the *mer* operon in MD736 doubled, compared to growth with just Km selection (Fig. 3C); MD767, containing the direct *mer* insertion did not show a change in copy number (Fig. 3C).

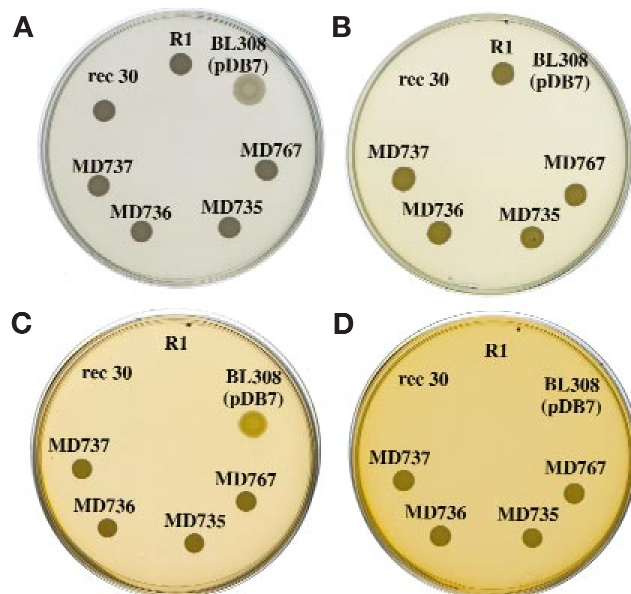

Figure 4. Effect of continuous exposure to γ-radiation and Hg (II) on the growth of strains, containing different copy numbers of the *mer* operon. Two TGY agar plates (A and B), and two TGY agar plates containing 30 μM Merbromin (C and D) were spotted with 1 x 10^5 cells of each of the indicated strains. Following plate inoculation, plates B and D were placed into the ^{137}Cs irradiator (60 Gy / h) for incubation for 5 days. The control plates (A and C) were incubated at the same temperature in the absence of radiation for the same time.

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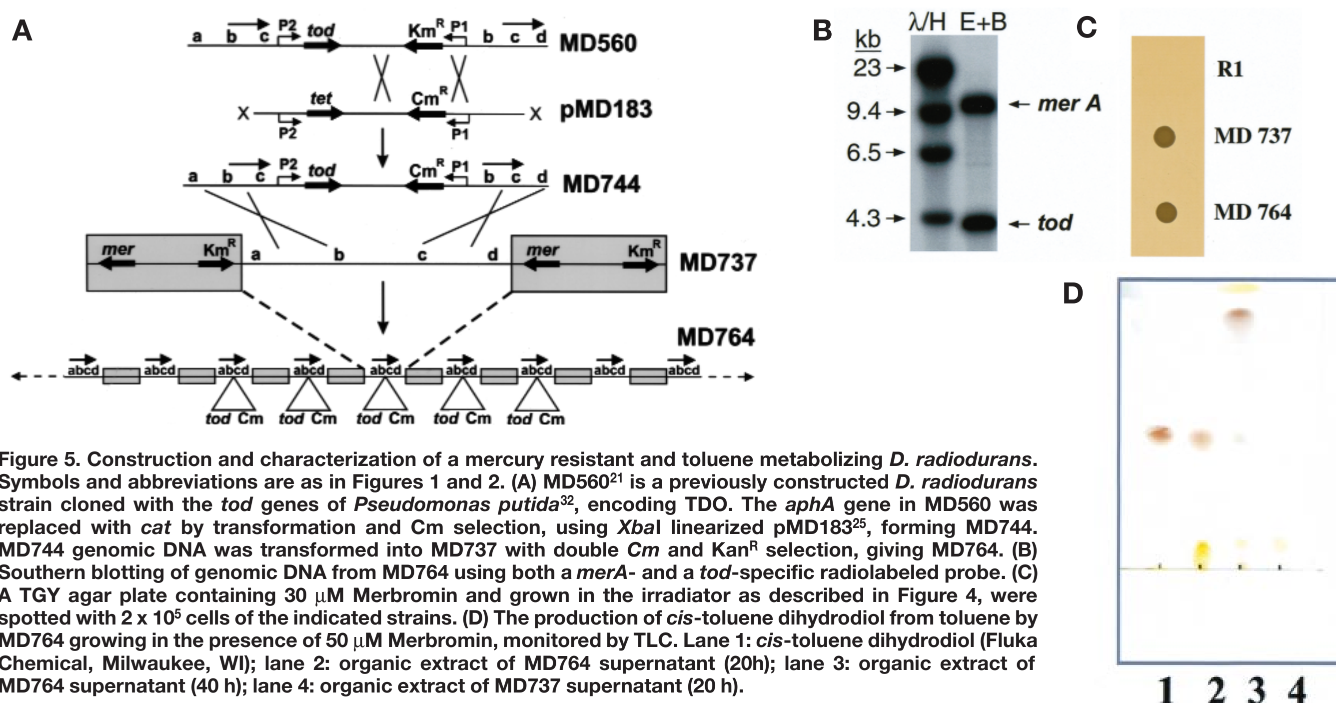

Figure 5. Construction and characterization of a mercury resistant and toluene metabolizing *D. radiodurans*. Symbols and abbreviations are as in Figures 1 and 2. (A) MD560[21] is a previously constructed *D. radiodurans* strain cloned with the *tod* genes of *Pseudomonas putida*[32], encoding TDO. The *aphA* gene in MD560 was replaced with *cat* by transformation and Cm selection, using *Xba*I linearized pMD183[25], forming MD744. MD744 genomic DNA was transformed into MD737 with double *Cm* and Kan^R selection, giving MD764. (B) Southern blotting of genomic DNA from MD764 using both a *merA*- and a *tod*-specific radiolabeled probe. (C) A TGY agar plate containing 30 μM Merbromin and grown in the irradiator as described in Figure 4, were spotted with 2 x 10^5 cells of the indicated strains. (D) The production of *cis*-toluene dihydrodiol from toluene by MD764 growing in the presence of 50 μM Merbromin, monitored by TLC. Lane 1: *cis*-toluene dihydrodiol (Fluka Chemical, Milwaukee, WI); lane 2: organic extract of MD764 supernatant (20h); lane 3: organic extract of MD764 supernatant (40 h); lane 4: organic extract of MD737 supernatant (20 h).

As seen in Figure 3D, we detected that increased growth rate correlated with greater copy number of the *mer* operon. However, there is a significant lag before growth ensues and the growth rates shown in Figure 3D are lower than what is observed in the absence of Hg (II). These observations are consistent with previous reports[31] that mercury causes cell death even with cells actively expressing the *mer* operon. As Hg (II) detoxification is occurring in the cytoplasm, we expect that there could be toxic effects on the outer cell wall and membranes that could effect cell transport and, therefore, limit growth. This would also explain the 5-day lag phase observed before the onset of growth (Fig. 3D), as it was previously reported that toxic effects of an initially high mercury concentration are most manifest at low cell densities[31]. The relatively small number of cells inoculated into medium containing mercury experience the effects of mercury toxicity while only slowly reducing the concentration of mercury. Once the cell concentration reaches a threshold level and the concomitant mercury detoxification activity increases, the rate of cell growth overtakes the rate of cell death caused by the toxicity of the mercury.

Effect of γ-radiation. To determine any effect of continuous exposure to γ-radiation on the growth of these strains in the presence of Hg (II), we tested the strains R1 (*recA*$^+$, *mer*$^-$), rec30 (*recA*$^-$, *mer*$^-$)[18], MD735 (*recA*$^+$, *mer*$^+$), MD736 (*recA*$^+$, *mer*$^+$), MD737 (*recA*$^+$, *mer*$^+$), MD767 (*recA*$^+$, *mer*$^+$), and the wild-type *E. coli* strain K12 (*recA*$^+$) containing pDB7 (BL308) (*mer*$^+$) (Fig. 4). After 5 days of incubation in an irradiating (60 Gy / h) and Hg (II)-containing environment, as expected, the only strains that could grow in the presence of both were MD735, MD736, MD737, and MD767 (Fig. 4D).

Construction of the toluene-metabolizing and Hg (II) resistant *D. radiodurans* MD764. To assess the potential for expressing several remediating functions, encoded on separate gene cassettes in *D. radiodurans*, a strain expressing both mercury resistance/reducing and toluene metabolizing functions was constructed (Fig. 5A).

MD764 was analyzed for its ability to resist (Fig. 3D) and reduce Hg (II) (Fig. 6) as well as metabolize the toluene dioxygenase (TDO) specific substrate toluene (Fig. 5D). The growth characteristics of MD764 in Hg (II), in the presence or absence of radiation (60 Gy/h) were indistinguishable from those expressed in the parent MD737 (Fig. 5C). MD764 could also reduce Hg (II) to Hg (0) (Fig. 6), in a manner similar to MD737.

Mercury (II), and the expression of *mer* operon genes, did not erode the ability of recombinant *D. radiodurans* cells to express functional TDO. This was demonstrated with MD764 when toluene was provided as a substrate, while cells were growing in the presence of Merbromin (Fig. 5D). Toluene is oxidized to *cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol) by TDO.

Thin layer chromatography (TLC) was used to assess the activity of TDO, which showed product formation in comparison to authentic *cis*-toluene dihydrodiol. After 20 h, a single metabolite (Fig. 5D,

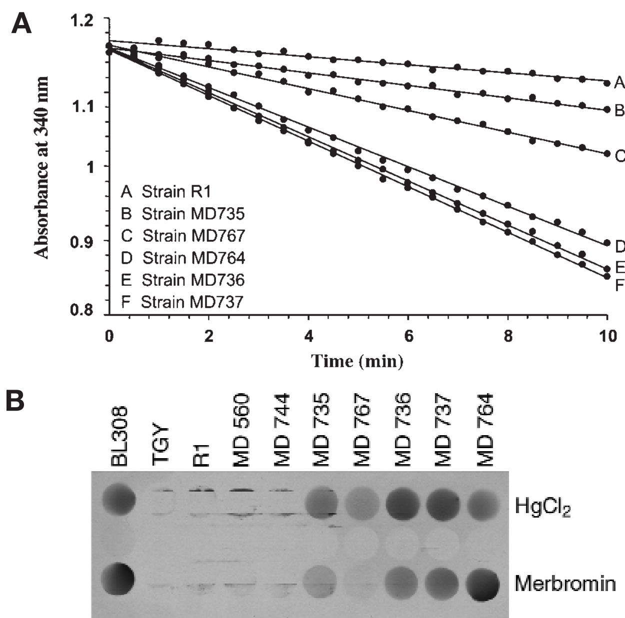

Figure 6. (A) Mercuric reductase assay. Hg(II)-dependent NADPH oxidation catalyzed by cell extracts prepared from the strains R1 (*mer*$^-$, *tod*$^-$; wild type), MD735 (*mer*$^+$), MD767 (*mer*$^+$), MD764 (*mer*$^+$, *tod*$^+$), MD736 (*mer*$^+$), and MD737 (*mer*$^+$) were monitored spectrophotometrically[30]. Decreasing absorbance at 340 nm corresponds to a decreasing NADPH concentration. (B) Mercury volatilization by engineered *D. radiodurans*.

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lane 2) comigrating with the dihydrodiol standard (Fig. 5D, lane 1) was observed. This is consistent with product data obtained by UV spectroscopy; *cis*-toluene dihydrodiol absorbs maximally at 264 nm, and substantial absorbance at this wavelength was observed in culture supernatants of *D. radiodurans* MD764, but not in control strains lacking the *tod* gene cassette (data not shown). Data obtained by TLC (Fig. 5D, lane 3) also clearly demonstrate that *D. radiodurans* further metabolizes the product dihydrodiol. Gas chromatography-mass spectrometry (GC-MS) data indicates that the product of this reaction is 3-methylcatechol produced by a native nonspecific dehydrogenase (data not shown).

Reduction of Hg (II) to Hg (0) by engineered strains. MerA activity was determined in cell extracts of *D. radiodurans* strains by following Hg (II)-stimulated NADPH oxidation spectrophotometrically (Fig. 6A). Mercury (II)-dependent NADPH oxidation was observed in cell extracts of recombinant strains containing the *merA* gene, but not in wild-type *D. radiodurans* strain R1 (Fig. 6A). In the absence of Hg (II), the rate of NADPH oxidation by the *mer*-containing strains was comparable to that of strain R1 (Fig. 6A, curve A). Also, there was a good correlation between the variable Hg (II)-dependent NADPH oxidation activity (Fig. 6A) and the resistance of strains to Hg (II) (Fig. 3D). This activity corresponded to strains shown to contain MerA, visualized by SDS–polyacrylamide gel electrophoresis. In all cases, strains containing the *mer* operon showed MerA, but not in *D. radiodurans* control strains lacking *mer* (data not shown).

The observed mercuric ion dependent NADPH oxidation suggests the concomitant production of volatile Hg (0) by our *mer*-containing *D. radiodurans* strains. We have used a simple X-ray film assay[33] (Fig. 6B) for detecting the production of Hg (0) vapor by the incubation of our *D. radiodurans* strains with Hg (II). There was a good correlation of results between the Hg (II) resistance profiles of these engineered strains containing the *mer* operon (Fig. 3D) and the X-ray film results (Fig. 6B); elemental mercury vapor reacts with the silver ions of X-ray film, causing film darkening[33]. Following 14 h of strain incubation with Hg (II) in the microplate, covered with an X-ray film, only those strains containing the *mer* operon caused film darkening (Fig. 6B). Incubation of Hg (II) with the controls, R1, MD744, MD560, or growth medium alone, did not show any evidence for Hg (0) volatilization. In a separate experiment using cold vapor atomic fluorescence spectroscopy, mercury depletion from medium was shown for MD764, but not for the control strain R1 (data not shown).

Discussion

The *E. coli* mercury resistance operon, *mer* (Fig. 1, Top), was chosen for expression in *D. radiodurans* because it is a highly characterized metal resistance operon[22,23] encoding metal reducing functions that are highly applicable to DOE clean-up efforts. Ionic Hg (II) is a frequent contaminant of radioactive DOE waste sites[1,2], with the highest reported concentration level reported in contaminated areas being 10 μM[1]. The extremely radiation resistant *D. radiodurans* strains expressing *mer*-encoded gene functions reported here were: (1) resistant to the bactericidal effects of ionic Hg (II) at concentrations (50 μM; Fig. 3D) well above the highest concentration reported for mercury-contaminated DOE waste sites; (2) able to reduce toxic Hg (II) to much less toxic elemental and volatile Hg (0) (Fig. 6); and (3) resistant to Hg (II) in highly irradiating environments (Fig. 4). Numerous other metal-resistance functions from other bacteria, specific for other metals[5–12], could be cloned in this way.

Modulating gene expression in *D. radiodurans* by varying the copy number of a cloned sequence. Gene expression could be regulated in *D. radiodurans* by varying the gene dosage between 1 and 150 copies per cell (Figs. 1, 2, 3B). These engineered strains show a good correlation between their *mer* copy number and their resistance to, and reduction of, Hg (II) (compare Figs. 3B, 3D, 6). The most mercury resistant *D. radiodurans* strains are MD737, MD764, and MD736; MD735 shows intermediate Hg (II) resistance, and the least resistant recombinant strain is MD767 (Fig. 3D).

It is noteworthy that MD737, with its 150 copies of the 20-kb vector (pMD731; Fig. 1C), has a genome with ~3 Mbp more DNA than wild type; and in MD764, the size was further increased, by the introduction of the *tod* cassette (Fig. 5A). This remarkable genome plasticity shows that *D. radiodurans* is able to maintain, replicate, and express extremely large segments of foreign DNA, and that it will probably be able to accommodate the large number of gene cassettes required for bioremediation of complex waste mixtures. MD736 is as resistant to Hg (II) as MD737 and MD764 (Fig. 3D) although MD736 has about 100 fewer copies of *mer* per cell; this is explained by the presence of a deinococcal promoter upstream of *mer* in MD736, that is missing in MD737 and MD764. Thus, constructing *D. radiodurans* strains with tandem duplications may be preferable to using amplification vectors, as tandem duplications are probably less of a burden on a cell's genome, and they can adapt to changing environmental conditions (Fig. 3C).

This mercury resistance work has also expanded another aspect of *D. radiodurans* genetic technology. In the past, we were restricted to using antibiotic resistance markers to isolate *D. radiodurans* transformants. We have shown that this constraint can be overcome by selecting for functions encoded by other genes; we were able to select for mercury resistant transformants using *mer*-containing donor DNA (Fig. 2B). As a result, the prospect of using other metal selection systems, arising from cloning other metal remediating genes[5–12] into *D. radiodurans*, is good.

Demonstrating how different gene clusters can be combined into a single *D. radiodurans* host. We are currently metabolically engineering[21] *D. radiodurans* for growth on some of the organic toxins prevalent in radioactive wastes (e.g., fuel hydrocarbons). We showed that we could introduce several remediating functions into a single *D. radiodurans* host by combining the TDO function of MD560[21] with the mercury remediating function of MD737. This generated a novel strain that could metabolize toluene (MD764; Fig. 5) or chlorobenzene (data not shown) while at the same time resist and reduce toxic ionic mercury to volatile elemental mercury (Fig. 6).

Experimental protocol

Growth of Cells. *D. radiodurans* and *E. coli* strains were grown as described previously[21], in the absence or presence of irradiation (^{137}Cs Gammacell 40 irradiation unit [Atomic Energy of Canada Limited, Ottawa, Canda) at 22ºC. Freshly prepared mercuric chloride or Merbromin (mercurochrome; 2',7'-Dibromo-5′[hydroxymercuri]-fluorescein) was used in the following concentrations (unless indicated otherwise in the text): for growth on solid medium, 30 μM; in liquid medium, 50 **μM**. We preferentially used Merbromin in our Hg (II) studies, over $HgCl_2$, because its red color (Fig. 2B) allowed us to track highly toxic Hg (II) waste more easily.

Strain Constructions. MD735 (Fig. 1A): The *Stu*I site of the *mer* operon was converted to a *Bgl*II site, yielding pMD725, followed by the conversion of the *Nco*I site to an *Mfe*I site, yielding pMD726 (Fig. 1, Top). The *Mfe*I-*Bgl*II (4.2 kb) fragment of pMD726 was cloned into the *EcoR*I-*BamH*I site of pMD66[18], yielding pMD727. pMD727 was transformed into *D. radiodurans* strain R1 (wild type). **MD736** (Fig. 1B): The *Mfe*I-*Bgl*II fragment of pMD726 was cloned into the *EcoR*I-*BamH*I site of pMD417[28], yielding pMD728. pMD728 was transformed into strain R1. **MD737** (Fig. 1C): The *Dra*I site of pS11[27] was converted to a *Nco*I site, yielding pMD729. The *Nco*I-*Bgl*II fragment of pMD725 was cloned into the *Nco*I-*Bgl*II site of pMD729, yielding pMD731. pMD731 was transformed into strain R1. **MD767** (Fig. 2): MD399[29] was transformed with MD736 genomic DNA. Petri plates of nonselective TGY (TGY = 1% bactotryptone, 0.5% yeast extract, 0.1% glucose) solid medium (30 cm^3/plate) were inoculated with 100 μl of transformed cells. Once dry, 8 μl of 0.1 M Merbromin were pipetted onto the plates' center. **MD764** (Fig.5): The construction is described in Fig. 5A.

Determination of *mer* operon copy number (Fig. 3A,B). Genomic DNA from exponentially growing strains selected with Kan was prepared[18]. Approximately 600 ng DNA samples were cut with *EcoR*I and elec-

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trophoresed at 60V for 16 h in a 0.6% agarose gel (Fig. 3A). The gel was blotted and hybridized to a radiolabeled probe (Fig. 3B). The number of disintegration counts in each of the hybridizing bands was determined using an Instant Imager (Packard Instrument Company, Downers Grove, IL) and adjusted for DNA content present in each of the corresponding gel lanes similarly scanned.

Assaying the production of *cis*-toluene dihydrodiol from toluene by MD764 (Fig. 5D). Fresh growth medium containing Merbromin was inoculated with 1×10^7 cells of MD764, pregrown in the presence of Merbromin. Toluene was introduced in the vapor phase, and the cells were incubated at room temperature with shaking at 200 rpm[34]. Accumulation of metabolites in the culture medium was periodically monitored by taking a 2-ml sample, removing the cells by centrifugation, and the supernatants extracted twice with equal volumes of ethyl acetate. The extracts were dried over anhydrous sodium sulfate and evaporated to dryness. Each residue was redissolved in methylene chloride for analysis by TLC on silica using methylene chloride: ethyl acetate (1:1) as the solvent. The metabolites were located on the TLC plate by reacting with iodine vapor.

Mercuric reductase assays (Fig. 6). Hg(II)-dependent NADPH oxidation catalyzed by cell extracts prepared from strains were monitored spectrophotometrically according to the method of Fox and Walsh[30] (Fig. 6 A). The protein fractions (0.2 mg) were preincubated with 2 μM, flavin adenine dinucleotide (FAD) in sodium phosphate buffer containing 2-mercaptoethanol and NADPH for 10 min, before initiating the reaction with 0.1 mM $HgCl_2$.

For mercury volatilization by strains[33] (Fig. 6B), cells were pregrown to OD_{600} 0.5 in the presence of 20 μM Merbromin, and also in 20 μM $HgCl_2$. Cells of each strain were harvested, washed twice in fresh medium lacking Hg (II), concentrated to OD_{600} 2.0 in fresh medium, followed by the inoculation of 1×10^7 cells (~50 μl) of each into 200 μl of medium containing 30 μM $HgCl_2$ or 30 μM Merbromin contained in 300-μl wells of a microplate. Cells pregrown in Merbromin or HgCl were tested for mercury volatilization in Merbromin-containing wells or $HgCl_2$-containing wells, respectively. Then, the plate was covered with a sheet of X-ray film, held together with a weight, and incubated in the dark at 32°C. Following exposure for 14 h, the film was developed.

DNA manipulation. DNA cloning, preparation, and transformations were as described previously[21, 28, 29].

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