Construction and Characterization of Recombinant *Deinococcus radiodurans* for Organopollutant Degradation in Radioactive Mixed Waste Environments

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ABSTRACT

Thousands of high priority waste sites around the world contain mixtures of toxic chlorinated solvents, hydrocarbon solvents and radionuclides. Because of the inherent danger and expense of cleaning up such wastes by physicochemical methods, other methods are being pursued for cleanup of those sites. One alternative method could be to engineer radiation resistant microbes that degrade or transform such wastes to less hazardous mixtures. Here, we report the construction and characterization of recombinant *Deinococcus radiodurans*, the most radiation resistant organism known, expressing toluene dioxygenase (TDO). Cloning of the *tod* genes, encoding the multicomponent TDO, into the chromosome of this bacterium imparted to the strain the ability to oxidize toluene, chlorobenzene, 3,4-dichloro-1-butene and indole. The recombinant strain was capable of growth and functional synthesis of TDO in the highly irradiating environment (60 Gray/hour) of a ^{137}Cs irradiator and 5 x 10 ^{8}cells/ml degraded 125 nmole/ml of chlorobenzene in 150 minutes under those conditions. *D. radioduranss*trains were also found to be tolerant to the solvent effects of toluene and trichloroethylene at levels exceeding those of many radioactive waste sites. These data support the prospective use of engineered *D. radiodurans* for bioremediation of mixed wastes containing both radionuclides and organic solvents.

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INTRODUCTION

In 1990 there were 26 countries that produced electricity from a total of 426 operating nuclear power plants[1] and many countries maintain or are developing nuclear weapons programs. Further, as a result of the last five decades of international nuclear proliferation, thousands of radioactive waste sites were generated with little regard for public safety or environmental concern[2]. These nuclear production wastes are highly toxic, recalcitrant, and require the development of new technologies for their cleanup.

A detailed 1992 survey of 91 (out of 3,000) U.S. Department of Energy (DOE) waste sites indicates that about 32% (960) of soils and 45% (1350) of groundwaters at these sites are contaminated with organopollutants (*e.g.*, toluene and trichloroethylene) plus radionuclides and heavy metals (*e.g.*, U, Pu, Cs, Pb, Cr, As)[3]. There is currently little prospect for cleanup of these wastes by physicochemical means alone because of the extreme expense, danger, and intensity of labor. Unless new cost-effective cleanup technologies are developed, these wastes increasingly will threaten human health as they leach into the environment. In the United States alone, of the 3,000 waste sites disclosed by DOE, the total cleanup by physicochemical methods was estimated in 1988 at about $90 billion[4] and more recently between $189 and $265 billion, over a 70 year period (information obtained from The 1996 Baseline Environmental Management Report [web site: http://www.em.doe.gov/bemr96/]). Such sites, therefore, represent defined targets for less expensive *in situ* bioremediation technologies utilizing specialized microorganisms that can degrade the organic component of radioactive mixed wastes.

Numerous microorganisms (particularly *Pseudomonas* spp.) have been described, and studied in detail, for their ability to degrade, transform, detoxify, or immobilize a plethora of organic and inorganic pollutants[5-11]. Biodegradation of the organic component of these sites is a logical first step in their detoxification. However, most microorganisms are sensitive to the damaging effects of radiation found in mixtures containing radionuclides. For example, *Pseudomonas* spp. are very sensitive to radiation (more sensitive than *E. coli*[12]) and are not suited to remediate mixed wastes. Therefore, radiation resistant microorganisms that can degrade organic toxins need to be found in nature or engineered in the laboratory to address this problem.

The most radiation resistant organism discovered to date is *Deinococcus radiodurans*[13-15]. *D. radiodurans* is a non-pathogenic, desiccation resistant[16], soil bacterium that can survive acute exposures to ionizing radiation of 15,000 Gy without lethality[17]; this dose induces about 130 double strand breaks (DSBs) per *D. radiodurans* chromosome[17]. This ability is extraordinary since most organisms cannot survive more than 2-3 DSBs per chromosome[18]. Previous studies on *D. radiodurans*' resistance to radiation describe its exposure to acute doses of radiation (*i.e.*, delivered as a single dose) followed by its recovery in the absence of radiation [17,19,20]. Here, we show that *D. radiodurans* can grow and express foreign genes while growing in the presence of continuous irradiation. This characteristic is critical given the continuous exposure to radiation and other DNA damaging agents a microorganism would be subjected to at radioactive DOE waste sites. Recent advances in our ability to genetically manipulate this bacterium[21] has led to insights into its DNA repair capabilities. The mechanism of radiation resistance has been shown to be due, in part, to exceedingly efficient *recA*-dependent[17, 19, 22, 23] as well as *recA*-independent DNA repair processes[20]. Likewise, *D. radiodurans* is also extraordinarily resistant to most chemical DNA damaging agents such as nitrous acid, 4-nitroquinoline-*N*-oxide, and mitomycin-C[14, 24, 25].

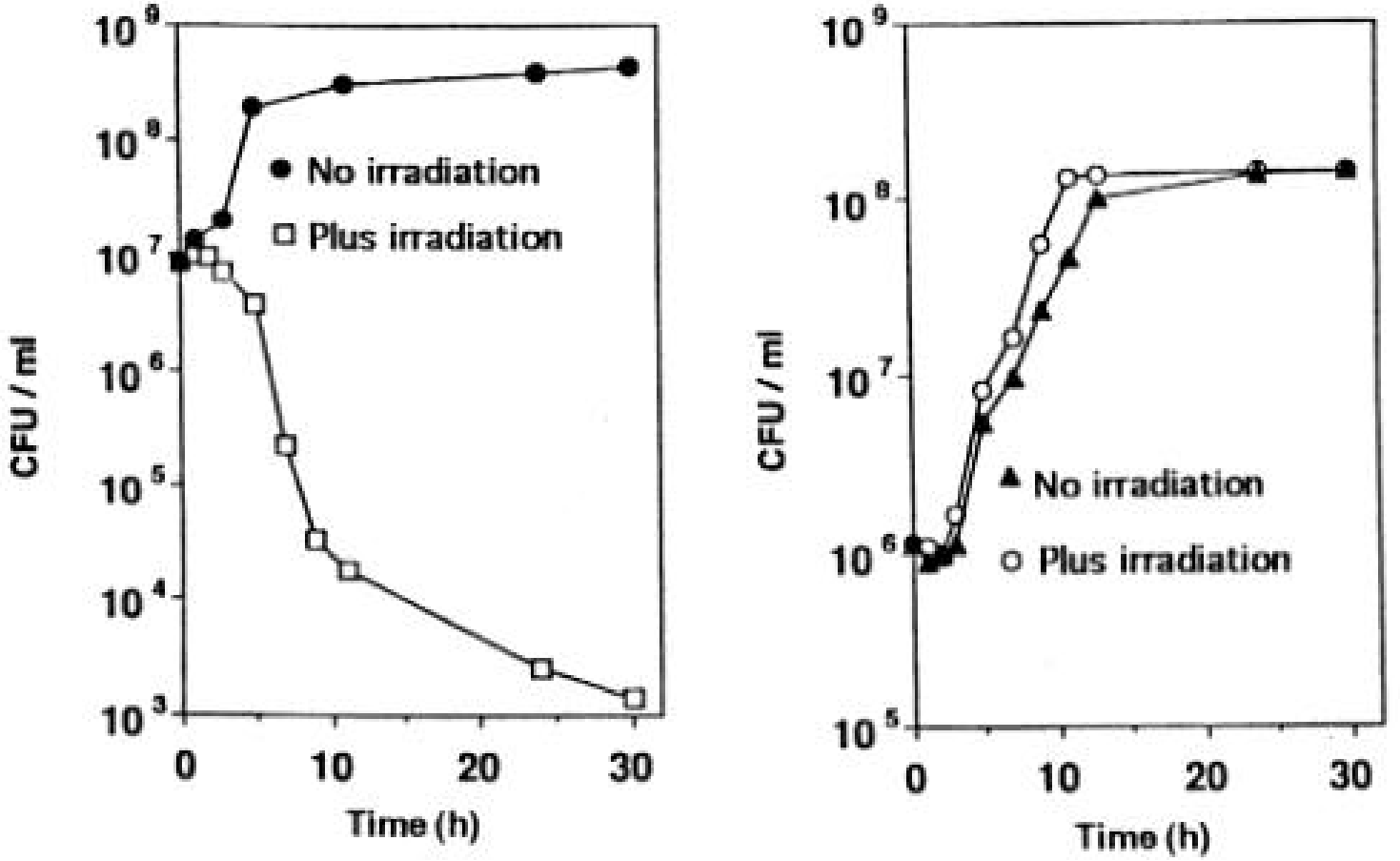


Figure 1. Effect of γ-irradiation on the growth of *E. coli* (left) and *D. radiodurans* R1 (right). *E. coli* (wildtype) and *D. radiodurans* R1 (wildtype) were both grown to the plateau phase of their respective growth cycles and diluted 150-fold in fresh growth media. The diluted cultures were divided into two parts and incubated with aeration in the presence and absence of γ−radiation (^{137}Cs; 60 Gy/hr) for a total of 30 hours. The survival rates were determined by plating appropriate dilutions of irradiated cells and counting the number of colony forming units (cfu) following incubation.

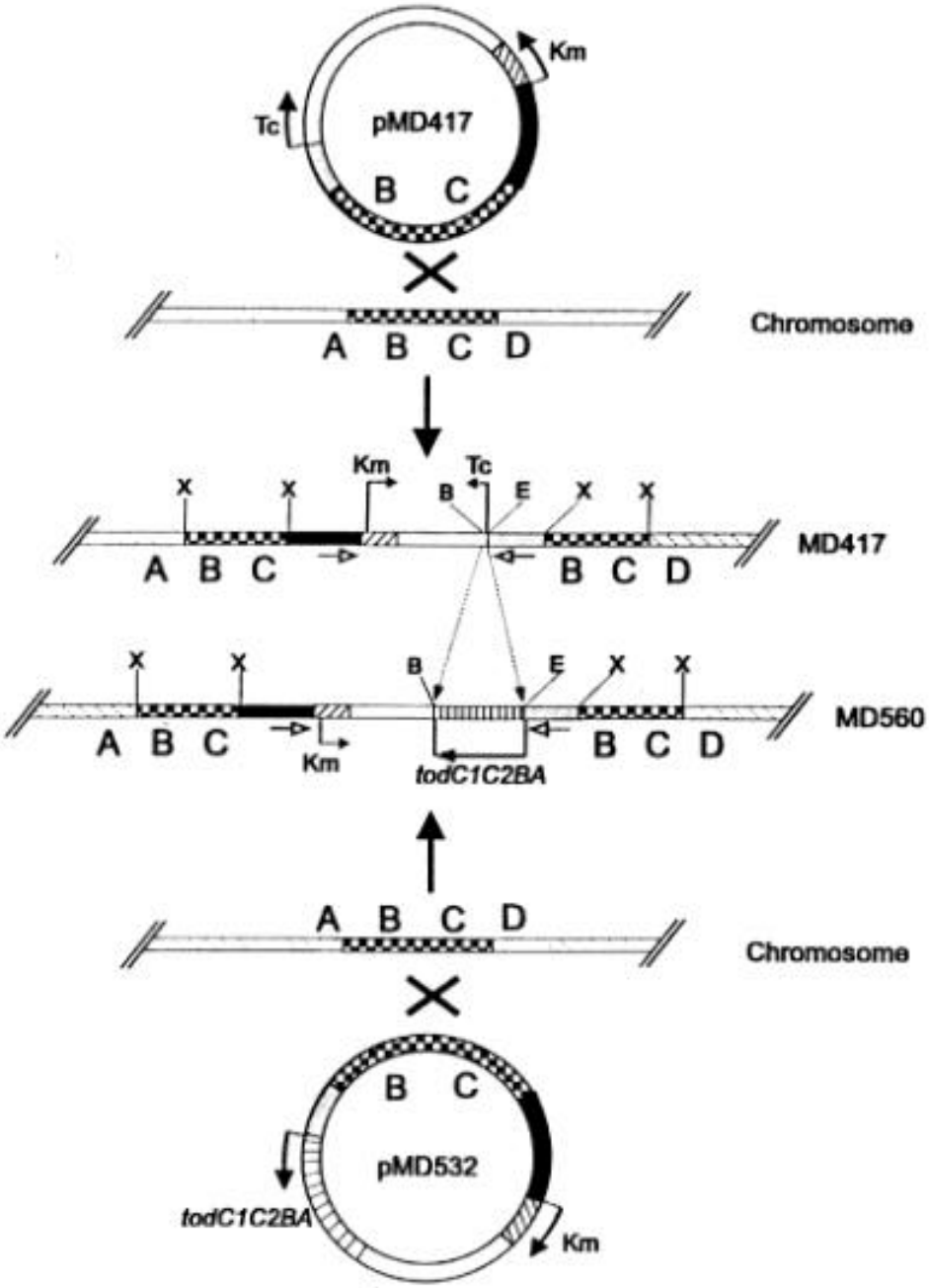


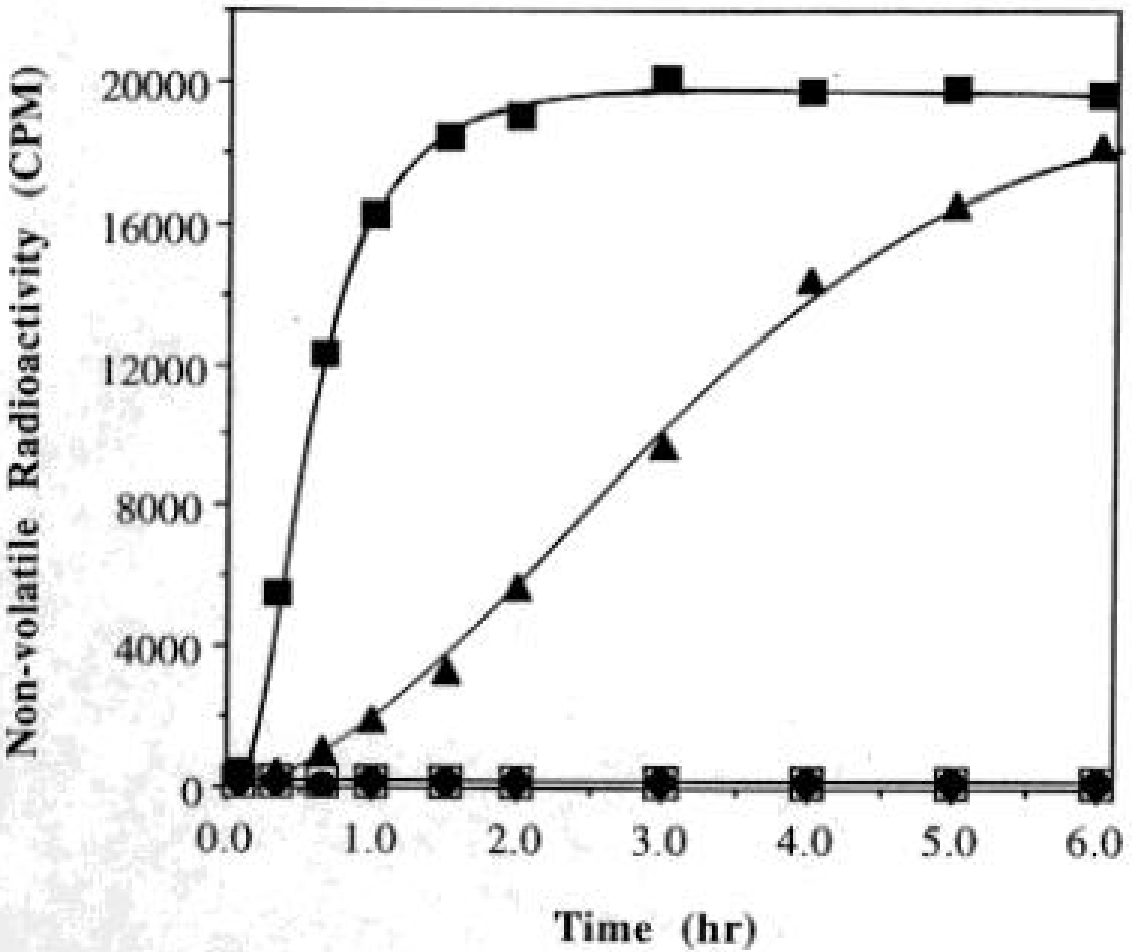
Figure 2. Regional chromosomal maps and functions of *D. radiodurans* strains MD417 (*tod*$^-$) and MD560 (*tod*$^+$). MD560 constitutively expresses TDO (encoded by *todC1C2BA*). MD417 is a control strain (lacking *tod* genes). The strains are the products of transformation of wildtype strain R1 with the circular plasmids pMD532 and pMD417, respectively. The two arrows drawn between chromosomal regions MD417 and MD560 show the location of the *tod* genes; the black arrow below the *tod* genes shows the direction of functional transcription. The checkered segment **BC** indicates the chromosomal integration sequence. **A** and **D** are chromosomal sequences flanking the integration site **BC**. Km (resistance to kanamycin) is encoded by the *aphA* gene [diagonally hatched segment]. Transcription of the *aphA* genes is driven by a deinococcal constitutive promoting sequence (open arrow) located in the black segments. Tc (resistance to tetracycline) is encoded by the *tet* gene [white region]. Transcription of the *tet* and *todC1C2BA* genes is driven by another deinococcal constitutive promoting sequence (open arrow) present in the light grey segments. Restriction sites: X, XbaI; B, BamHI; E, EcoRI.

Table 1. Mass spectra and GC retention time (R_t) of products identified from incubations of *D. radiodurans* MD560 (*tod*$^+$) or purified toluene dioxygenase with the listed substrates.

Starting Compound	Transforming Organism or Enzyme	Product Data	
		GC R_t (minutes)	*m/z* (% relative intensity)
toluene *cis*-dihydrodiol standard	------	9.22	126 (10), 108 (100), 107 (49), 80 (67), 79 (84)
toluene	*D. radiodurans* MD560	9.25	126 (10), 108 (100), 107 (50), 80 (65), 79 (84)
chlorobenzene	toluene dioxygenase	11.15	148 (10), 146 (30), 130 (25), 128 (71), 117 (19), 102 (39), 100 (100)
chlorobenzene	*D. radiodurans* MD560	11.14	148 (8) , 146 (27), 130 (19), 128 (57), 117 (19), 102 (39), 100 (100)
3,4-dichloro-1-butene	toluene dioxygenase	NA	163 (3), 161 (16), 159 (19), 145 (14), 143 (61), 141 (100)
3,4-dichloro-1-butene	*D. radiodurans* MD560	NA	163 (2), 161 (11), 159 (20), 145 (9) , 143 (66), 141 (100)

NA: not applicable, compounds analyzed by direct probe insertion

A .


Non-volatile Radioactivity (CPM)
20000
16000
12000
8000
4000
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Time (hr)

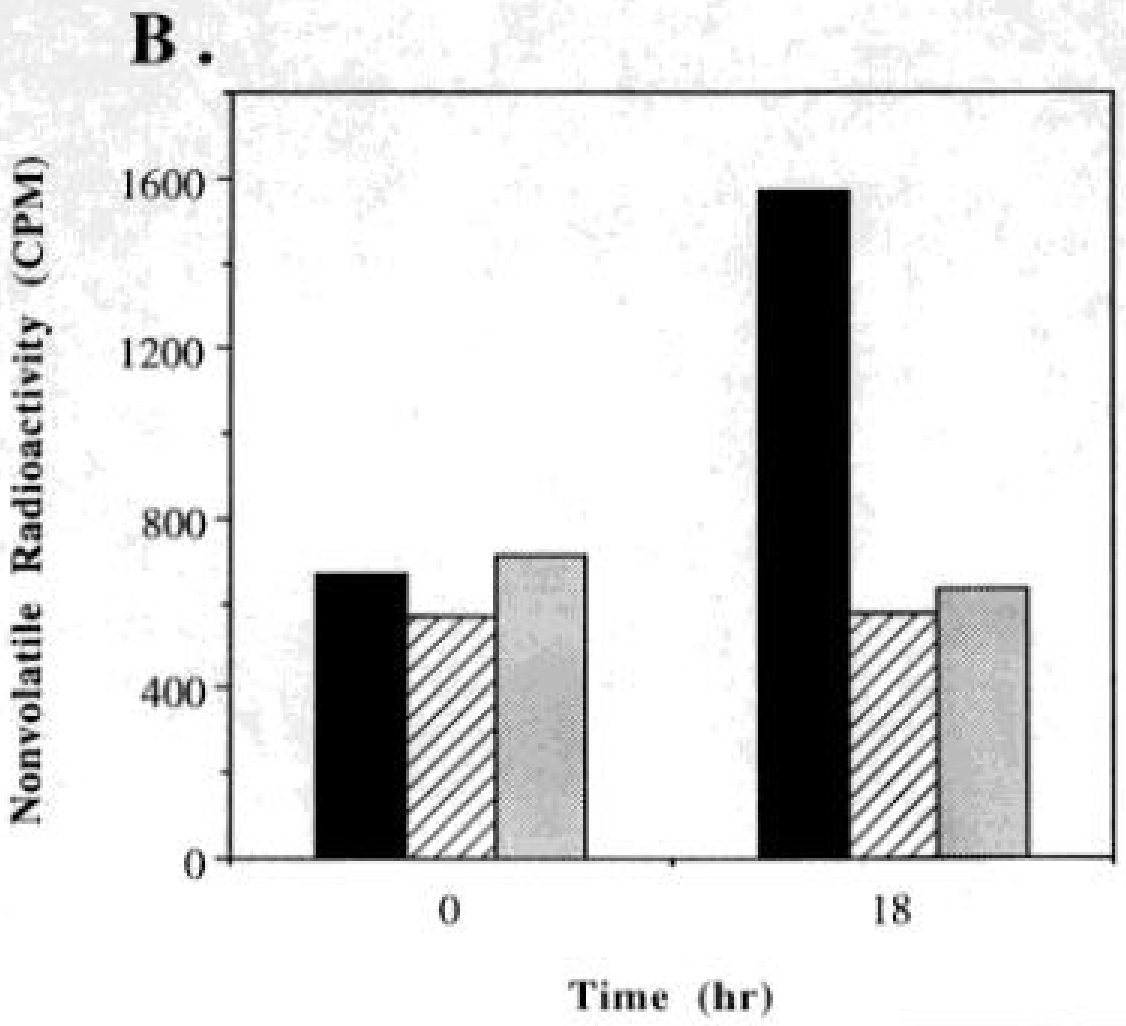


Figure 3. (A) Non-volatile radioactive products formed upon incubation of ^{14}C-labeled toluene with *D. radiodurans* strains MD560 (*tod*$^{+}$; closed triangle) and MD417 (*tod*$^{-}$; open square), *E. coli* (pDTG351; *tod*$^{+}$; solid square), and negative control of TGY medium alone (solid circle).

(B) Non-volatile radioactive products formed upon incubation of ^{14}C-labeled trichloroethylene with *D. radiodurans* strains MD560 (*tod*$^{+}$; black), MD417 (*tod*$^{-}$, diagonally hatched), and TGY medium control (grey).

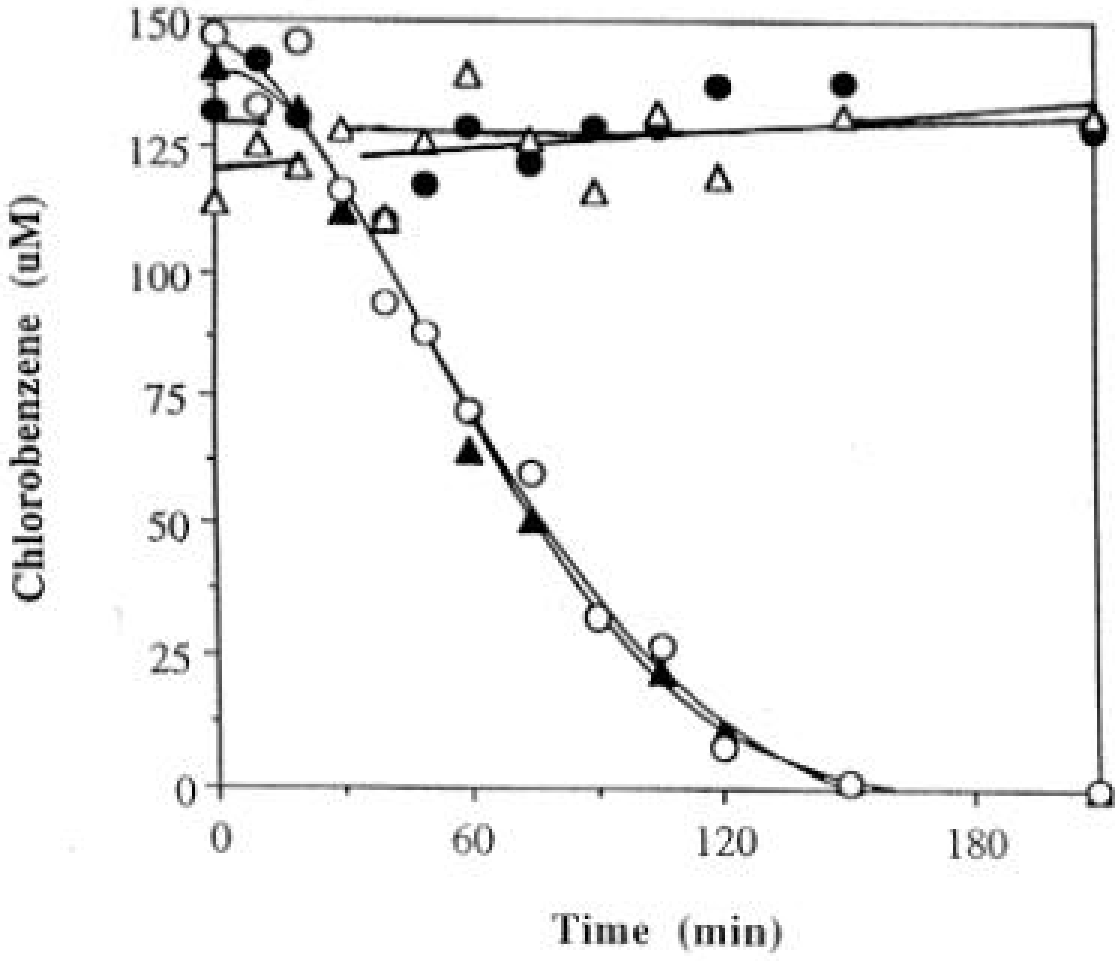


Figure 4. Effect of γ-irradiation (60 Gy/hr) on the function of TDO expressed in *D. radiodurans*. Strain MD560 (*tod* $^{+}$) in the presence of (solid triangle) and absence of (open circle) irradiation; and MD417 (*tod*$^{-}$) in the presence of (solid circle) and absence of (open triangle) irradiation.

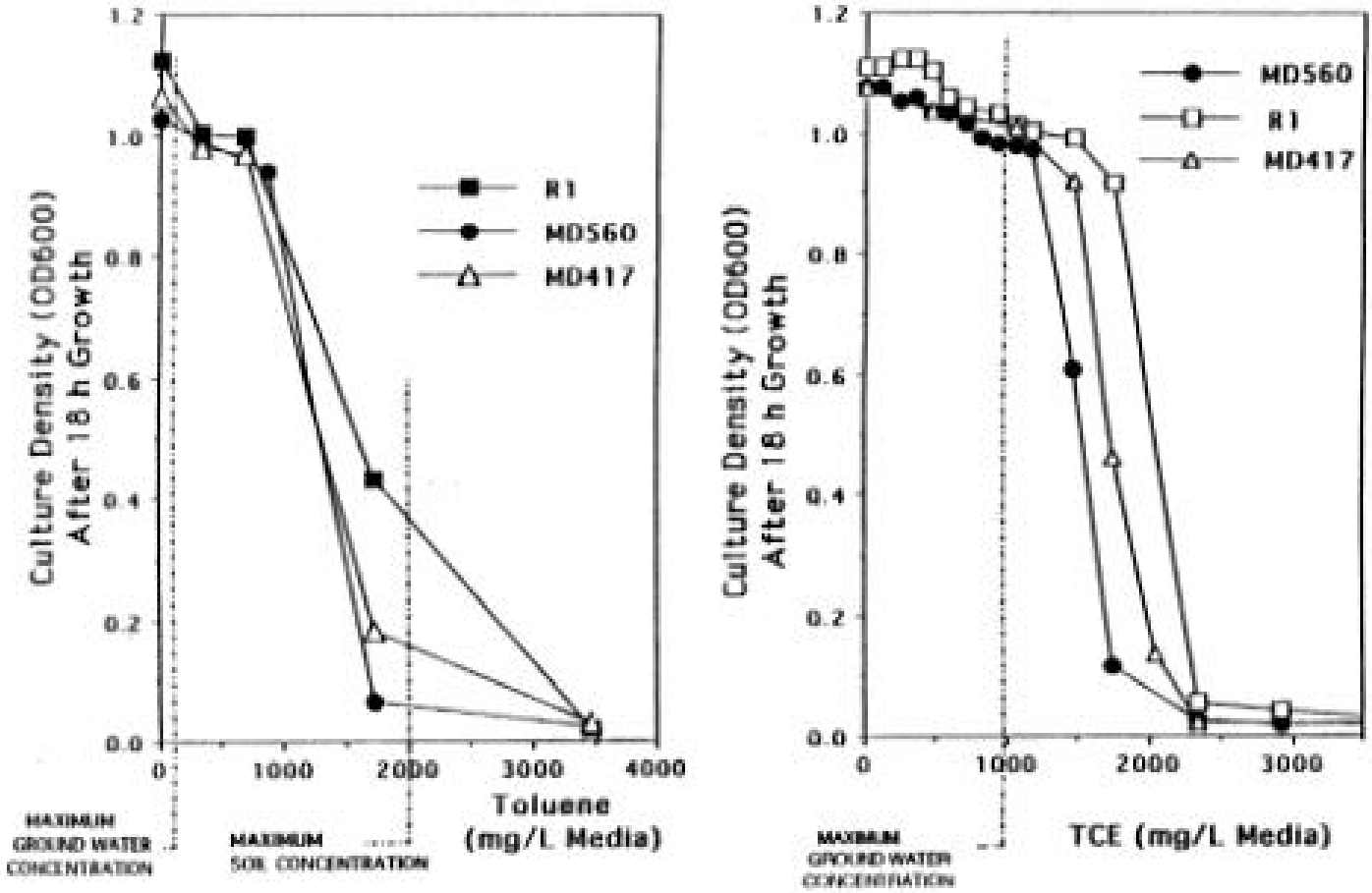


Figure 5. Effect of toluene and TCE on the growth of *D. radiodurans* strains R1, MD417 (vector control, *tod*$^-$) and MD560 (*tod*$^+$). Strains were first grown overnight in liquid growth medium (to 1.1 OD_{600}) followed by dilution into fresh growth medium (to 0.02 OD_{600}) containing varying amounts of toluene (left) and TCE (right). After 18 hours of incubation at 32°C, the cell densities of the cultures were determined and plotted as a function of solvent concentration.

DISCUSSION

TDO was chosen for expression in *D. radiodurans* because it is prototypic of a large class of bacterial dioxygenases and has a broad substrate range that includes compounds present at sites containing organic and radioactive mixed wastes. Furthermore, TDO is comprised of three protein components with their attendant metal and organic cofactors[26] and, thus, its successful expression in *D. radiodurans* suggests that many less complex biodegradative enzyme systems could be expressed.

Strain MD560, expressing TDO, oxidized indole, toluene, chlorobenzene, and 3,4-dichloro-1-butene, all known substrates for TDO, to the anticipated oxidation products (Table 1). *D. radiodurans* strains grew under continuous irradiating conditions of 60 Gy/hr in a ^{137}Cs irradiator (Fig. 1). Furthermore, strain MD560 synthesized functional TDO under those conditions and degraded 125 nmole/ml chlorobenzene while being exposed to radiation (Fig.4). Before this work, no studies regarding *D. radiodurans* growth in the presence of continual high level radiation had been reported.

The cell envelope of *D. radiodurans* includes an outer and inner lipid membrane that surrounds the cell wall[29, 30]. It was unknown before this study if the membrane architecture of this organism might result in sensitivity or resistance to organic solvents. Organic solvents are generally toxic to bacteria by making their membranes porous[31, 32]. Toluene and TCE are two of the most common organopollutants at radioactive DOE waste sites[3]; toluene has been reported as high as 26 mg/L groundwater and 2,000 mg/Kg soil, and TCE as high as 1,000 mg/L and 12,000 mg/Kg. In this study, *D. radiodurans* strains R1, MD560 and MD417 were all found to be naturally tolerant to toluene and TCE groundwater concentrations well above those found at most sites, and resistant to about half the highest toluene concentrations reported in contaminated soils (Fig. 5).

The lack of TCE oxidation, beyond that measured using sensitive a ^{14}C assay, has been attributed to turnover-dependent TDO inactivation[28]. Enzymatic TCE oxidation is known to generate reactive acyl chlorides that bind covalently to proteins and other macromolecules[7]. Sustained biological TCE oxidation may, thus, require the intracellular biosynthesis of a scavenging nucleophile, such as glutathione, to protect against enzyme inactivation. Our analysis of genome sequences failed to detect DNA homologues to *E. coli* genes *gshA* and *gshB* (not shown), but it may be possible to clone and express these glutathione biosynthetic genes in *D. radiodurans*.

Previous to this report, we are not aware of any reported expression of genes for biodegradative purposes in *D. radiodurans*. Likewise, information regarding effects of radiation on *D. radiodurans*' growth and protein expression, or solvent effects, were not available. This report presents data that strongly support the idea that recombinant *D. radiodurans* strains can be constructed for biodegradation in radioactive aqueous/organic mixed wastes.

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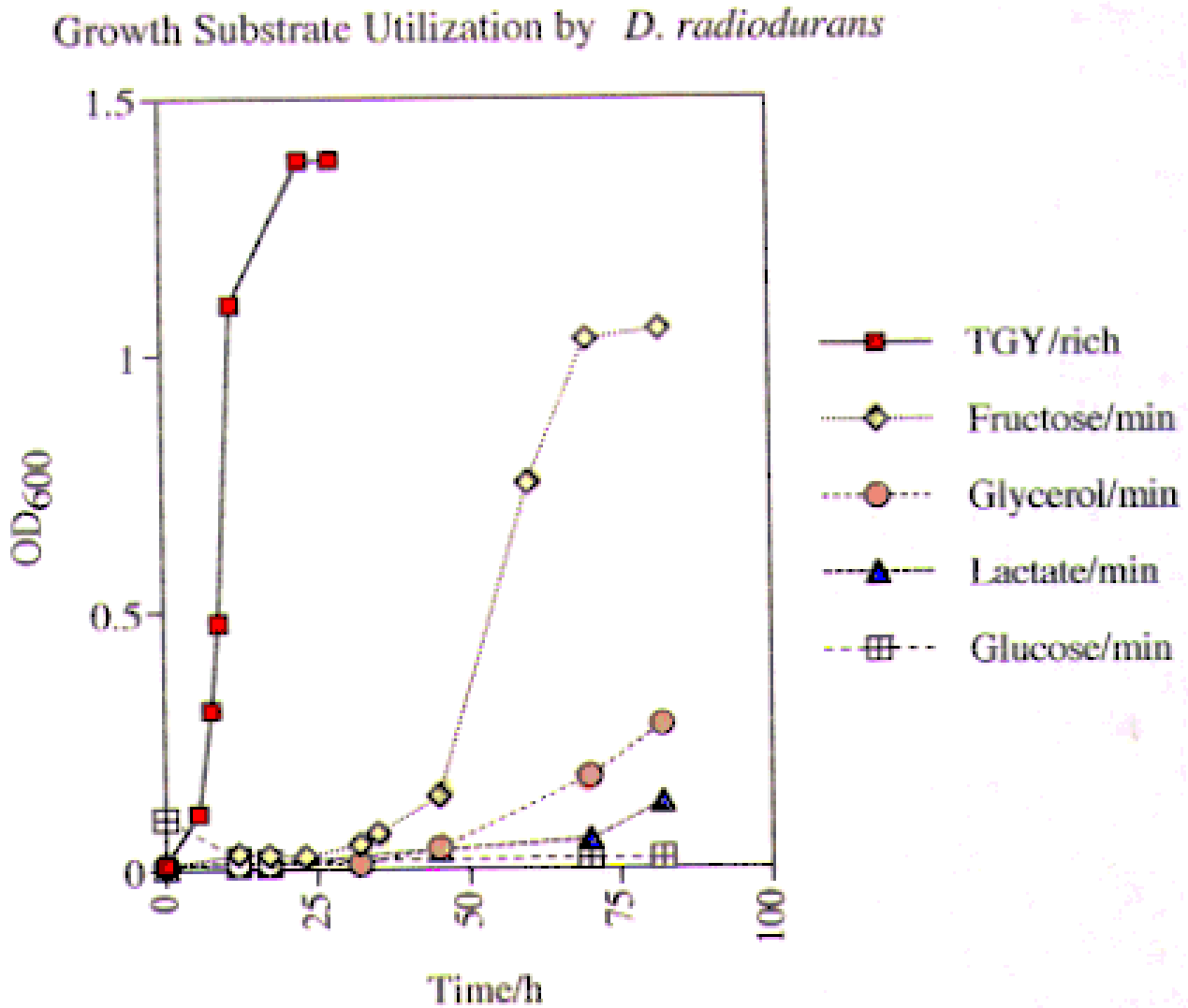
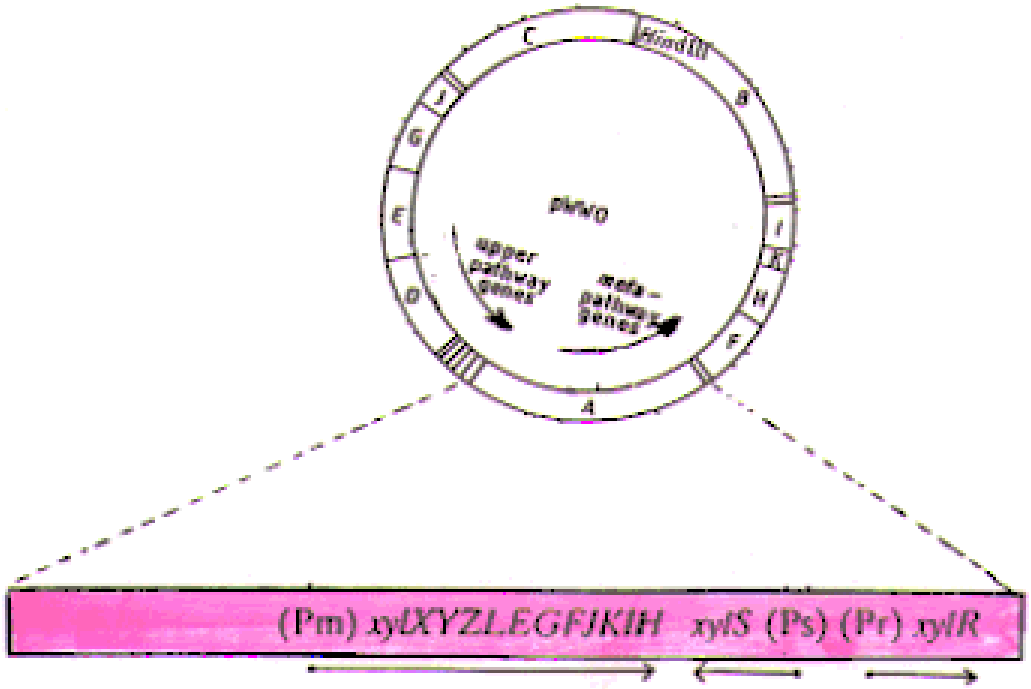
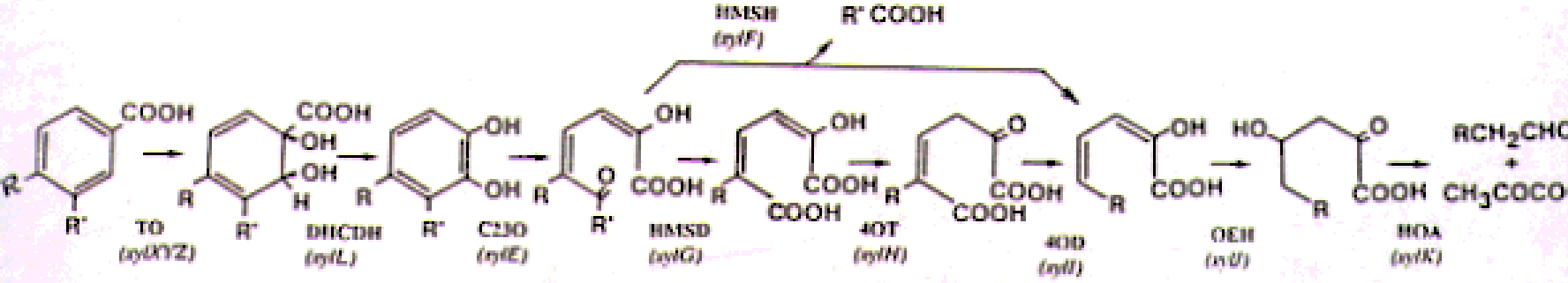
CURRENT WORK ON STRAIN MD560

The catabolic degradative capabilities of the genetically engineered strain MD560 are being expanded.

The absence of information on well-defined synthetic minimal medium led us to investigate the capability of *D. radiodurans* to use various hexoses, trioses, dicarboxylic and tricarboxylic acids as growth and energy substrates. The development of a defined minimal medium, together with fundamental knowledge on its metabolic pathways, will allow us to unambiguously test genetically engineered *D. radiodurans* for growth on organic toxins. Among the TCA cycle components tested, pyruvate was found to be the most effective growth substrate. Lactate, acetate, gluconate and oxaloacetate were found to support modest growth. Among the hexoses and trioses, only fructose was very effective at supporting growth while glycerol supported moderate growth. Of the other substrates tested, acetate was found to be less effective than lactate and glycerol. The following order represents the effectiveness of a substrate to support *D. radiodurans* growth: Pyruvate > Fructose > Glycerol > Lactate > Acetate (See Below).

Glucose and other aldoses were totally ineffective at supporting growth. Preliminary observations indicate that there is a permeability barrier or lack of a transport mechanism of these carbon sources; this may explain the narrow growth substrate range of this organism.



Growth of *D. radiodurans* on different carbon sources. Pre-induced (on Noble agar + C-source) *D. radiodurans* cells were inoculated into 100 ml minimal medium containing the indicated carbon sources, and incubated at 32°C with vigorous aeration. At the indicated time-points OD_{600} measurements were made of the cultures.

The meta-cleavage pathway genes of plasmid pA15/pWWO encode all the enzymes that lead to conversion of toluene to pyruvate/acetaldehyde. The whole genetic cassette is located on a 25kb *Hind*III fragment that has been purified and cloned. Currently, we are introducing this cassette into the *D. radiodurans* chromosome by transformation. Our target sequence is the previously cloned *todC1C2BA* cassette present in strain MD560. Successful, or even limited, expression of these Tol meta-pathway genes should permit the host to grow on toluene as a sole carbon and energy source.