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THE ROLE OF IRON IN *Deinococcus radiodurans* ENGINEERED FOR GROWTH ON TOLUENE AND THE ROLE OF MANGANESE IN THE EXTREME RADIATION RESISTANCE PHENOTYPE

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Abstract

Toluene and other fuel hydrocarbons are commonly found in association with radionuclides at numerous Department of Energy (DOE) sites, frequently occurring together with Cr(VI) and other heavy metals. In this study, the extremely radiation resistant bacterium *Deinococcus radiodurans* was engineered for complete toluene mineralization by cloned expression of tod and xyl genes of *Pseudomonas putida*. The recombinant Tod/Xyl strain showed significant incorporation of carbon from the toluene aromatic ring into cellular macromolecules and carbon dioxide, in the absence or presence of chronic radiation. We have shown that intracellular iron concentrations in wild-type *D. radiodurans* in minimal medium are exceptionally low and not sufficient to support growth on toluene using Fe-dependent oxygenases cloned from *P. putida*. Introducing the fur mutation into *D. radiodurans* increased intracellular Fe levels, and imparted on the engineered strain the ability to grow on meta-toluate as the sole carbon and energy source. The organism's native Cr(VI) reduction capabilities were facilitated by toluene when present as the sole carbon and energy source in natural sediment analogues of DOE contaminated environments. The engineered bacteria were able to oxidize toluene under both minimal and complex nutrient conditions, which is important since both conditions have environmental equivalents in the context of bioremediation processes. As such, the Tod/Xyl strain is providing a model for understanding the role of Fe and reduction of metals coupled to organic contaminant oxidation in aerobic radionuclide contaminated sediments. For an overview of this work see http://www.usuhs.mil/pat/deinococcus/index_20.htm. We have shown that *D. radiodurans* contains high intracellular manganese levels, and that Mn restriction sensitizes cells to irradiation. We propose that the unusually high Mn/Fe ratio of *D. radiodurans* facilitates survival by quenching oxidative stress during recovery.

Objective

A. Engineer *D. radiodurans* for growth on toluene

B. Characterize the role of Manganese in *D. radiodurans*.

A. Growth of *D. radiodurans* on Toluene.

Introduction

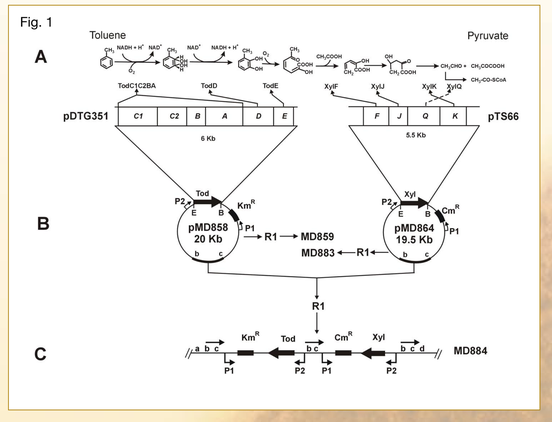
Immense volumes of soil and groundwater at numerous U. S. Department of Energy (DOE) sites have low levels of widespread contamination that include mixtures of heavy metals (e.g., Hg & Cr), radionuclides (e.g., U & Tc), and toxic organic compounds (e.g., toluene). The remediation of such contaminated sites constitutes an immediate and complex waste management challenge for DOE, particularly in light of the costliness and limited efficacy of current physical and chemical strategies for mixed wastes. The goal of in situ bioremediation via natural microbial processes (e.g., metal reduction & aromatic compound degradation) remains a potent, potentially cost-effective approach to the reductive immobilization or detoxification of environmental contaminants.

For bioremediation to be effective in such areas, microorganisms must be able to withstand cellular toxicity caused by heavy metals, solvents, and chronic radiation during vegetative growth. These requirements have not been met by any single known organism, nor are they likely to be achieved in the foreseeable future by engineering genetic components of radiation resistance into other bacteria that are radiation sensitive. Therefore, our approach has been to express cloned genes in the naturally radiation resistant Deinococcus bacteria, extending their intrinsic metabolic functions.

The fuel hydrocarbon toluene is a contaminant in hundreds of DOE mixed waste sites. This contaminant is a growth substrate for a number of organisms, including *P. putida* strains F1 and mt-2, for which the genetics and biochemistry have been studied in great detail. *P. putida* F1 and mt-2 express tod and xyl genes, respectively, for the catabolism of fuel-derived aromatic hydrocarbons and represent two of the most proficient toluene degraders yet reported. With respect to *P. putida* genes encoding degradation of toluene, our goal has been to construct a pathway in *D. radiodurans* that allows it to completely degrade this solvent and use carbon and energy derived from its catabolism to help drive cellular processes. To demonstrate the applicability of this strategy, we cloned genes of the *P. putida* tod and xyl operons into *D. radiodurans*, to generate a strain capable of completely mineralizing toluene and other fuel hydrocarbons, and showed that energy derived from toluene catabolism is coupled to *D. radiodurans*' native Cr(VI) reducing capabilities.

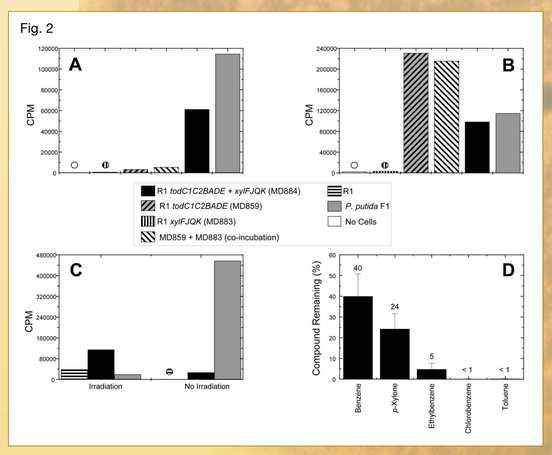
1) Construction of toluene-mineralizing *D. radiodurans*. (A) Intermediates of toluene degradation encoded by the indicated genes. (B) Co-integration of the tod and xyl gene cassettes into *D. radiodurans* involved the construction of two different plasmids derived from the tandem duplication vector pMD417. Both constructions placed the two degradation cassettes downstream of a constitutively expressed promoter P2 that is distinct from the constitutive P1 promoter upstream of the resistance genes for kanamycin (Kmr) and chloramphenicol (Cmr). (C) Co-transformation of pMD858 and pMD864 into strain R1 with double Km and Cm selection yielded strain MD884. Abbreviation: R1, *D. radiodurans* strain R1 (ATCC BAA-816).

Fig. 1



2) Fate of ^{14}C-toluene in engineered *D. radiodurans* (resting cells). (A) Generation of $14CO^2$. (B) Production of non-volatile radioactive products. (C) Incorporation into macromolecular cellular components with and without ionizing irradiation (^{137}Cs [g, b-]E, 23 Gy/hour). Cells were adjusted to OD_{600} = 5.0 for (A) and (B) and assayed after 48 h. Analyses shown in (C) utilized cells adjusted to OD600 = 3.5, with ^{14}C-toluene added to 4.1 µM. (D) Transformation of BTEX and chlorinated hydrocarbons by MD884 relative to *D. radiodurans* R1. When a column-value is low in Panels A, B, or C, see inset circle for pattern-designation and correspondence to key. Abbreviation: R1, *D. radiodurans* strain R1.

Fig. 2



3) Increased Fe levels in *fur* mutant

Strains	^{59}Fe accumulation atoms/cell	ICP-MS/nmol Fe/mg protein
D. radiodurans wild-type (ATCC BAA-816)	2.7 x 10^3	0.079 (±0.005)
D. radiodurans fur mutant	Not done	0.23 (±0.005)
E. coli 4.6 Mbp (MG1655)	7 x 10^3	4.2
S. oneidensis 5.1 Mbp (MR-1)	2.7 x 10^4	7.4 (±1.7)

Conclusion A

The development of viable in situ bioremediation applications is a long-term goal of the DOE, including the use of engineered organisms, and a variety of DOE field research efforts are currently underway (http://public.ornl.gov/nabirfrc/PITableMarch2003.pdf). Genetically-engineered microorganisms have already been used successfully in non-DOE, regulatory agency-approved, field-scale bioremediation. Recombinant organisms have been considered as an option when naturally-occurring organisms do not provide the set of overlapping functions needed to deal with contaminant mixtures and sites. *D. radiodurans* is non-pathogenic and indigenous to some contaminated DOE sites. In the present example *D. radiodurans* has been engineered for growth on toluene in radioactive, heavy metal contaminant mixtures.

4) Growth of MD884/fur with meta-toluate as the sole carbon source in deinococcal minimal medium. Plate incubation was for 7 days at 32°C. No growth was observed on Tol plates for the controls (wild-type, fur-, MD884) after 4 weeks.

Fig. 4

B. High Intracellular Manganese and Low Iron Levels of *Deinococcus radiodurans* Facilitate Recovery from Ionizing Radiation

Introduction

Since the amount of DNA damage inflicted in *D. radiodurans* by exposure to γ-radiation is the same as other organisms, we propose that the high Mn/Fe ratio of *D. radiodurans* (1) facilitates survival (2) by quenching oxidative stress during recovery. *Escherichia coli* and *Shewanella oneidensis* have very low Mn/Fe ratios (1) and are substantially less radiation resistant (2). We show that *D. radiodurans* is dependent on Mn (3), and Mn restriction inhibits recovery of irradiated cells (2). Among the most radiation resistant bacterial groups reported that do not form resistant endospores, deinococci, enterococci, and lactobacilli share physiologic and metabolic traits including Mn accumulation and luxuriant growth in Fe-limiting conditions in the presence of chronic radiation (2).

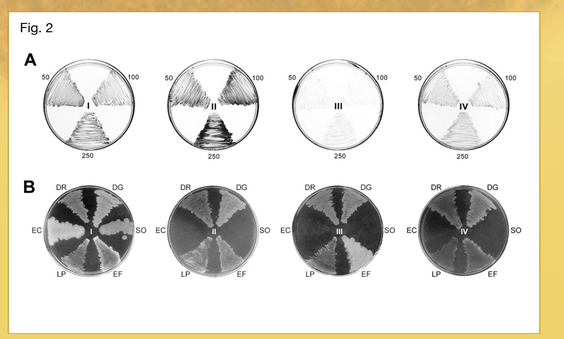
1) Summary of intracellular Mn and Fe levels.

Strains	^{59}Fe accumulation atoms/cell	ICP-MS/ nmol Fe/mg protein
D. radiodurans wild-type (ATCC BAA-816)	2.7 x 10^3	0.079 (±0.005)
D. radiodurans fur- mutant	Not done	0.23 (±0.005)
E. coli 4.6 Mbp (MG1655)	7 x 10^3	4.2
S. oneidensis 5.1 Mbp (MR-1)	2.7 x 10^4	7.4 (±1.7)

2) A. Effect of Mn(II) on growth of wild-type *D. radiodurans* on DMM (12) under chronic radiation. Cells were pre-grown on DMM supplemented with 50 nM Mn(II), 100 nM Mn(II), or 250 nM Mn(II) and inoculated onto DMM plates containing the indicated Mn(II) concentrations. I, no irradiation control, [DMM + 25 nM Mn(II)]; II, [DMM + 2.5 µM Mn(II)] + 47 Gy/hour; III, [DMM + 25 nM Mn(II)] + 47 Gy/hour ; and IV, [DMM + 25 nM Mn(II) + 250 µg/ml each of Met, Cys, His, Ala, Gly, Pro, Ser] + 47 Gy/hour. Approximately the same number of cells (~10^6 CFUs) was inoculated onto each segment

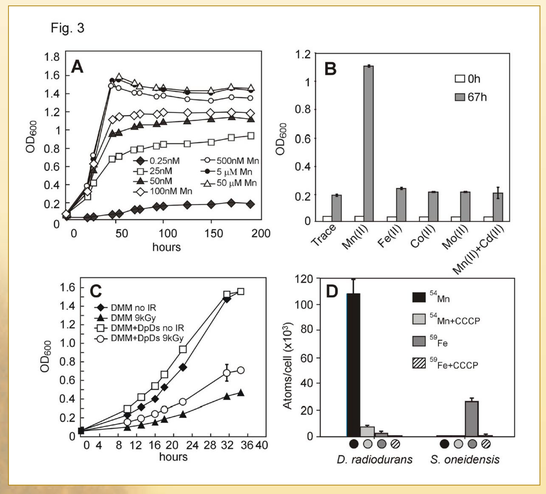
B. Growth of bacteria on TGY plates under genotoxic conditions. I, control, TGY; II, TGY + 47 Gy/hour; III, TGY + Dp + Ds (each, 250 µM); IV, TGY + 47 Gy/hour + Dp + Ds (each, 250 µM). Abbreviations: DR, *D. radiodurans* (ATCC BAA-816); DG, *D. grandis* (DSM 3963); SO, *S. oneidensis* (MR-1); EF, *Enterococcus faecium* (ATCC 19434); LP, *L. plantarum* (ATCC 14917); EC, *E. coli* (K-12) (30). Growth under chronic irradiation was tested in a ^{137}Cs Gammacell 40 irradiation unit [Atomic Energy of Canada Limited]) for 7 days at 30°C.

Fig. 2



3) Role of transition metals in wild-type *D. radiodurans*. A, Growth dependence on Mn(II) in DMM. **B,** Dependence on transition metals. *D. radiodurans* (ATCC BAA-816) was inoculated at 10^6 CFU/ml into DMM (12) containing 2.5 µM Mn(II) (manganous chloride), Fe(II) (ferrous sulfate), Co(II) (cobalt chloride), Mo(II) (ammonium molybdate), or [Cd(II) (cadmium sulphate) (2.5 µM) + Mn(II) (2.5 µM)]. For each trial, three independent incubations were at 32°C for 67 h prior to measuring cell density at OD_{600}, with standard deviations shown. Note, Cd(II) is a competitive inhibitor of Mn(II). Trace, 0.2 µM each of Mo, Cu, Cr, Bo, Zn, & I. **C,** Comparison of growth of cells following irradiation (^{60}Co, 9 kGy). For Fe-chelator studies, cells were pre-cultured in DMM to OD_{600} 0.9, irradiated and inoculated (1/20 dilution) into DMM +/- 50 µM Dp and 50 µM Ds. Non-irradiated control cells were pre-cultured and inoculated as for irradiated cells. Growth was monitored at OD_{600}. Values are from three independent trials, with standard deviations shown. **D,** *D. radiodurans* accumulated ^{54}Mn, but not ^{59}Fe, in an energy-dependent manner. When a column-value is low, see inset circle for designation and correspondence to key. Standard deviations are shown. ^{59}Fe and ^{54}Mn accumulation was as described by Posey and Gheradhini (2000) (16). Radiolabeled metal was added to cell suspensions of 6.3 x 10^7 and 1 x 10^9 CFU/mL for *D. radiodurans* and *S. oneidensis*, respectively, at a final concentration of 0.018 and 0.0057 µCi/ml, respectively, for ^{59}Fe or ^{54}Mn (Isotope Products Laboratories; ^{59}Fe 100 Ci/g; ^{54}Mn 10 Ci/g). CCCP (100 µM) was added and cells were incubated at 30°C for 1 h before adding either ^{59}Fe or ^{54}Mn. Cell density was determined by direct microscopic counting after staining with acridine orange to allow resolution of individual cells, whether they occurred singly, in pairs, or in tetrads.

Fig. 3



Conclusion B

We propose that non-enzymatic Mn(II)-based protection systems in *D. radiodurans* dominate enzymatic defences against ROS such as SOD and catalase, which together with low intracellular Fe levels help prevent oxidative stress from reaching toxic levels during recovery from radiation and desiccation. Consistently, we have previously reported that irradiated *D. radiodurans* actively repressed expression of parts of the tricarboxylic acid (TCA) cycle, particularly the $O_2^{\cdot-}$ radical-generating step (sdhB) (Liu et al., 2003), that could dampen oxidative stress production. At the same time, the glyoxylate bypass of the TCA cycle was strongly induced early in recovery, which could reinforce metabolism-conferred protection by providing biosynthetic intermediates needed for survival without generating high levels of ROS. Genomic analyses show that the repair systems identified in *D. radiodurans* appear less complex and diverse than those reported for *E. coli* or *S. oneidensis* (http://www.usuhs.mil/pat/deinococcus/index_20.htm), and few novel genes involved in radiation resistance have been identified in *D. radiodurans* so far (Table 2). Viewed in this context, it appears that *D. radiodurans* uses a relatively conventional set of DNA repair functions, but with greater efficiency than other organisms. The basis for this efficiency remains poorly defined, but might depend on non-enzymatic protection and metabolic strategies during recovery as described here, in combination with the multiple haploid genome copies per cell utilized during homologous recombination. The high intracellular Mn and low Fe levels of *D. radiodurans* are previously unrecognized components of the extreme radiation resistance phenotype, and point to potential approaches that could be used to augment recovery of sensitive organisms from radiation injury.

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