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 cloning expression of toluene and xylene genes from Pseudomonas putida. The recombinant Tol/Xy l 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 concentration and metabolism of toluene 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 improved on the engineered strain’s ability to grow on meta-toluene 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 Tol/Xy l strain is providing a model for understanding the role of Fe and reduction of toxicants coupled to organic contaminant oxidation in aerobic radionuclide contaminated sediments. For an overview of this work see http://www.usgs.gov/earth/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

Innervous volumes of soil and groundwater at numerous U.S. Department of Energy (DOE) sites have low levels of widespread materials including mixtures of heavy metals (e.g., Hg & Cu), radionuclides (e.g., U & C Tc), and toxic organic compounds (e.g., toluene). The remediation of such contaminated sites involves 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 degradation) is to recover metals and organic compounds into potable, reusable products. Recombinant organisms have been considered as cost-effective approaches 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 host bacteria that are radiation sensitive. Therefore, our approach has been to express cloned genes in the naturally radiotolerant Deinococcus bacterium, 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 m-2, for which the genetics and biochemistry have been studied in great detail. P. putida F1 and m-2 express tol 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 toluene and xylene 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.

Conclusion A

The development of viable in situ bioremediation applications is a long-term goal of the DOE, the inclusion of engineered organisms, and a variety of DOE field research efforts are currently underway (http://publik.cordm.org/publicf/PITB0March/2003.pdf). Genetically-engineered microorganisms have already been used successfully in non-DOE, regulatory agency-approved, field-scale bioremediation trials, and could be 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.

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 Streptococca ssp 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 lacticobacilli 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.

2) Affect 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 μM Mn(II), 100 μM Mn(II), or 250 μM Mn(II) and inoculated onto DMM plates containing the indicated Mn(III) concentrations. I, no irradiation control, [DMM + 25 μM Mn(II)]; II, [DMM + 2 μM Mn(II)] + 47 Gy/hour; III, [DMM + 25 μM Mn(II)] + 47 Gy/hour; and IV, [DMM + 25 μM Mn(II)] + 250 μM Mn(II) each of Me, Cu, Zn, & I. Approximately the same number of cells (~10^6 CFU/ml) were inoculated onto each segment.

3) Increased Fe levels in fur mutant

A. Engineer D. radiodurans for growth on toluene

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

1. Construction of toluene-mineralizing D. radiodurans. (A) Intermediates of toluene degradation encoded by the indicated genes. (B) Co-integration of the tol 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 (Cam). (C) Co-transformation of pMD98 and pMD864 into strain R1 with double Km and Cam selection yielded strain MD884. Abbreviation: R1, D. radiodurans strain R1 (ATCC BAA-810).

2. Rate of C₃-toluene in engineered D. radiodurans (resting cells). (A) Generation of 14CO₂. (B) Production of non-volatile radioactive products. (C) Incorporation into macromolecular cellular components with and without irradiation (137Cs, [g, 6-32P]Gy/hour). Cells were added to OD₅₀₀ = 5.0 for (A) and (B) and assayed after 48 h. Analyses shown in (C) utilized cells adjusted to OD₀₀₀ = 3.5, with C₃-toluene added to 4.1 μM. (D) Transformation of BTX 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.

3. Growth of MD884 fur/mut with meta-toluene as the sole carbon source in deionized 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.

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