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Design and Construction of Deinococcus radiodurans for Biodegradation of Organic Toxins at Radioactive DOE Waste Sites

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Research Objective

A 1992 survey of DOE waste sites indicates that about 32% of soils and 45% of groundwaters at these sites contain radionuclides and metals plus an organic toxin class. The most commonly reported combinations of these hazardous compounds being radionuclides and metals (e.g., U, Pu, Cs, Pb, Cr, As) plus chlorinated hydrocarbons (e.g., trichloroethylene), fuel hydrocarbons (e.g., toluene), or polychlorinated biphenyls (e.g., Arochlor 1248). These wastes are some of the most hazardous pollutants and pose an increasing risk to human health as they leach into the environment.

The objective of this research is to develop novel organisms, that are highly resistant to radiation and the toxic effects of metals and radionuclides, for in-situ bioremediation of organic toxins. Few organisms exist that are able to remediate such environmental organic pollutants, and among those that can, the bacteria belonging to the genus Pseudomonas are the most characterized. Unfortunately, these bacteria are very radiation sensitive. For example, Pseudomonas spp. is even more sensitive than Escherichia coli and, thus, is not suitable as a bioremediation host in environments subjected to radiation. By contrast, D. radiodurans, a natural soil bacterium, is the most radiation resistant organism yet discovered; it is several thousand times more resistant to ionizing radiation than Pseudomonas. The sophisticated gene transfer and expression systems we have developed for D. radiodurans over the last eight years make this organism an ideal candidate for high-level expression of genes that degrade organic toxins, in radioactive environments. Our ultimate aim is to develop organisms and approaches that will be useful for remediating the large variety of toxic organic compounds found in DOE waste sites that are too radioactive to support other bioremediation organisms.

Research Progress and Implications

This report summarizes work after the first 6 months of a 3-year project. The work described here has been submitted for publication. 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 have the ability to degrade or transform those 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).

Genes encoding TDO (todC1C2BA, cloned form Pseudomonas putida) were chosen for expression in D. radiodurans because TDO 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 four protein components with their attendant metal and organic cofactors and, thus, its successful expression in D. radiodurans suggests that many less complex biodegradative enzyme systems could be expressed.

Sequence Analysis of the D. radiodurans Genome: The nearly completed D. radiodurans genomic DNA sequence was searched for similarity to TDO sequences using the Basic Local Alignment Search Tool (BLAST). No D. radiodurans sequences were found to have significant homology at the

DNA level nor at the translated peptide level, suggesting that a TDO homolog does not exist in D. radiodurans strain R1 (wildtype).

Construction of D. radiodurans Strains MD560 and MD417: The todC1C2BA genes cloned into D. radiodurans (strain MD560) are constitutively expressed to make functional TDO. D. radiodurans strains R1 (wildtype), MD417 (control strain with vector sequences lacking tod genes, tod-) and MD560 (tod+) were incubated with indole and only strain MD560 yielded indigo. Incubation of 1.5 x 10+9 cells/ml of strain MD560 resulted in complete degradation of 25 nmole/ml of toluene and chlorobenzene in 30 minutes and at near equal rates. A similar reaction with 25 nmole/ml 4-dichloro-1-butene resulted in oxidation of about 40% of the substrate in 80 minutes. Incubations of overnight grown cultures of MD560 (1 x 10+8 cells/ml) with chlorobenzene, toluene and 3,4-dichloro-1-butene yielded the anticipated diol products, as determined by GC/MS analysis of culture supernatant extracts. All of the in vivo products were identical by GC/MS analysis to products obtained in vitro using purified toluene dioxygenase. Although chlorobenzene, toluene, and 3,4-dichloro-1-butene were detectably oxidized by headspace analysis, 25 nmole/ml trichloroethylene (TCE), a substrate known to inactivate TDO, was not detectably oxidized under these conditions. However, incubation of MD560 with volatile ^{14}C-TCE yielded a detectable increase in ^{14}C-nonvolatile material that was associated with the cells. This was consistent with previous studies in vivo and in vitro in which ^{14}C-TCE oxidation inactivates TDO and becomes covalently attached to cell materials. Strain MD417, lacking tod genes, was uniformly negative in the metabolism of all the TDO substrates tested.

In strain MD560, the toluene dioxygenase expression is low level constitutive (TDO concentration is undeterminable by SDS-PAGE). Our closest comparison was with E. coli DTG351 which is also constitutive, but is plasmid encoded and not chromosomal, like MD560. We have determined that the oxidation rate of ^{14}C -toluene to non volatile ^{14}C -labeled products by strain MD560 is about half of that observed for E. coli strain DTG351.

Resistance of D. radiodurans to Toluene and TCE: The cell envelope of D. radiodurans includes an outer and inner lipid membrane that surrounds the cell wall. 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. Toluene and TCE are two of the most common organopollutants at radioactive DOE waste sites; 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. Our work shows that D. radiodurans wildtype and toluene metabolizing strains 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.

Before this work, no studies regarding D. radiodurans' growth in the presence of continual radiation had been reported to our knowledge. Likewise, information regarding effects of radiation on D. radiodurans' growth and protein expression, or solvent effects, were not available. Our work strongly supports the idea that recombinant D. radiodurans strains can be constructed for biodegradation in radioactive aqueous/organic mixed wastes.

Reference

Lange C., L. Wackett, K. Minton, and M.J. Daly. 1998. Construction and characterization of recombinant Deinococcus radiodurans for organopollutant degradation in radioactive mixed waste environments, submitted.

Planned Activities

Our ultimate aim is to design novel D. radiodurans strains that can not only degrade organic toxins, but also derive carbon and energy from these compounds while at the same time surviving the effects of co-contaminating toxic metals and radionuclides. To this end, we will be working on the following projects over the next year:

1) Cloning the companion genes in the toluene catabolic pathway into D. radiodurans strain MD560 (see Research Progress). Once cloned, we intend to test the expression of those genes and determine if aromatic compounds can be catabolized to products that can be fed into the

organism's carbon-assimilating and energy-yielding pathways. A parallel investigation of ours is now sytematically mapping D. radiodurans' metabolic pathways by a combination of genomic informatics and biochemical/growth assays.

2) Since DOE radioactive sites contain high concentrations of toxic metals, D. radiodurans will have to be designed for resistance to metals (e.g., U, Co, Cd, As, Zn). We have cloned the Desulfovibrio vulgaris cytC3 gene into D. radiodurnas and have obtained a series of metal resistance genes (e.g., cnr and czc; Alcaligenes eutrophus) for introduction into our strains. Metal resistance profiles of the engineered strains will be determined.
3) We have developed an assay for D. radiodurans using a newly engineered strain that can distinguish between lethality as the result of DNA damage and lethality that is the result of cellular toxification by non-DNA damaging agents (such as solvent toxicity). This assay will be used to assess the relative toxicity of organics, metals and their potentially interacting combinations in simulated DOE waste soils. Results of this assay may help us to better understand and engineer the organism for survival in actual DOE waste environments.