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Project Title: **Design and Construction of Deinococcus radiodurans for Biodegradation of Organic Toxins at Radioactive DOE Waste Sites**

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

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A Masters student, Sara McFarlan, at the University of Minnesota, contributed to this work.

Research Objective:

Immense volumes of radioactive waste, generated from nuclear weapons production during the Cold War, were disposed directly to the ground. The current expense of remediating these polluted sites is driving the development of alternative remediation strategies using microorganisms. The bacterium *Deinococcus radiodurans* is the most radiation resistant organism known and can grow in highly irradiating (>60 Gray/h) environments (1).

Numerous microorganisms (*e.g.*, *Pseudomonas* sp.) have been described, and studied in detail, for their ability to transform and degrade a variety of organic pollutants (*e.g.*, toluene), present at many radioactive DOE waste sites. Detoxification of the organic toxins at these sites is an important goal in remediating or stabilizing contaminated sites as well as preventing their further dissemination.

The aim of this project is to engineer strains of *D. radiodurans* that are capable of degrading organic/aromatic hydrocarbons present in radioactive mixed waste sites - sites that contain mixtures of toxic organic compounds, radionuclides and heavy metals. Conventional bioremediating organisms are unable to survive at many of these sites because of their sensitivity to radiation. Generally, microorganisms are sensitive to the damaging effects of ionizing radiation, and most of the bacteria currently being studied as candidates for bioremediation are no exception. For example, *Pseudomonas* sp. is very sensitive to radiation (more sensitive than *E. coli*) and is not suited to remediate radioactive wastes. Therefore, radiation resistant microorganisms that can remediate toxic organic compounds need to be found in nature or engineered in the laboratory to address this problem.

Research Progress and Implications:

Our current research effort is directed at introducing, and expressing, degradative genes in *D. radiodurans* using well-established genetic manipulation techniques (1, 2, 3). Our efforts also continue to focus on studying the genetic mechanisms responsible for extreme radiation resistance (4); a remarkable phenotype that underlies our current work on designing this organism for remediation of radioactive waste sites.

The degradative genes/operons we are introducing into *D. radiodurans* are well-characterized, and encode enzymes/biochemical pathways that catalyze oxidative reactions. The encoded meta-cleavage pathway reactions convert unsubstituted aromatic compounds (*e.g.*, toluate) to pyruvate and other TCA cycle intermediates (*e.g.*, acetate, oxaloacetate, malate, succinate, *etc.*,); these intermediates could potentially be used by *D. radiodurans* as carbon/energy sources. To date, we have constructed and characterized a recombinant *D. radiodurans* expressing toluene dioxygenase (TDO) (1). Cloning of the multicomponent TDO into 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 Gy/hour) of a ^{137}Cs irradiator and 5 x 10^8 cells/ml degraded 125 nmole/ml of chlorobenzene in 60 minutes under those conditions. *D. radiodurans* strains were also found to be resistant to the solvent effects of toluene and trichloroethylene at levels exceeding those of many radioactive waste sites.

Because the metabolic repertoire of *D. radiodurans* was unexplored, we have investigated the ability of *D. radiodurans* to utilize the expected catabolic products of the operons targeted for cloning into *D. radiodurans*. This informed approach guided us in selecting biodegradative genes for expression in *D. radiodurans* that may yield strains capable of deriving carbon and energy from toxic organic compounds. We

are currently introducing into *D. radiodurans* catabolic genes derived from:
1) the upper part of the pAW15 TOL pathway, for the conversion of toluate/benzoate to catechol.
2) the lower part of the pAW15 TOL pathway, for the conversion of catechol to pyruvate.
3) the *tfd* fragment of JP4, for the conversion of halogenated aromatics to beta-ketoadipate.

This systematic approach was made possible by our development of a minimal medium suitable for diagnostic metabolic studies (previously published minimal media compositions were flawed and not suited to such studies). In addition to an appropriate carbon source, an exogenous source of amino acids (specifically, cysteine and histidine) was determined to be essential for *D. radiodurans* growth. The hexoses fructose and glucose, and glycolytic end-products pyruvate and lactate were also shown to be good carbon/energy sources. By contrast acetate and glycerol are poor substrates. Among TCA cycle intermediates, only oxaloacetate supported growth.

These two avenues of *D. radiodurans* research, one in designing pathways, the other in investigating its global metabolism, have converged in a third distinct area important to bioremediation of radioactive waste sites. Namely, we have shown the occurrence of phenotype reversal, from radiation resistance to sensitivity, when *D. radiodurans* cells are grown in a chemically defined medium under amino acid limiting conditions (2) - conditions that are likely to prevail in soils and sediments surrounding waste sites. The inability of the organism to utilize most TCA cycle intermediates as growth substrates, and the absolute dependence on certain exogenous amino acids for growth (even in the presence of an abundance of carbon/energy sources), suggests a defect in the global metabolic regulation that integrates carbon and nitrogen metabolism. This hypothesis is supported by our analysis of the complete *D. radiodurans* genomic sequence. Furthermore, this analysis reveals how this defect could be overcome readily by genetic engineering - a task that is important if *D. radiodurans* is to maintain its biotic potential and its global physiologic integrity in the restricted environments of radioactive waste sites. Details of this work are being prepared for publication (2) and experiments on overcoming this defect are underway.

We have recently developed and characterized four different expression systems suitable for engineering *D. radiodurans* for bioremediation of waste sites (3). Previously, we developed genetic systems to manipulate the copy number of cloned DNA in *D. radiodurans*. Now, we have used these systems to correlate a gene's expression with its cellular copy number; gene expression could be regulated by varying the gene dosage between 1 and 150 copies per cell. In short, we constructed recombinant *D. radiodurans* strains with varying copy numbers of the mercuric reductase operon (3). *D. radiodurans* strains expressing cloned mercury (II) resistance functions were constructed and shown to be highly effective at reducing toxic ionic mercury, a frequent constituent of radioactive wastes, to volatile elemental mercury - all in the context of growth at 60 Gy/h. During exponential growth, these strains supported different numbers of the *mer* operon per cell when cloned as: 1] an autonomously replicating plasmid (~1 copy per cell); 2] a chromosomal tandem duplication (~10-20 copies per cell); 3] an amplified chromosomal duplication insertion (~150 copies per cell); and 4] a chromosomal direct insertion (~10 copies per cell). These engineered strains show a close correlation between their *mer*-copy number and their resistance to Hg (II).

Published information on DOE facilities reports numerous examples of waste mixtures that resulted from co-disposal of radionuclides and toxic metals with one or more organic compound classes. To demonstrate the ease by which multiple remediating functions can be introduced into a single *D. radiodurans* host, we combined the TDO function of a recombinant *D. radiodurans* strain (1) with the mercury remediating function of another recombinant strain (3), generating a novel strain that could metabolize toluene or chlorobenzene while at the same time resisting and reducing toxic ionic mercury to volatile elemental mercury.

Radioactive waste sites containing mixtures of radionuclides, heavy metals, and organic toxins are

potentially good targets for bioremediation using non-pathogenic microorganisms that are radiation resistant, capable of degrading organic chemicals, and resisting toxic metals. The development of a series of flexible genetic expression systems for *D. radiodurans* together with our accompanying *tod* and *merA* expression data, strongly support the idea that *D. radiodurans* can be engineered for degradation of organic toxins in radioactive aqueous/organic wastes co-contaminated with heavy metals.

Planned activities (1999 - 2000):

1) Degradation of, and growth on toluene by *D. radiodurans*.
2) Integration of carbon and nitrogen metabolism in *D. radiodurans*.
3) *In vivo* gene shuffling to generate novel catabolic functions in *D. radiodurans*.

Information Access: Publications:

1) C. Lange, L. Wackett, K. Minton and **MICHAEL J. DALY** (1998) Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. Nature Biotech., **16**, 929-933.
2) D. Ghosal and **MICHAEL J. DALY** (1999) Physiologic determinants of radiation resistance in *Deinococcus radiodurans*. Manuscript in preparation.
3) H. Brim, S. McFarlan, L. Wackett, J. Fredrickson, K. W. Minton, M. Zhai, and **MICHAEL J. DALY** (1999) Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments, submitted.
4) J. Lin, R. Qi, C. Aston, J. Jing, T. S. Anantharaman, B. Mishra, O. White, K. W. Minton, J. C. Venter, **MICHAEL J. DALY**, and D. C. Schwarzt (1999) Whole genome shotgun optical mapping of *Deinococcus radiodurans* using genomic DNA molecules, submitted.