FINAL Project Report

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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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Objective:

Seventy million cubic meters of ground and three trillion liters of groundwater have been contaminated by leaking radioactive waste generated in the United States during the Cold War. A cleanup technology is being developed based on the extremely radiation resistant bacterium *Deinococcus radiodurans* that is being engineered to express bioremediating functions. Research aimed at developing *D. radiodurans* for organic toxin degradation in highly radioactive waste sites containing radionuclides, heavy metals, and toxic organic compounds was started by this group in September 1997 with support from DOE EMSP grant DE-FG07-97ER20293. Work funded by the existing grant has already contributed to eleven papers on the fundamental biology of *D. radiodurans* and its design for bioremediation of highly radioactive waste environments.

Approach:

This final report summarizes work after 2.8 years of a 3-year project dedicated to engineering the radiation resistant bacterium *Deinococcus radiodurans* for toxic organic compound degradation in radioactive DOE waste sites. Our approach matches the Aims proposed in our 1997 EMSP application and is summarized as follows. We have shown that *D. radiodurans* can be genetically engineered to express organic toxin-degrading functions using expression systems that were tested during growth at 6,000 rads/hour. While *D. radiodurans* is naturally tolerant to the concentrations of uranium and plutonium at most radioactive DOE waste sites, its growth will likely be inhibited by the concentrations of heavy metals at those sites. To demonstrate how *Deinococcus* could be engineered for both toxin degradation and metal remediation, we expressed metal resistance/reducing functions in *D. radiodurans* also engineered for toxin-degradation. For example, we have cloned the *Escherichia coli merA* locus into engineered *D. radiodurans* strains capable of degrading chlorobenzene, 3,4-dichloro-1-butene, and toluene. These *merA*-containing strains were resistant to highly toxic Hg(II), and could reduce Hg(II) to much less toxic and volatile Hg(0). An assortment of genes encoding resistance to Hg(II), Pb(II), and Cr(VI) is now being tested in *D. radiodurans* in combination with organic toxin-degrading genes. Characterization of organic compound degrading strains was aided by our development of a synthetic minimal medium for *D. radiodurans*. In combination with our genomic informatic analyses, this medium has enabled us to identify the minimum nutrient requirements necessary to support growth in nutrient poor radioactive environments, and to explain how radiation resistance relates to this organism's metabolic repertoire. Together, these studies have facilitated our ongoing experimental efforts to engineer *D. radiodurans* for growth on toxic organic compounds present in most radioactive, heavy metal-contaminated environments. It now appears likely that we will be able to engineer *D. radiodurans* to use chlorinated hydrocarbons as carbon/energy sources that could provide electron donors for growth of toxin-degrading *Deinococcus* and, possibly, metal reduction; we have shown that anaerobic cultures of wildtype *D. radiodurans* can reduce U(VI), Tc(VII), and Cr(VI).

Results: Engineering *D. radiodurans* for organic toxin degradation, 1997-Present (The reader is directed to the published data listed below Refs. 1-11)

Our research aimed at developing *D. radiodurans* for toxin degradation in radioactive environments began in 1997 with the demonstration that this bacterium can grow in the presence of ionizing radiation at 6,000 rads/hour **(Ref. 1, 2)**, comparable to the most radioactive DOE waste sites. In fact, all reported members of the *Deinococcaceae* can grow at this dose rate and are poised to contribute their individual characteristics to this developing technology. For example, *D. geothermalis* grows optimally at about 50°C, and we have recently shown that the expression systems developed for *D. radiodurans* work in this thermophile and that its growth is superior to *D. radiodurans*' in radioactive environments **(Ref. 3, 4)**. As such, it is likely that the genetic technology being developed for *D. radiodurans* will be readily transferable to *D. geothermalis*, and could be useful in thermally insulated radioactive environments (*e.g.*, within or beneath leaking tanks) where temperatures can be elevated due to radioactive decay. Initially, growth of the *Deinococcaceae* during high-level chronic irradiation exposure was thought to be unlikely since it had been reported that DNA replication in *D. radiodurans* ceases upon DNA damage and RecA expression **(Ref. 8)**. However, as manifested by their growth in a ^{137}Cs irradiator, these bacteria are proficient at

simultaneous semi-conservative DNA replication and homologous recombination **(Ref. 4)**, as modeled previously. We have tested *D. radiodurans* with a variety of cloned bioremediating gene functions that can be expressed during growth at 6,000 rads/hour; and these engineered strains are now being used in the design of more complex organic compound degradation systems that exploit our recent progress.

Growth on Toxic Organic Compounds During Metal/Radionuclide Remediation:

It now appears likely that we will be able to engineer *D. radiodurans* to use chlorinated hydrocarbons as carbon/energy sources that could provide electron donors for growth of toxin-degrading *Deinococcus* and, possibly, metal reduction; we have shown that anaerobic cultures of wildtype *D. radiodurans* can reduce U(VI), Tc(VII), and Cr(VI) **(Ref. 5)**. With respect to DOE facilities, there has been no adequate method for microbiological treatment of contaminant waste sites containing both hazardous radioactive metal and organic components since organisms like *Pseudomonas* spp. are very radiation sensitive (**Ref. 3**). High concentrations of toxic organic compounds (*e.g.*, toluene) in mixed radioactive wastes pose as viable carbon/energy sources for engineered *Deinococcus* **(Ref. 2, 3)**. Such metabolic capabilities would not only potentially provide electron donors for growth of metal-remediating *Deinococcus* **(Ref. 3)**, but would provide an effective alternative to conventional physicochemical treatments of toxic organic compounds.

In the presence of toluene, *D. radiodurans* expressing *todC1C2BA* (TDO) produces toluene-*cis*-dihydrodiol that is further metabolized to 3-methylcatechol by a native non-specific dehydrogenase **(Ref. 1, 2)**. Once formed, catechols readily polymerize to form insoluble polymers and this has been observed in *tod*-engineered *D. radiodurans* strains, that turn dark brown in the presence of toluene over the course of several days to weeks. Other *Pseudomonas* catabolic genes that convert 3-methylcatechol to pyruvate have been introduced into *todC1C2BA*-containing *D. radiodurans*, that may yield a strain that is able to mineralize toluene and related chlorinated compounds.

Engineering *D. radiodurans* for Resistance to Common Metallic Waste Constituents:

While *D. radiodurans* is naturally tolerant to the uranium and plutonium concentrations at most DOE sites, growth of these bacteria will likely be inhibited by the concentrations of heavy metals prevalent at those sites (mercury, chromium, and lead). A series of genetic vectors that encode resistance to these metals have been constructed and are being examined in *D. radiodurans* strains also expressing organic toxin degrading genes **(Ref. 1)**. For example, the highly characterized *merA* locus from *Escherichia coli* has been cloned into *D. radiodurans* also expressing genes encoding toluene dioxygenase (*tod*) **(Ref. 1, 2)**. *tod*-containing *D. radiodurans* expressing *merA* were resistant to the bacteriocidal effects of highly toxic, thiol-reactive mercuric ion, Hg(II), at concentrations (50 μM) well above the highest concentration reported for mercury-contaminated DOE waste sites (10 μM). These strains were also very effective at reducing Hg(II) to much less toxic and nearly inert elemental and volatile Hg(0). We have very carefully characterized these *D. radiodurans* strains expressing both organic-degrading and metal-reducing functions and have shown how expression of these genes can be regulated. These studies have formed the foundation of our ongoing efforts to expand the remediating capabilities of this bacterium and to ensure survival of these constructs in environments co-contaminated with radionuclides, heavy metals and toxic organic compounds.

Physiology, Radiation Resistance, and Degradation of Organic Toxins:

Adding to the challenge of surviving the harsh radioactive, metallic, and organic properties of DOE waste sites is the likelihood that *D. radiodurans* may be limited by several inherent physiologic constraints. For example, genomic informatics shows that the amino acid biosynthetic pathways for serine, cysteine and lysine are incomplete in wildtype *D. radiodurans*. One major thrust of our research involves characterization of its physiology and optimization of the external parameters for growth and survival in adverse radioactive environments (**Ref. 4**). For example, under optimal growth conditions *D. radiodurans*' DNA repair capabilities are extremely well suited to survive either acute (**Ref. 10**) or chronic irradiating exposures (**Ref. 1, 4**). However, *D. radiodurans* is unable to grow and is rapidly killed in certain nutrient poor radioactive environments that support luxuriant *D. radiodurans* growth when radiation is absent (**Ref. 4**). This phenotypic reversal from radiation resistance to sensitivity is of great interest and concern to us since it questions the suitability of *D. radiodurans* as a bioremediation host in radioactive waste sites. A combination of growth studies (**Ref. 4**) and analysis of the complete *D. radiodurans* genomic sequence (**Refs. 6, 7, 9, 11**) has identified several defects in *D. radiodurans*' global metabolic regulation that limit carbon, nitrogen and DNA metabolism. In nutrient-restricted conditions, DNA repair was found to be limited by this organism's metabolic capabilities and not by any nutritionally induced defect in genetic repair; and, this information has been used successfully as a guide to identify key nutritional constituents that restore luxuriant growth of *D. radiodurans* in nutritionally restricted radioactive environments (**Ref. 4**). Analyses like these, coupled to the possible integration of native and cloned metabolic pathways in *Deinococcus*, will facilitate the design of *in situ* remediation protocols for this organism.

Genome Flexibility and its Impact on Constructing Bioremediating *D. radiodurans* :

The preeminent factors that are allowing rapid progress in the area of genetic engineering of *D. radiodurans* are: 1) *D. radiodurans* is highly transformable using DNA with homology to its genome (**Ref. 1, 2**); 2) it is prolific in its ability to amplify DNA sequences that are flanked by direct repeats (**Ref. 2, 6**); 3) a large variety of *D. radiodurans* vectors has already been developed (**Ref. 1**); and 4) the entire genomic sequence of *D. radiodurans* is now available and is being subjected to analysis (**Ref. 6, 7, 9, 11**).

Upon transformation, if a gene is integrated between direct repeats of *D. radiodurans* genomic DNA, selection pressure can yield recombinant strains with about 20 duplicated copies per chromosome, 8-10 identical chromosomes per cell. We have used this strategy to amplify vectors as large as 20kb, encoding organic toxin degradation in *D. radiodurans*, resulting in a genome that contains about 4 Mbp more DNA than wildtype (**Ref. 1**); these expansions are stable and readily maintained for many generations, even without selection. We have exploited this organism's ability to amplify genes and have engineered a strain that can detoxify both toluene (and related compounds) and Hg(II) ions efficiently during growth in the presence of chronic radiation (**Ref. 1, 2, 3**).

Summary of Publications: EMSP Grant DE-FG07-97ER20293: 1997-Present

1 H. Brim, S. McFarlan, L. Wackett, K. W. Minton, M. Zhai, J. Fredrickson, and **MICHAEL J. DALY** (2000) Engineering *Deinococcus radiodurans* for metal remediation in radioactive

mixed waste environments. *Nature Biotechnology*, **18**, 85-90.

2 C. Lange, L. P. Wackett, K. W. Minton and **MICHAEL J. DALY** (1998) Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. *Nature Biotechnology*, **16**, 929-933.

3 **MICHAEL J. DALY** (2000) Engineering radiation-resistant bacteria for environmental biotechnology. *Current Opinion in Biotechnology* **11**, 280-285.

4 A. Venkateswaran, S. C. McFarlan, D. Ghosal, K. W. Minton, A. Vasilenko, K. Makarova, L. P. Wackett, and **MICHAEL J. DALY** (2000) Physiologic determinants of radiation resistance in *Deinococcus radiodurans*. *Appl. Environ. Mirobiol.* **66**, 2620-2626.

5 J. K. Fredrickson, H. M. Kostandarithes, A. W. Li, A. E. Pyle, and **MICHAEL J. DALY** (2000) Reduction of Fe(III), Cr(VI), U(VI), and Tc(VIII) by *Deinococcus radiodurans*. *Appl. Environ. Microbiol.* **66**, 2006-2011.

6 K. S. Makarova, Y. I Wolf, K. W. Minton, O. White, and **MICHAEL J. DALY** (1999) Short repeats and insertional elements in *Deinococcus radiodurans* and comparison to other bacterial species. *Res. Microbiol.*, **150**, 711-724.

7 K. S. Makarova, L. Aravind, **MICHAEL J. DALY**, and E. Koonin (2000) Specific expansion of protein families in the radioresistant bacterium *Deinococcus radiodurans*. *Genetica,* In Press.

8 A. K. Sharma, A. Jambura, M. M. Cox, R. B. Inman, K. W. Minton, and **MICHAEL J. DALY** (2000) Expression and characterization of the RecA protein from the extremely radioresistant bacterium *Deinococcus radiodurans*. Submitted to *J. Biol. Chem.*

9 K. S. Makarova, E. V. Koonin, L. Aravind, L. Tatusov, Y. I. Wolf, O. White, J. R. Battista, and **MICHAEL J. DALY** (2000) The genome of the extremely radioresistant bacterium *Deinococcus radiodurans*: Comparative genomics and applications. *Microbiology and Molecular Biology Reviews*. Submitted.

10 J. Lin, R. Qi, C. Aston, J. Jing, T. S. Anantharaman, B. Mishra, O. White, K. W. Minton, **MICHAEL J. DALY**, J. C. Venter, and D. C. Schwarzt (1999) Whole genome shotgun optical mapping of *Deinococcus radiodurans* using genomic DNA molecules. *Science,* **284**, 1558-1561.

11 O. White, J. A. Eisen, J. F. Heidelberg, E. K. Hickey, J.D. Peterson, R. J. Dodson, D. H. Haft, M. L. Gwinn, W. C. Nelson, D. L. Richardson, K. S. Moffat, H. Qin, L. Jiang, W. Pamphile, M. Crosby, M. Shen, J. J. Vamathevan, P. Lam, L. McDonald, T. Utterback, C. Zalewski, K. S. Makarova, L. Aravind, **MICHAEL J. DALY**, K. W. Minton, R. D. Fleischmann, K. A. Ketchum, K. E. Nelson, S. Salzberg, H. O. Smith, J. C. Venter, C. M. Fraser (1999) Sequencing and functional analysis of the *Deinococcus radiodurans* genome. *Science*, **286**, 1571-1577.