Genetic Engineering of a Radiation Resistant Bacterium for Bioremediation of Mixed Waste in Bioreactors

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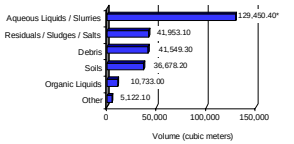
Abstract

The mixture of toxic chemicals, heavy metals, halogenated solvents and radionuclides in many DOE waste materials presents a challenging remediation problem. One approach for dealing with mixed wastes would be to selectively remove classes of hazardous wastes producing a less complex mixture. Recent work with the deinococci suggests a new approach to this problem. *Deinococcus radiodurans* R1 is a bacterium that is the most highly radiation resistant organism known. It can survive 5Mrad of gamma radiation and is also highly resistant to UV radiation and desiccation. Although the deinococci do not appear to naturally degrade toxic organics, such systems are present in less radiation resistant species, and are well characterized at the genetic level. The goal of this project is to develop a suite of *D. radiodurans* strains for treatment of toxic organics in mixed waste streams in an optimized above-ground reactor system. Genetic tools have been developed for the construction of strains meeting the requirements for successful treatment: 1.) High expression activity, 2.) Regulated expression, and 3.) Stable expression in the absence of antibiotic selection

Background

Low level mixed waste (LLMW) contains a toxic component in combination with low-level radioactive waste. An estimated 226,000 m^3 of DOE LLMW will need to be disposed of, as a result of research, development, and production of nuclear weapons over the next 20 years (8). Because of the initial chemical complexity of mixed waste mixtures, these treatment systems should be carried out at relatively low temperatures and pressures and without the addition of chemicals, to avoid possible secondary reactions that might generate new toxic species. Therefore, a microbiological treatment system is an attractive possibility for separation and treatment.



*This waste volume includes 114,600 cubic meters of mixed low level waste water anticipated to be generated from the treatment of HLW from Hanford's tank waste evaporator

Figure 1: DOE Low Level Mixed Waste Types. Most LLMW stored in 1995, or predicted to be created by 2000 is in the form of aqueous liquids or slurries. Adapted from DOE mixed waste overview (7)

Enzymes exist in bacteria that detoxify halogenated organics by cometabolism (non-growth) mechanisms, and theoretically these could be used to selectively remove these compounds from mixed wastes under mild conditions. However, bacteria containing these systems would not be expected to survive in the mixed waste streams that must be treated.

Figure 2: *Deinococcus radiodurans* R1. Photo by John Battista. http://www.nsf.gov/od/lpa/news/publicat/frontier/10-96/10bugs.htm

Deinococcus radiodurans R1 is a bacterium that can survive 5 Mrad doses of gamma-irradiation, and is also highly resistant to desiccation and to peroxides. This resistance is apparently due to highly active DNA repair systems, high chromosome copy number, and an unusual RecA protein (4,5). *D. radiodurans* is a candidate strain for genetic engineering to survive in and detoxify DOE's mixed waste streams. The desiccation-resistance of this organism suggests that once successful treatment strains are developed, they could be grown in a production facility, dried down, and used to seed contaminant mixtures on site, thereby providing a potentially convenient and economic solution to selective removal of contaminants in such waste materials.

The desired characteristics of bioreactor strains are 1.) high biodegradative activity, 2.) regulated expression of biodegradative ability, and 3.) stable phenotype in the absence of selection or expression. In order to construct such strains, more information is needed on promoters in deinococci and stable expression systems are needed (2).

References:

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Goals

1. *Develop D. radiodurans strains meeting requirements for bioreactor treatment*
   - High biodegradative activity
   - Regulated expression
   - Stable phenotype in the absence of selection
2. *Test strains in simulated waste*
   - Evaluate pertinent process variables
   - Develop optimized treatment strategy

Approach to Date

1. *Develop new genetic tools for D. radiodurans* R1
   - Stable integrative vector
   - *Deinococcus* shuttle vector with minimal replicon and multiple unique cloning sites.
2. *Identify and characterize promoters*
   - Collect a suite of promoters with varying strengths for expression
   - Investigate basic promoter structure in *D. radiodurans* R1.
   - Develop and test expression vectors

Conclusions

- New genetic tools were created for use in *D. radiodurans*.
- Promoters were identified and characterized.
- Promoters with varying strengths were identified, and one was tested in an expression vector.
- Strains are being constructed to express broad range oxygenases such as soluble and particulate methane monooxygenase
- These strains will be tested for bioremediation activity and stable expression.

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Results

Vector Development

I. Stable chromosomal insertion vectors (Figure 3)
- Double crossover insertion in non-essential amylase (*amyE*) gene.
- Linear vector, cut at *Sca*I site, favors double crossover insertion.
- 100% stability of marker insertion into chromosome after 100 generations without selection.

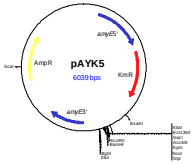

Figure 3: Stable chromosomal insertion vector. This vector inserts a marker (kanamycin resistance) and target DNA into the *D. radiodurans* R1 chromosomal gene, *amyE*.

II. Convenient, versatile *D. radiodurans* shuttle vectors
- Minimum replicon was identified for the large *Deinococcus* shuttle vector, pI3 (2). (Figure 4)
- Analysis of chloramphenicol resistance without selection showed that the gene *resU*, named for its similarity to resolvase genes was necessary for stability. (Figure 5)
- Unique cloning sites were added to minimal replicon for flexibility in cloning. (Figure 6)

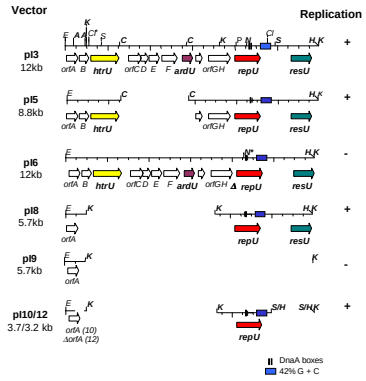

Figure 4: Deletion derivatives of *Deinococcus* derived portion of the large shuttle vector pI3. This region was sequenced and restriction deletions were made in pI3 to sequentially disrupt or remove open reading frames, and to determine the minimal replicon, contained in pI12.

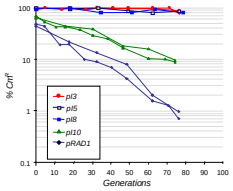

Figure 5: Stability of Shuttle Vectors in *D. radiodurans* R1. After 75+ generations in the absence of selection (chloramphenicol) dilutions were plated on Cm to determine plasmid stability

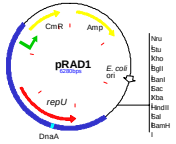

Figure 6: Shuttle vector pRAD1. Showing: Sequence derived from Deinococcus plasmid (■), repU gene necessary for replication, (■) location of putative *Deinococcus* promoter driving CmR (■).

Deinococcus Promoters

I. *Deinococcus* promoters identified
- Directed search for several genes from genome sequence
- Random search using clone bank and new integrative promoter probe vector (similar to Figure 3).

II. Several selected for activity screening and transcription start site mapping
- Activity of promoters determined using *lacZ* expression; weak, medium, and strong promoters characterized.
- Transcription start site mapping (Figure 7) revealed preliminary vegetative sigma consensus sequence (Figure 8).

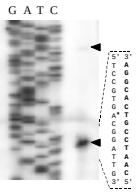

Figure 7: Major and minor vegetative transcription start sites for *D. radiodurans* R1 *groESL* operon promoter.

$groESL_M$	TTGACATTTTTCTTATCGGCGCTCTACCATCCGTGA*
$groESL_m$	GTCCCAGCGCCCCTTGAGCGTCATAGACTCAGA*
aceR	GGAACAGCCGGCCTCATTTGCTGTAAAATGAAATC*
lexA	TCCTCGTAGGCCAATTCTGACGGTTGGGCCGCCGCTTG*
pI3 *resU*	GGAACCCCGCGCCGACCCCCCTTAATGCGGC*
rpoBC	TTGACAGGGAATCATGAGCGCCCTATACTTTC*
Consensus	KKNMCA - $N_{17/18}$ - TANNNT
E. coli σ^{70}	TTGACA - N_{17} - TATAAT

Figure 8: Transcription start sites (*), promoter region, and preliminary consensus sequence for *D. radiodurans* R1 sigma 70 promoters. $groESL_M$ and $groESL_m$ are major and minor start sites respectively. See Figure 7.

III. Promoter tested in integrative expression vector
- Strong constituitive promoter tested in new integrative expression vector using cometabolic genes of toluene dioxygenase (*todC1C2BA*) from *Pseudomonas putida*, previously shown to be expressed in *D. radidurans* (1).
- Activities similar to *P. putida* mutant blocked after *todC1C2BA* genes (9) were obtained.