Progress Report 6/02 – 6/03

Research Objective: The specific aims of this project are to: 1) Clone and express broad spectrum oxygenases in *D. radiodurans* with a target TCE degradation rate of 1 nmol/min/mg protein, 2) analyze and upregulate stress response systems in *D. radiodurans*, and 3) test survival and activities of these strains in artificial mixtures of contaminants, designed to simulate DOE mixed waste streams, using bench-scale treatment reactors. In addition, we proposed to generate a set of new genetic tools to carry out this work. In this report, progress is described in the development of new tools and in the study of solvent resistance, heat shock regulation, and polyP metabolism.

Research Progress and Implications

This work summarizes work in year 3 of a 3-year project.

I. New Tools: System for Generation of Unmarked Mutants

For strain construction it is extremely useful to be able to generate clean unmarked deletions, and in addition this allows the construction of successive multiple mutations. We have modified the *cre-lox* insertion system we developed for *Methylobacterium* (Marx & Lidstrom, 2002) for successful usage in *D. radiodurans*, including adding a *Deinococcus* promoter upstream of the kanamycin marker and addition of a chloramphenicol resistance marker driven by a second *Deinococcus* promoter. This has been an essential tool for this project.

II. Solvent Resistance in *D. radiodurans*

As reported previously, we have used our expression systems to generate a stable insertion strain of *D. radiodurans* with toluene dioxygenase (TOD) expressed to give a toluene degradation rate of 24 nmol/min/mg protein under standard assay conditions (0.4 mM substrate). However, when rate was assessed vs. toluene concentration, we found the rate increased up to a toluene concentration of 1.5mM (Fig. 1). At this high toluene concentration, the rate was 70 nmol/min/mg protein, higher than any previously reported strain. 1.5 mM toluene is toxic to most bacteria, but surprisingly, we have found that *D. radiodurans* is quite solvent-resistant.

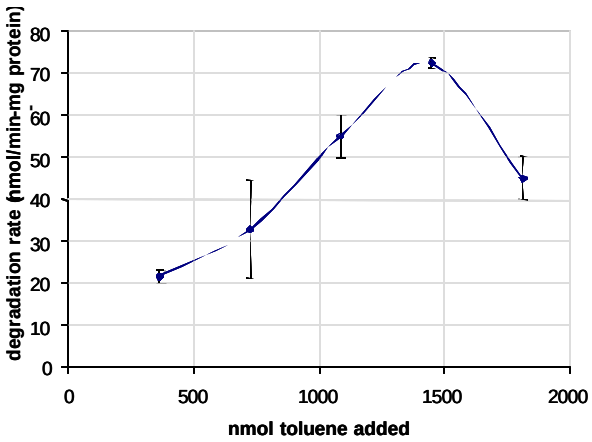

Fig. 1. Initial degradation rate of toluene by TOD-expressing construct with varying amounts of toluene added to a 1ml culture

The extremely solvent resistant bacterium *P. putida* DOT-T1E shows 5 orders of magnitude loss of survival when exposed to 0.3% (28mM) toluene for 30 minutes unless it has been pre-grown with low levels of toluene (Ramos et al., 1998). *D. radiodurans*, however, showed no loss of viability after 30 minutes or 1 hour contact with 0.3% toluene with or without pre exposure to toluene. This solvent-resistant characteristic will be highly advantageous for a treatment strain. The TOD-expressing strain degrades TCE at 2 nmol/min/mg protein, double our target rate. Therefore, we now have a strain in which the target degradation rates for both toluene and TCE have been exceeded.

III. Stress response systems in *D. radiodurans*

The mixed wastes that might be treated by our process strains are varied in composition, but all involve harsh conditions including high ionic strength, non-neutral pH, and in some cases, high temperature, high solvents, and/or high heavy metals. Therefore, a desired characteristic of effective process strains is survival and activity in such harsh chemical conditions. Our approach

has been to survey native resistance to a variety of stresses, analyze genes involved in response to such stresses, and manipulate those genes to increase resistance as needed. As reported previously, we have found *D. radiodurans* to be remarkably resistant to a variety of stresses. However, *D. radiodurans* is not especially heat-resistant. We have found that it shows a similar heat shock-dependent viability loss as other bacteria with a similar optimum growth temperature, and that a putative RpoE-class of sigma factors (Sig1) is involved in regulation of heat shock response (Schmid & Lidstrom, 2002).

A. Heat Shock Reponse: proteomics. To define the heat shock regulon more fully, we have pursued proteomics approaches, to define Sig1-regulated genes.

For the proteomics, we have taken two approaches. First, we have worked with Kendrick Laboratories, Inc. to carry out 2D gel analysis of extracts of Sig 1 and Sig 2 mutants and wild-type grown at 30C (non-heat shock conditions) and after 1 hr exposure to 48C (heat shock conditions). Excellent gels were obtained, with about 300 spots resolved. Of these, 67 were induced more than 2-fold at 48C, as compared to 30C, and of those, 20 were reduced either in the Sig1 or Sig2 mutant, or in both. In general, the Sig2 mutant showed fewer proteins changing and the differences were less distinct than in the Sig1 mutant. We are in the process of analyzing these 20 spots by LC-MS via a facility at the Fred Hutchinson Cancer Research Center in Seattle, and have data on the 7 spots showing the greatest change. In addition, we have included one spot that is heat-inducible with no change in the mutants. That protein turned out to be Ctc, a general stress protein. Two other proteins were clear heat shock proteins, trigger factor and Hsp20, a small heat shock protein. We did not identify GroES or DnaKJ in this screen, but the pI of GroES would have made it difficult to detect. It is likely that GroEL, DnaK, and DnaJ will be found in the other 12 spots. The other 5 spots all contained more than one protein, a not uncommon phenomenon with 2D gels. We are currently using quantitative RT-PCR with SYBR Green (Earl et al., 2002a) to determine which of the proteins in each of those spots shows heat shock-inducible changes in mRNA. So far, we have worked out the protocol with *groES* and *dnaK*, using a gene for the predicted S-layer protein (*hpi*) as a standard. This transcript is produced at high levels and is not affected by heat shock, so it makes a good standard for comparison. So far our data show the expected increase in expression for *dnaK* and *groEL* after exposure to 48C than at 30C. Of more significance are the proteins of unknown function we have identified from the 2D gels, which may represent novel heat-shock genes in *D. radiodurans*. If the RT-PCR results indicate that any of these are heat-shock inducible, we will generate mutants in these and test the heat-shock phenotype.

B. PolyP Metabolism. As part of this specific aim, we proposed to engineer metal resistance in *D. radiodurans* by manipulating polyP metabolism. Since the only bacterium for which polyP metabolism has been studied in detail is *E. coli*, it is important to understand how this system is organized in *D. radiodurans* to determine the best approach for developing a strain in which polyP could first be induced to accumulate and then be degraded. Our approach has been to analyze the genome sequence for putative phosphate regulon genes, generate mutants in those genes involved in polyP metabolism, assess mutant phenotypes, and measure expression of these genes.

1. Genome analysis. *D. radiodurans* contains genes predicted to encode all of the key genes of the phosphate regulon, except *phoR*, encoding a sensor kinase that acts with PhoB. It is possible that *D. radiodurans* uses a different sensor kinase for Pho regulation, as it is likely that a partner for PhoB does exist. Not only have we identified putative Pho boxes in front of these genes, we have shown phosphate-starvation induction of one of these promoters (see below), suggesting PhoB/R-like regulation. PhoB and another known regulatory gene, PhoU, are adjacent and transcribed in the same direction. The two key genes for manipulating phosphate precipitation are *ppk* encoding polyP kinase (the enzyme that synthesizes polyP) and *ppx*

encoding the polyphosphatase. In addition, it is likely the Pit transporter is important. One of the unusual features of the sequence is that *ppk* is predicted to contain a frameshift, but when we analyzed this gene in chromosomal DNA, it did not exist, and is presumably a mistake in the genome sequence. We have confirmed that active enzyme is expressed from this gene in *E. coli* (see below).

2. Minimal medium. In order to analyze the response of *D. radiodurans* to phosphate stress, it is necessary to have an effective minimal medium lacking phosphate. Unfortunately, the only minimal medium in the literature contains a phosphate buffer and produced a 20 hr doubling time (Venkateswaran et al., 2000). For a bacterium that grows with a 1.5-2 hr doubling time in rich medium, such slow growth is indicative of high nutritional stress. Therefore, we have developed an alternate medium with no added phosphate that reproducibly gives a 4-5 hr doubling time. This is based on a medium used for phosphate limitation in other bacteria, which contains a MOPS buffer (Neidhardt et al., 1974), and it appears that one reason for the significantly enhanced growth is the use of this buffer. In addition, this medium contains nicotinic acid, shown previously to be required (Venkateswaran et al., 2000), and a few amino acids. Although the literature indicates that cysteine and histidine are required (Venkateswaran et al., 2000), there is some ambiguity since amino acids are used as both nitrogen and sulfur sources. In our medium neither cysteine nor histidine is absolutely required. Instead, a sulfur-containing amino acid is required as well as one or two of a set of amino acids that serve as a nitrogen source. In our medium, the best set is methionine, serine, and asparagine. Finally, the best carbon source in our hands is glycerol. We are now using this medium in all experiments involving nutritional stress.

3. *ppx* and *ppk* mutants. Using our new system for generating unmarked chromosomal deletions, we have generated deletions of *ppk* and *ppx*. These mutants do not show a growth phenotype in either rich or minimal medium, and they also grow similar to wild-type under amino acid or carbon limitation. These results are in contrast to those found for these mutants in *E. coli*, in which cells grow slowly on minimal medium plates, show long lags upon inoculation, and show reduced survival in stationary phase (Kornberg et al., 1999). However, in *E. coli* these responses involve the RpoS regulon, and *D. radiodurans* does not contain an RpoS homolog. Therefore, we expect the regulatory system to be different from *E. coli*.

4. *ppx* and *ppk* promoters. Promoter regions have been cloned upstream of these two genes from *D. radiodurans* using our *lacZ* reporter vector, and the expression of the promoters has been assessed under different nutritional conditions, including limitation by phosphate, amino acids, and carbon source. Compared to controls that were resuspended in complete medium, the only major difference in expression was in the phosphate-limited cells. Under these conditions, the *ppk* promoter is induced about 10-fold, which is in contrast to *E. coli*, where *ppk* is not induced by phosphate limitation. The *ppx* promoter is not induced under these conditions. Although we have not yet measured polyP in the cells (see below), these results would suggest that during phosphate limitation polyP should accumulate, while in the other nutrient limitation conditions, we would expect much less polyP accumulation. These preliminary results suggest that the regulation of this system is significantly different from *E. coli*, and is not tuned to general stress in the same way. We have found putative Pho box sequences within 500 bp upstream of three phosphate regulon genes (*ppk*, *ppx*, and *phoA*), see below, consistent with regulation in response to phosphate level.

Pho Box consensus sequence (Wanner, 1993): GA(A/C)AGTA(T(A/T)T(A/T))GA(A/C)A(G/T)T(G/A)

D. radiodurans ppk (15/18 agreement): **GAAA**C**T**T**TTTA**G**GACAGT**

D. radiodurans ppx (13/18 agreement): **AAGTATTTG**G**C**G**GTG**

D. radiodurans phoA (10/18 agreement): **GACAG**CGC **ATT**C **A**GC **G**

5. Biochemistry of Ppx, Ppk, and polyP. In order to follow the biochemistry of the Pho system in *D. radiodurans*, we have set up assays for Ppx, Ppk, and polyP (Kornberg et al., 1999;

Mullan et al, 2002). The best versions of these assays require purified Ppk, which is not available commercially. We have made a His-tagged construct for Ppk from *E. coli*, purified large quantities of the enzyme, and are now using this to assay Ppx, Ppk (reverse reaction), and polyp in *D. radiodurans* wild-type and mutants. In addition, we have His-tagged versions of both Ppx and Ppk from *D. radiodurans* and have expressed those in *E. coli*. We are currently purifying them also to have protein for carrying out biochemical analyses, for instance, determining kinetic constants and the extent of the forward and reverse reactions.

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Planned Activities: a renewal proposal has been submitted to continue this project.

Information Access:

Schmid AK, Lidstrom ME. 2002. Involvement of two putative alternative sigma factors in stress response of the radioresistant bacterium *Deinococcus radiodurans*. J Bacteriol. 184(22):6182-9.

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