Final Report

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University of Washington

Genetic Engineering of a Radiation-Resistant Bacterium for Biodegradation of Mixed Wastes

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Executive Summary

Aqueous mixed low level wastes (MLLW) containing radionuclides, solvents, and/or heavy metals represent a serious current and future problem for DOE environmental management and cleanup. In order to provide low-cost treatment alternatives under mild conditions for such contained wastes, we have proposed to use the radiation-resistant bacterium, *Deinococcus radiodurans.* This project has focused on developing *D. radiodurans* strains for dual purpose processes: cometabolic treatment of haloorganics and other solvents and removal of heavy metals from waste streams in an above-ground reactor system. The characteristics of effective treatment strains that must be attained are: a) high biodegradative and metal binding activity; b) stable treatment characteristics in the absence of selection and in the presence of physiological stress; c) survival and activity under harsh chemical conditions, including radiation. The result of this project has been a suite of strains with high biodegradative capabilities that are candidates for pilot stage treatment systems. In addition, we have determined how to create conditions to precipitate heavy metals on the surface of the bacterium, as the first step towards creating dual-use treatment strains for contained mixed wastes of importance to the DOE.

Goals, Objectives, and Accomplishments

The specific aims of this project period were to: 1) Clone and express broad spectrum oxygenases in *D. radiodurans* with a target TCE degradation rate of 1nmol/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. We have achieved the goals of the first two specific aims, and have achieved preliminary progress on the third specific aim.

Project Activities

I. Develop new genetic tools

All of the vectors existing at the start of this project period suffered from disadvantages of large size, lack of sequence information, lack of information on promoters, and instability in the absence of selection. Therefore, they were unsuitable for the construction of effective strains for treatment of compounds that must be degraded by cometabolism, such as many of the problem haloorganic contaminants at DOE sites. Since these compounds do not support growth, their presence does not provide positive selection for the biodegradative systems, and it is clearly not reasonable to include selective antibiotics in a mixed waste treatment system. Therefore, in the early part of this project period we finished up work started in the previous project period, the development of a suite of convenient and stable gene replacement (insertion) vectors and replicating vectors to aid in the genetic manipulation of *D. radiodurans*.

Two types of genetic tools were developed, integration vectors and replicating vectors. We have sent these new vectors out to dozens of laboratories world-wide, and it is clear the development of these systems is having a major impact on the use of *D. radiodurans* to study a wide variety of topics.

A. Integration vectors. A series of vectors have been developed that target integration of expressed genes to non-essential sites in the chromosome via double crossover recombination. The sites that have been chosen and found to be successful are genes encoding amylase and pullulanase. The vectors include derivatives for cloning and expression and for analysis of

promoter activity using two reporters, catechol diooxygenase (XylE) and beta-galactosidase (LacZ). The vectors have also been tested with green fluorescent protein. All of the integration vectors have been shown to generate stable constructs, with no detectable loss of the insertion after 60 generations in the absence of selection. The work describing the amylase insertion vectors has been published (Meima et al., 2001), and a manuscript is under review that describes the suite of pullulanase vectors (Rothfuss & Lidstrom, submitted.; Fig. 1). Each insertion site has advantages, so both are useful genetic tools, and the two allow us to generate strains with two different insertions in neutral sites. We also have other candidate sites, and can generate more insertions in a single strain, if necessary.

B. pI3-based replicating vectors. A second series of replicating vectors has been developed based on the previously reported *D. radiodurans* plasmid, pI3. The plasmid has been completely sequenced and potential replication functions identified by deletion analysis. A minimal replicon has been cloned and used to generate a suite of small and convenient shuttle vectors, including vectors for general cloning, expression, and promoter analysis with LacZ as a reporter. These vectors are useful cloning and screening tools, but are not stable in the absence of antibiotic selection and will not be used for strain construction. A manuscript describing this work has been published (Meima & Lidstrom, 2000).

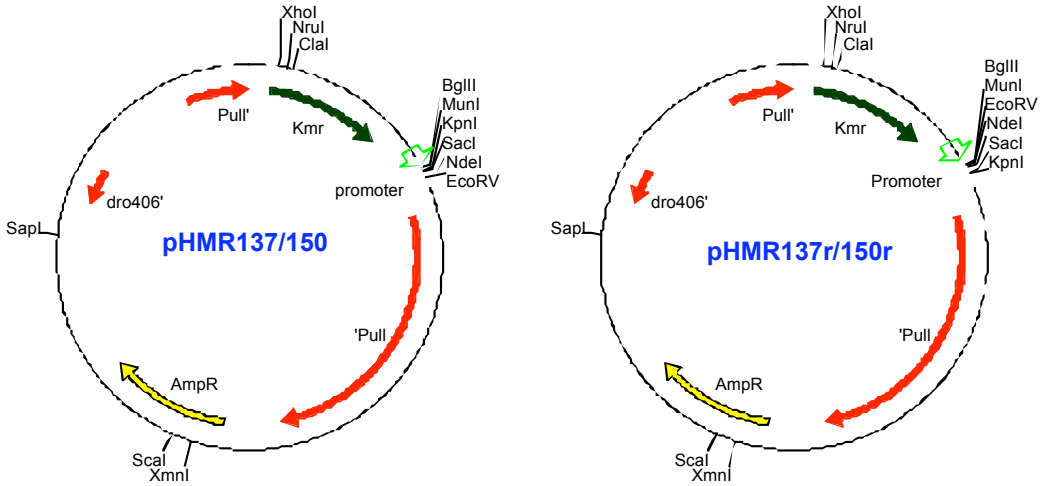

Fig. 1. Basic insertion vectors in *D. radiodurans* pullulanase locus. Promoters are P_{gro} for the 137 vectors, and P_{amy} for the 150 vectors.

C. Expression systems. In order to optimize the construction of process strains for biodegradation, a suite of expression systems are needed, preferably regulated and capable of being modulated at different promoter strengths. Since virtually nothing was known about promoters in this strain, we cloned and characterized a variety of promoters from *D. radiodurans.*

Two different approaches were used to isolate *D. radiodurans* promoters. First, random *D. radiodurans* clone banks were generated in our new promoter screening vector and tested for activity in *E. coli*. Those showing activity were then tested in *D. radiodurans.* This screen resulted in several fragments with promoter activity in both strains. Second, genes were chosen from the genome sequence and the pI3 plasmid that were expected to contain strong promoters, and upstream regions were cloned into the promoter screening vector and tested in both *D. radiodurans* and *E. coli*. A number of promoters showing activity in *D. radiodurans* were isolated in this way, and a subset also showed activity in *E. coli*.

We mapped transcriptional start sites for several of these promoters and have developed a consensus promoter sequence. Surprisingly, this sequence is similar to the

standard *E. coli* s^{70} promoter sequence, with modifications to the –10 region. A manuscript describing this work has been published (Meima et al., 2001).

D. Mutation systems. 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 a *cre-lox* insertion system we developed for *Methylobacterium* 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, and a manuscript is under review describing this tool (Schmid et al, submitted).

E. General technique. We have also developed efficient methods for electroporation and colony PCR that have greatly facilitated our strain construction.

II. Clone and express broad spectrum oxygenases in *D. radiodurans*

A. Expression strains. We have cloned a number of broad spectrum oxygenases into *D. radiodurans,* inserting them into the pullulanase site under control of moderate and high promoters: toluene dioxygenase (TOD) from *Pseudomonas putida* F1, toluene-2-monoxygenase (T2MO) from *Burkholderia cepacia* JS150, and toluene-3-monoxygenase (T3MO) from *Ralstonia pickettii* PKO1 a hybrid toluene dioxygenase/biphenyl dioxygenanse (T/BiPDO) with high activity towards halogenated solvents and the soluble methane monooxygenase (sMMO) from *Methylococcus capsulatus.* In each case, activity was verified in *E. coli* prior to insertion in *D. radiodurans*, because these oxygenases are toxic in *E. coli* and we discovered that many of the clones have acquired down mutations. As a way to partially circumvent this problem, we have used copy-down strains of *E. coli*, which maintain ColE1 replicon-based vectors at a few copies per cell. Clones were screened for activity by both a toluene and TCE disappearance assay (measured by GC), and ^{14}C-TCE or ^{14}C-tolulene assays. Although trace amounts of activity were detected in several cases, the only constructs with activities near the target amount were TOD with the strong *groES* promoter and the hybrid oxygenase with the same promoter. These have been characterized (see below) and a paper has been submitted describing this work (Rothfuss and Lidstrom, submitted).

B. TCE and Toluene degradation by *D. radiodurans::TOD*. Under standard assay conditions (0.4 mM substrate), a toluene degradation rate of 24 nmol/min/mg protein was achieved, significantly higher than an *E. coli* TOD-expressing strain (16 nmol/min/mg protein) or previous *D. radiodurans* constructs (4 nmol/min/mg protein). However, when rate was assessed vs. toluene concentration, we found the rate increased up to a toluene concentration of 1.5mM (Fig. 2). 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.

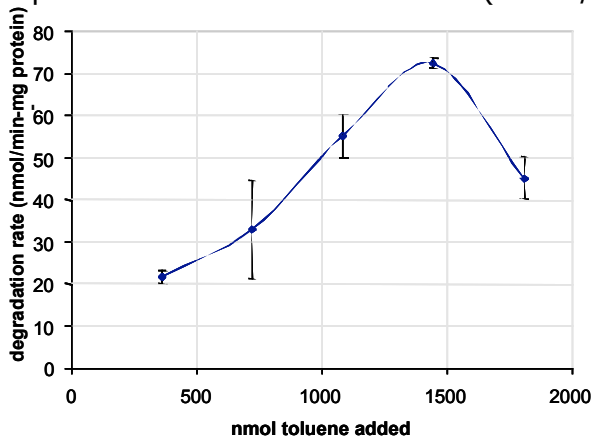

Fig. 2. Initial degradation rate of toluene by TOD-expressing construct with varying amounts of toluene added to a 1ml culture

The extremely solvent resistant bacterium *P. putida* DOT-T1E shows 5 orders of maganitude loss of survival when exposed to 0.3% (28mM) toluene for 30 minutes unless it has been pre-grown with low levels of toluene. *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. In addition, the strain containing the hybrid enzyme has significantly higher activities with TCE, but the current strain is a single-crossover construction. In order to develop a stable, highly expressing strain, it will be necessary to test a stronger promoter and develop a double crossover insertion strain (Rothfuss and Lidstrom, submitted).

III. Analyze and upregulate 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.

A. Survey stress resistance in *D. radiodurans*. We have analyzed the ability of *D. radiodurans* to survive a variety of stresses. The protocol we follow for these analyses is similar to the standard in the stress response field, we grow the cells to mid-exponential phase, shift the cells to a stress condition, then follow both growth and viability over time. A range of increasing stress was tested, and in each case, conditions were discovered in which growth stopped but viability remained virtually 100% for 24 hrs. At stress levels higher than this, viability began to drop and conditions were chosen for stress that resulted in 10- to 100-fold loss of viability over a 4-8 hr time period. Remarkably, *D. radiodurans* is extremely resistant to ionic, pH, and solvent stress (Table 1). Compared to *B. subtilis, D. radiodurans* is many times more resistant to these three stresses. As noted above, *D. radiodurans* is also much more resistant to toluene than most other bacteria. These characteristics suggest that the natural tolerance of the strains will greatly aid the development of effective treatment processes.

Table 1. Stress resistance (loss of viability) of *D. radiodurans*, compared to *B. subtilis*

Stress Condition	*D. radiodurans*	*B. subtilis*
Ethanol	20%: 10X loss in 8hr	9%--100X loss in 4h
NaCl	1.2M: 10X loss in 4h	0.2M: 550X loss in 3h
Acid	pH4.2: 10X loss in 8h	pH4.3: 1000X loss in 2h

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. Therefore, we have investigated heat shock response in more detail.

B. Heat shock in *D. radiodurans*

1. Sigma factors. No orthologs for the two major stress sigma factors (*rpoH*, for heat shock and *rpoS*, for starvation and other stresses or the known negative regulators of stress response are present in the *D. radiodurans* genome sequence. However, a number of familiar heat shock genes are present, including *groESL*, *dnaJK*, and trigger factor. In addition, two putative alternative sigma factors predicted to be of the RpoE class are present in addition to a putative vegetative sigma factor. We have designated these two putative alternative sigma

factors Sig1 and Sig2, and we have analyzed their role in heat shock survival. Null deletion mutants have been generated for each of these using our new genetic tools, and the heat shock phenotypes have been analyzed. As shown in Fig. 3, both mutants show impaired survival, but the Sig1 mutant appears to be especially deficient. Solvent stress and heat shock often share common mechanisms, as both involve detection and handling of misfolded proteins.

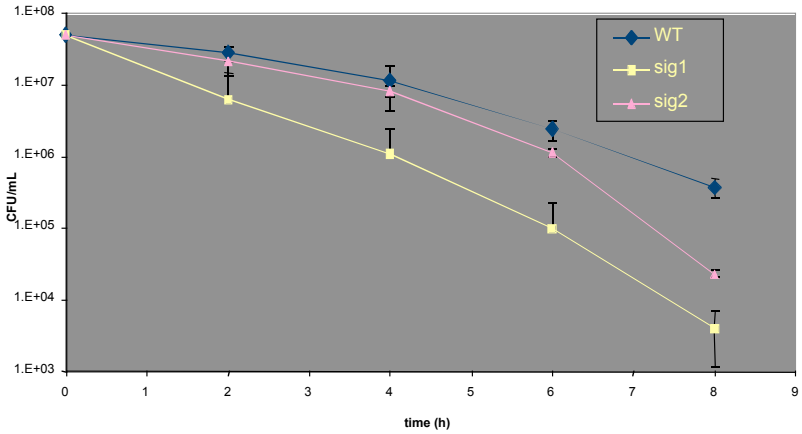

Fig. 3. Heat shock survival in *D. radiodurans* wild-type and in the sig1 and sig2 mutants (Schmid & Lidstrom, 2002). Therefore, we tested survival under ethanol stress and a similar pattern was obtained. These results suggested that Sig1 might play a major role in heat shock response, perhaps similar to RpoH in other bacteria, with Sig2 playing a more minor role.

We next tested the activity of promoters for *groESL* and *dnaJK* using our *lacZ*-reporter based promoter vector, and as shown in Figs. 4 and 5, these promoters are about 3-fold inducible following heat-shock in the wild-type, but not in the Sig1 mutant and less so in the Sig2 mutant.

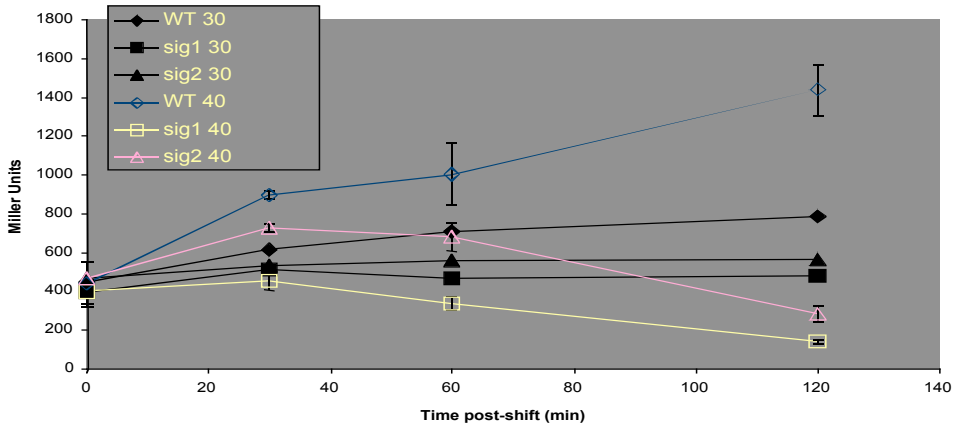

Fig. 4. Expression of pgroESL in wild-type and Sig1, Sig2 mutants, as *lacZ* fusions (Schmid & Lidstrom, 2002).

These data support the hypothesis that Sig1 plays a major role in heat shock response, while Sig2 plays a more minor role. This work has been published (Schmid and Lidstrom, 2002).

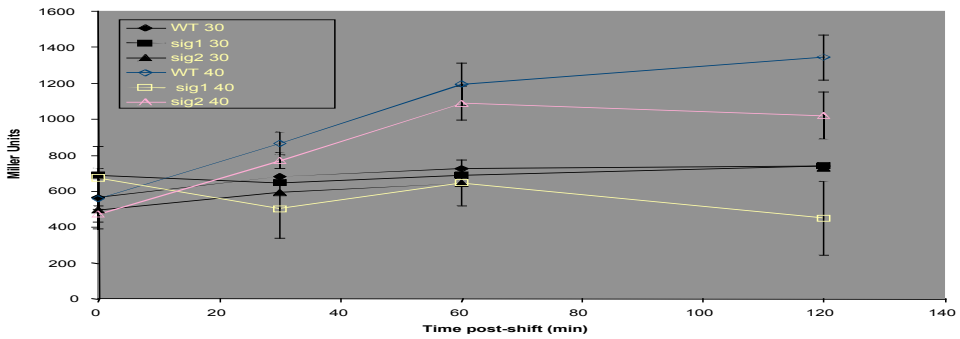

Figure 5. Expression of pdnaKJ in wild-type and Sig1, Sig2 mutants, as *lacZ* fusions (Schmid & Lidstrom, 2002).

2. Global regulon: microarrays and proteomics. As a way to define the heat shock regulon more globally, we are pursuing both proteomics and microarray approaches.

Although either one alone would provide us with information on regulons, it is clear from other systems that both approaches are complementary and a better picture of complex regulatory systems can be obtained by carrying out both. In all cases, we will compare heat shock response in the wild-type and Sig1 mutant. We are not pursuing the Sig2 mutant at this time, as we are focused on defining the role of Sig1 first.

a. Microarrays. We are working with John Battista at LSU and his collaborator (Scott Peterson) at TIGR on their whole genome microarray for *D. radiodurans*, and Amy Schmid, the graduate student working on this project, will spend 3 weeks at TIGR in January 2004 to carry out these experiments. She has successfully carried out their protocols for the isolation of RNA from *D. radiodurans*, and she will take RNA samples with her. Our collaborators at TIGR have kindly offered to teach Amy how to carry out the cDNA generation, labeling, and hybridization parts of the analysis, and will also assist with the data analysis. The results from these experiments should provide evidence for the genes that are transcriptionally affected by the Sig1 regulon.

b. Proteomics. 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. 12 of these spots were identified, some of which were known heat shock genes and others that appear to be novel heat shock-regulated proteins (Table 2; Schmid et al., submitted).

Table 2. Identification of proteins in the Sig1-affected heat-inducible spots.

Spot	Gene name	Annotated ORF number[b]	Putative function
1	*tig*	DR1948	trigger factor
2	*groEL*	DR0607	chaperone hsp60
3		DR2195	l-glucan phosphorylase
4		DR1314	conserved hypothetical
5	*groEL*	DR0607	chaperone hsp60
6	*ctc*	DR0427	general stress response
7	*dnaK*	DR0129	chaperone hsp70
8	*dnaK*	DR0129	chaperone hsp70
9	*dnaJ-1*	DR0126	protein folding
10		DR1306	conserved hypothetical
11	*hsp20*	DR1114	small heat shock protein
12		DR1768	hypothetical protein

[a]Spot numbers correspond to spots in 2D gels.
[b]Annotated ORF numbers come from the *D. radiodurans* complete genome sequence at www.tigr.org.

Quantitative real-time PCR experiments verified the transcriptional requirement of Sig1 for the heat shock induction of the mRNA of four of these genes—*dnaK, groES, DR1314* and *hsp20*. *hsp20* appears to encode a new member of the small heat shock protein superfamily and *DR1314* is predicted to encode a hypothetical protein with no recognizable homologs. Deletion mutant analysis demonstrated the importance in heat shock protection of *hsp20* and *DR1314*. These results expand the *D. radidodurans* Sig1 heat shock regulon to include 9 potential new members, at least one of which, *DR1314*, appears to encode a novel heat shock protective protein.

In addition to this 2D gel approach to proteomics, we are working with Mary Lipton in Dick Smith's group at PNNL, to carry out whole cell proteomics analyses of mutant and wild-type, similar to that published by this group for wild-type *D. radiodurans*. They have generated a dataset for the wild-type and are now working with the Sig1 mutant.

C. PolyP Metabolism. As part of this specific aim, we proposed to engineer metal resistance in *D. radiodurans* by manipulating polyP metabolism. It had previously been shown by Jay Keasling at UC-Berkeley that in some bacteria, metal-phosphates could be generated at the cell surface by alternately inducing the cells to overproduce polyP and then inducing the system that releases phosphate from polyp. Such cells not only remove the metals from solution, they also become more metal-resistant, so it seemed to be a highly attractive system for our treatment strain. However, during the course of our project we learned that Jay Keasling and Heino Nitsche at LBNL had also been awarded a grant from DOE through the NABIR program to manipulate metal precipitation activities in *D. radiodurans*. Therefore, we joined forces a year ago and have been carrying out a highly effective collaboration. This collaboration is enhanced by the presence in my laboratory of a graduate student, Alexandra Holland, who was an undergraduate in Jay Keasling's laboratory working on this *Deinococcus* project. Our part of this project involves characterizing polyP metabolism in *D. radiodurans* and generating the genetically manipulated strains, while the Berkeley groups are analyzing the metal precipitate in terms of chemistry, kinetics, and mechanism. They already have interesting preliminary data showing that the wild-type *D. radiodurans* will bind uranium on its surface. However, the amount and rate are small, pointing out the importance of further genetic manipulation. We have now providee them with our manipulated strains, and they are carrying out the characterization of the uranyl phosphate complex.

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 (Table 3). It is possible the *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).

Table 3. Predicted phosphate regulon genes in *D. radiodurans.*

Annotated name	Locus	Strain of reference		identity	homology	P value
Pit	DR0925	R. solanacearum		50.9%	67.9%	6.60E-50
		E.coli O157:H7	pitA	38.2%	60.8%	8.30E-20
PstS	DRA0157	E.coli O157:H7	pstS	46.4%	63.2%	5.40E-64
PstC	DRA0158	E.coli O157:H7	pstC	40.9%	65.7%	2.40E-30
PstA	DRA0159	E.coli O157:H7	pstA	41.2%	61.9%	1.40E-38
PstB	DRA0160	E.coli O157:H7	pstB	51.0%	71.9%	3.50E-67
PhoU	DRA0161	E.coli O157:H7	phoU	24.0%	47.5%	5.90E-07
PhoA	DRB0046	E.coli O157:H7	phoA	33.2%	48.8%	2.20E-21
PhoB	DR2245	E.coli O157:H7	phoB	40.8%	60.5%	3.10E-29
PhoU	DR2243	E.coli O157:H7	phoU	32.9%	54.1%	2.20E-21
PPX	DRA0185	Nostoc.sp		34.0%	54.5%	4.80E-63
		E.coli O157:H7	ppx	28.9%	48.7%	4.00E-29
PPK	DR1939	Annotated authentic frameshift annotated by TIGR				
		disproved by restriction analysis and alignments [5]				

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. 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 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 and a few amino acids. Although the literature indicates that cysteine and histidine are required, there is some ambiguity since amino acids are apparently 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. 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: 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**TTTTT**AG**GACAGT**

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

D. radiodurans phoA (12/18 agreement): **GGACAG**CGC**ATTCAG**C**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 using purified Ppk generated from His-tagged constructs expressed in *E. coli*. We are currently purifying the enzymes from *D. radiodurans* also to have protein for carrying out biochemical analyses, for instance, determining kinetic constants and the extent of the forward and reverse reactions.

6. Protocols for polyP accumulation. We have developed a suite of starvation protocols in both wild-type and manipulated strains that result in high initial accumulation of polyP with subsequent degradation of the polyP. We are currently testing the release of Pi into the medium during the degradation phase, with subsequent metal precipitation, in collaboration with the Keasling laboratory.

IV. Test survival and activities of these strains in artificial mixtures of contaminants

Since only at the end of this project did we obtain appropriate *ppk/ppx* manipulated strains, we have not combined this capability with the biodegradative ability, and so we have not yet carried out this part of the project. However, we have carried out some studies involving multiple stresses.

We have carried out initial studies of starvation response. We have determined that the Sig1 and Sig2 mutants show similar starvation response to the wild-type, suggesting that the heat-shock regulon does not overlap significantly with starvation response. However, we also noticed that cells experiencing a nutrient downshift were markedly more sensitive to heat shock than cells growing at maximum growth rate, suggesting that multiple stresses are far more damaging that the sum of the individual stresses. In collaboration with the Keasling and Nitsche groups at UC-Berkeley/LBNL, we have carried out studies of irradiation response. In these experiments, we have shown that the Sig1 and Sig2 mutants also have a radiation survival response comparable to wild-type cells grown and irradiated under identical conditions, suggesting that the heat shock regulon does not overlap significantly with radiation-resistance.

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