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This project was a one-year addition of funds to a previous project (ER20294). The specific aims were to finish up the work from the previous project on stress response in *Deinococcus radiodurans*, involving heat shock and phosphate starvation, and publish the final five papers.

I. Heat shock in *D. radiodurans*

A. Global regulon: microarrays and proteomics

As a way to define the heat shock regulon more globally, we pursued 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 compared heat shock response in the wild-type and Sig 1 mutant.

1. Microarrays. We worked with John Battista at LSU and his collaborator (Scott Peterson) at TIGR on their whole genome microarray for *D. radiodurans*, and copublished a paper defining genes that are transcriptionally affected by the Sig1 regulon (Schmid et al., 2005b).

2. 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 2X 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 analyzed these 20 spots by LC-MS via a facility at the Fred Hutchinson Cancer Research Center in Seattle, and obtained data on the 7 spots showing the greatest change (Schmid et al., 2005b). 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.

The other 5 spots all contained more than one protein, a not uncommon phenomenon with 2D gels. We used quantitative RT-PCR with SYBR Green to determine which of the proteins in each of those spots shows heat shock-inducible changes in mRNA. Our results corroborated the LacZ fusion data for the promoters, in that the Sig1 mutant does not show an increase in expression of either *groES* or *dnaK* mRNA, while the wild-type shows a 2.5-3-fold increase.

As part of this work we identified a negative regulator of heat shock, HspR, and showed that the heat shock regulatory system in *D. radiodurans* has some aspects of gram-positive systems (Schmid et al, 2005a).

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

spot #	protein ID	gene name	predicted function
181	DR0427	ctc	general stress protein
141	DR1314		conserved hypothetical
	DR1124		SLH family protein
60	DR1948	tig	trigger factor
280	DR1768		hypothetical protein
	DR2111	rplF	ribosomal protein L6
	DR2113	rpsE	ribosomal protein S5
	DR2127	rpsD	ribosomal protein S4
236	DR1114	hsp20	small heat shock protein
67	DR0363		ABC transporter subunit
	DR1571		ABC transporter subunit
125	DR0386	gcpE	isoprenoid biosynth.
	DR1124		SLH family protein
255	DR2067	nusB	transcription antitermination f
	DR1306		hypothetical protein

In addition to this 2D gel approach to proteomics, we worked with Mary Lipton in Dick Smith's group at PNNL, to carry out whole cell proteomics analyses of mutant and wild-type (Schmid et al, 2005c). This approach does not provide quantitative information, it only indicates whether the protein is above or below their detection limit. Therefore, highly expressed proteins such as GroES that increase 3X after heat shock do not show up as heat-inducible, because they are above the detection limit in both conditions. However, we identified a number of proteins that are affected by the Sig1 regulon.

II. Solvent tolerance in *D. radiodurans*. We also analyzed the role of the *D. radiodurans* S-layer proteins and showed they were important for solvent tolerance, an important characteristic for a treatment strain. This work has been published (Rothfuss et al., 2006).

III. Phosphate Regulon in *D. radiodurans*

As part of this specific aim, we had proposed to engineer metal resistance in *D. radiodurans* by manipulating the phosphate regulon. 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 polyphosphate and then inducing the system that releases phosphate from polyphosphate . 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. As part of this work, we characterized the phosphate regulon of *D. radiodurans*. Our approach has been to analyze the genome sequence for putative phosphate regulon genes, generate mutants in those genes, assess mutant phenotypes, and measure expression of these *pho* 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 phosphate regulation, as it is likely that a partner for PhoB does exist.

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

Annotated name	Locus	Strain of reference		identity	homology	P value	Remark
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	putative
PhoA	DRB0046	E.coli O157:H7	phoA	33.2%	48.8%	2.20E-21	Alkaline Pas
PhoB	DR2245	E.coli O157:H7	phoB	40.8%	60.5%	3.10E-29	operon
PhoU	DR2243	E.coli O157:H7	phoU	32.9%	54.1%	2.20E-21	operon
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]					

The two key genes for manipulating phosphate precipitation are *ppk* encoding polyphosphate kinase (the enzyme that synthesizes polyphosphate) and *ppx* encoding the polyphosphatase. 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.

2. Minimal medium.

In order to analyze the response of *D. radiodurans* to nutritional stress, such as phosphate stress, it is necessary to have an effective minimal medium. Unfortunately, the only minimal media in the literature produce an 18-20 hr doubling time, and for a bacterium that grows with a 1 hr doubling time in rich medium, such slow growth is indicative of a highly nutritionally stressed condition. Therefore, we have developed an alternate medium that reproducibly gives a 4-5 hr doubling time. Part of the difficulty appears to be the buffer, and we have had best success with a MOPS 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 and in our hands they are not required but methionine, alanine, and lysine appear to be required. Finally, the best carbon source in our hands is glycerol. This work has been published (Holland et al., 2006). This medium was used in all experiments involving nutritional stress.

3. *ppx* and *ppk* mutants.

Using our new system for generating unmarked chromosomal deletions, we 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 similar to those found for mutants in *E. coli*, in which growth phenotypes are only observed under rapid shift-up or shift-down conditions or during extended stationary phase incubations.

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. Polyphosphate measurements show that during phosphate limitation polyphosphate accumulates, while in the other nutrient limitation conditions, much less polyphosphate accumulation occurs. Our results suggested that the regulation of this system is significantly different from in *E. coli*, and is not tuned to general stress in the same way. However, we found putative Pho box sequences upstream of three phosphate regulon genes (*ppk, ppx,* and *phoA*), see below, consistent with regulation in response to phosphate level.

Pho box consensus :

GA(A/C)AGTA[T(A/T)T(A/T)]GA(A/C)A(G/T)T(**G/A**)

The scanned regions are -500 to +50 of the translational start:

Deinococcus ppk (15/18 agreement): GAAACTTTTTAGGACAGT

Deinococcus ppx (13/18 agreement): AAGTATTTGGCGGTG

Deinococcus phoA (12/18 agreement): GACAGCGCATTCAGCG

5. Polyphosphate accumulation. Using an "overplus" procedure similar to that identified by the Keasling group that involves phosphate starvation, then phosphate excess, we were able to demonstrate accumulation of polyphosphate to levels similar to those reported for other phosphate-metal treatment systems. However, although the polyphosphate was degraded when cells were placed again under phosphate limitation, phosphate was not excreted. We obtained evidence that the phosphate control system operates differently in *D. radiodurans*, in that the main polyphosphatase appears to be PhoU, and phosphate released from polyphosphate accumulation is rapidly reincorporated. It is likely that this difference in phosphate metabolism is linked to the rapid synthesis of DNA in this strain under stress conditions. Our results suggest that significant redesign of the phosphate regulon in *D. radiodurans* would be required to develop a treatment strain that would be able to use phosphate release for metal precipitation.

Publications:

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