Final Report

Title: Identifying the Proteins that Mediate the Ionizing Radiation Resistance of Deinococcus Radiodurans R1

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PROJECT GOAL

The primary objectives of this proposal was to define the subset of proteins required for the ionizing radiation (IR) resistance of *Deinococcus radiodurans* R1, characterize the activities of those proteins, and apply what was learned to problems of interest to the Department of Energy.

Summary of the Most Significant Results

1. Identifying the proteins required for ionizing radiation resistance in *D. radiodurans* R1: At the outset of these studies, we knew very little about why *D. radiodurans*' was so much more IR resistance than other species, but it was clear that proteins needed for cell survival were synthesized in cultures exposed to IR. Irradiated cultures cannot recover in the presence of chloramphenicol, this antibiotic preventing restitution of IR-induced single-strand and double-strand DNA breaks. During the first three years of the funding period, we made identifying the proteins induced in response to ionizing radiation a priority in our efforts to better define *D. radiodurans*' mechanisms of IR resistance. We described the genomic expression profile of *D. radiodurans* R1 cultures as they recover from a sub-lethal dose of IR, comparing that profile with R1 cultures recovering from desiccation and with irradiated cultures of LSU2030, an IR sensitive strain of R1 that lacks the transcriptional activator IrrE.

We compared unirradiated R1 cultures in exponential phase growth with age matched R1 cultures during the first hour after exposure to a non-lethal 3 kGy dose of γ radiation. This time course was deemed appropriate because it was determined that most DNA repairs had been completed. To determine how global transcription in *D. radiodurans* R1 changes in response to IR, competitive hybridizations were repeated for 6 independent experimental trials. We identified those genes whose mean Cy5/Cy3 ratios increased 3-fold or higher in the irradiated population relative to the unirradiated population. Seventy-two genes (2.2% of the genome) respond with increased expression within the first hour after exposure. A detectable protein product, an accurate mass tag, has been reported for 65% of these loci, verifying that these open reading frames encode a protein product.

The genes induced in response to IR were grouped into nine categories based on their similarity to known proteins with 54 of the 72 loci falling into one of three categories. The largest group (44%) encodes proteins of unknown function. These loci are among the most highly induced; 20 being induced greater than 5-fold in the irradiated population. Six genes (*recA,*

ruvB, uvrA, uvrB, gyrA, and gyrB) make up a second group that encodes proteins associated with DNA metabolism. The third group consists of five loci (*katA, terB, terZ, mrsA,* and *dps*) that encode proteins that are associated with alleviating the effects of oxidative stress in other species.

The process of desiccation is inherently DNA damaging and dried bacterial cells exhibit a substantial number of DNA double-strand breaks, single-strand breaks and DNA crosslinks. Mattimore and Battista established a link between the desiccation resistance and the radiotolerance of *D. radiodurans* by demonstrating that a collection of IR sensitive strains were also sensitive to desiccation. They argued that *D. radiodurans'* ability to repair extensive DNA damage rendered this organism resistant to both stresses. The gene expression programs of R1 cultures recovering from IR and desiccation share 33 loci, indicating a common response to these stresses that should facilitate identifying proteins necessary for DNA double-strand break repair.

The transcriptome of an R1 culture was compared with a culture recovering from 2 weeks desiccation at 5% relative humidity. Samples were obtained from six independent cultures over a 3 point time course (0, 0.5 and 1 h) following re-hydration. Under these non-lethal conditions, 73 genes were induced during this time course. Although *D. radiodurans* maintains distinct inducible responses to each stress, there is substantial overlap in the gene expression profile observed. Thirty-two (45%) of the 73 loci responding to desiccation were also observed during R1's recovery from IR. Included in *D. radiodurans*' common response to these stresses are all of the genes linked to DNA metabolism, and 21 loci that do not have an identifiable function. The Battista group has documented its analysis of the transcriptional responses of *D. radiodurans* R1 to IR and desiccation and a manuscript describing that work was published in *Genetics* in 2004.

2, The IrrE protein is a transcriptional activator of genes induced following *D. radiodurans* exposure to ionizing radiation: LSU2030 is a strain that was created by disrupting the *irrE* gene (DR0167) of *D. radiodurans* R1 by transposon insertion. *irrE* defective (Def) strains are extremely sensitive to IR and desiccation, and loss of IrrE results in a 12-fold reduction in the level of *recA* transcript induced following a culture's exposure to 3 kGy gamma radiation, indicating that IrrE is a regulatory protein that enhances *recA* expression. This was published in the *Journal of Bacteriology* in 2002. Hua et al. confirmed these observations and demonstrated that re-establishing *recA* expression was not sufficient to restore IR resistance to an *irrE* (Def) strain, suggesting that IrrE regulates additional loci that must be expressed before R1 can recover from IR-induced damage. By comparing the expression profile of R1 and LSU2030, we have established that greater than 30% of those genes responding to IR in R1 are not up-regulated in LSU2030. Presumably, the failure to express proteins encoded by these genes, in addition to *recA*, explains the radiosensitivity of the *irrE* (Def) strain.

To establish whether IrrE regulates more than *recA* expression, a series of competitive hybridizations were performed comparing R1's response to IR during the hour following irradiation with that of the *irrE* (Def) strain. Initially, we compared global transcription in unirradiated cultures of R1 and LSU2030, and found three transcripts (DR1185, DR2126, and DR0383) elevated in R1 relative to LSU2030, and one transcript (DR0931) slightly suppressed. The levels of these four transcripts, which do not correspond to any of those identified as part of the cell's response to ionizing radiation, differed from the wild type by less than 4-fold.

Inactivation of IrrE does not appear to affect the expression of the majority of the 72 genes induced in response to IR. IrrE inactivation has little effect on the induction of genes involved in energy metabolism, transport, RNA processing, or protein synthesis. However, 23 of the genes that respond when the wild type is irradiated do not exhibit IR-induced increases in expression in an *irrE* (Def) background, indicating that IrrE, directly and/or indirectly, regulates expression of these loci. Thus, the loss of IrrE reduces or eliminates the increases in expression in greater than 30% of those transcripts induced when the R1 culture is exposed to IR. Among the loci whose expression is affected by loss of IrrE are *recA*, *ruvB*, *uvrA*, *uvrB*, *gyrA*, *gyrB*, and ten of the 26 genes encoding proteins of unknown function.

3. Five hypothetical proteins identified by microarray analyses confer resistance to killing by ionizing radiation: To establish the utility of our microarray studies, the genes encoding the five most abundant transcripts induced in response to IR and desiccation were deleted and the resulting strains evaluated for their ability to tolerate exposure to IR. None of these genes are essential and all confer enhanced radioresistance on *D. radiodurans* R1. In addition, all possible pairs of double mutants were generated using the five alleles generated by deletion. The genes (DR0003, DR0070, DR0326, DR0423, and DRA0346) corresponding to these transcripts were deleted, both alone and in all possible two-way combinations. Characterization of the mutant strains defines three epistasis groups that reflect different cellular responses to ionizing radiation-induced damage, and DR0070, DR0423, and DRA0346 are the founding members of these groups. The DR0070 and DR0423 gene products have complementary activities and inactivating both loci generates a strain that is more sensitive to ionizing radiation than strains in which either single gene has been deleted. These proteins appear to mediate efficient RecA-independent processes connected to IR resistance. The DRA0346 gene product is not necessary for homologous recombination during natural transformation, but nevertheless seems to participate in a RecA-dependent process during recovery from radiation damage. These characterizations clearly demonstrate that novel mechanisms significantly contribute to the IR resistance in *D. radiodurans*. A manuscript describing the purification and characterization of DR0423p was published in *PLOS Biology* in February 2004, and the epistasis study was included in the manuscript published in *Genetics* in 2004. The DRA0346 and DR0070 proteins have also been purified in the laboratory of M. M. Cox at the University of Wisconsin. The function of DRA0346 remains elusive, but DR0070 is now recognized as a previously uncharacterized single strand binding protein that appears to function in conjunction with SSB and appears to be a component in a novel double strand break repair mechanism.

4. A ring-like nucleoid is not necessary for radioresistance in the *Deinococcus* R1: Levin-Zaidman et al. reported that the genome of *D. radiodurans* assumes a tightly packed ring-like structure that may represent an alternative mechanism for protecting *D. radiodurans* from DSBs. These authors suggested this structure contributes to *D. radiodurans* radioresistance by preventing fragments formed by DSBs from diffusing apart during repair. Having the capacity to maintain the linear continuity of its genome in the face of the extensive fragmentation resulting from high dose ionizing radiation would provide obvious advantages to *D. radiodurans*. We tested this hypothesis by examining the nucleoid morphologies of seven recognized species of *Deinococcus*, the radioresistant bacterium *Rubrobacter radiotolerans*, and the more radiosensitive deinococcal relative *Thermus aquaticus.* All were evaluated using epifluorescence microscopy with deconvolution techniques. Although the nucleoids of *Deinococcus murrayi*, *Deinococcus proteolyticus*, *Deinococcus radiophilus*, and *Deinococcus grandis* have structures similar to *D. radiodurans*, the majority of nucleoids found in *Deinococcus radiopugnans* and *Deinococcus geothermalis* lack any specific organization. The nucleoid of *R. radiotolerans* consists of multiple highly condensed spheres of DNA scattered throughout the cell. The genomic DNA of *Thermus aquaticus* is uniformly distributed throughout the cell. These observations indicate there is no obvious relationship between the shape of a species' nucleoid and extreme radioresistance. However, the genomes of all extremely radioresistance species examined are highly condensed relative to more radiosensitive species. Whether DNA in this tightly packed configuration contributes to the radioresistance of these bacteria remains unknown, but this common structural feature appears to limit diffusion of fragments generated post-irradiation even in cells incapable of repairing strand breaks.

5. Directed Evolution of Ionizing Radiation Resistance in *Escherichia coli*: There are several dozen known bacterial species that display an extraordinary resistance to the effects of ionizing

radiation. The best studied of these is *Deinococcus radiodurans.* Although the literature is replete with well-constructed hypotheses, the molecular basis of the extreme ionizing radiation resistance of *Deinococcus* is not well understood. The evolution of ionizing radiation resistance has been linked to the evolution of desiccation tolerance. By evolving ionizing radiation resistance in a relatively sensitive bacterial species, we tried to focus on key mechanisms of ionizing radiation resistance as illustrated by the genomic changes in the evolved strains. Four populations of *Escherichia coli* K12 were independently derived from MG1655, each specifically adapted to survive exposure to high doses of ionizing radiation. D37 values for strains isolated from two of the populations approached that exhibited by *Deinococcus radiodurans.* Complete genomic sequencing was carried out on nine purified strains derived from these populations. Clear mutational patterns were observed that both pointed to key underlying mechanisms and guided further characterization of the strains. Desiccation tolerance was tested in the radioresistant populations. In these evolved populations, passive genomic protection is not in evidence. Instead, enhanced recombinational DNA repair plays a prominent role in genome reconstitution. Multiple lines of evidence indicate that multiple genes, multiple alleles of some genes, multiple mechanisms, and multiple evolutionary pathways all play a role in the evolutionary acquisition of extreme radiation resistance. Several mutations in the *recA* gene and a deletion of the e14 prophage both demonstrably contribute to and partially explain the new phenotype. Mutations in additional components of the bacterial recombinational repair system and the replication restart primosome are also prominent, as are mutations in genes involved in cell division, protein turnover and glutamate transport. At least some evolutionary pathways to extreme radiation resistance are constrained by the temporally ordered appearance of specific alleles.

PUBLICATIONS (supported in part or in total by this project)

Copies of the manuscripts are appended to this report.

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PATENTS (supported in part or in total by this project)

Method to Protect DNA Ends: US patent Number 7,211,393