Final Report: Grant DE-FG02-04ER63918 (2004-2007):

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PI: Michael J. Daly, Ph.D. Associate Professor, Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799**,** Tel: 301-295-3750, Fax: 301-295-1640.
E-mail: mdaly@usuhs.mil

Program DE-FG01-04ER04-06: *Natural and Accelerated Bioremediation Research Program*: **Integrative Studies element of NABIR, combining the Biomolecular Sciences and Engineering, and Community Dynamics and Microbial Ecology elements.**

Title: Characterizing the Catalytic Potential of *Deinococcus*, *Arthrobacter* and other Robust Bacteria in Contaminated Subsurface Environments of the Hanford Site

c/o Dr. Paul Bayer (paul.bayer@science.doe.gov), U.S. Department of Energy, Office of Science, Grants and Contracts Division, SC-64, 19901 Germantown Road, Germantown, MD 20874-1290

Principal Investigator:

Michael J. Daly, Ph.D.
Associate Professor
Department of Pathology
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799
Tel: 301-295-3750
Fax: 301-295-1640
E-mail: mdaly@usuhs.mil

Co-Investigators:

James K. Fredrickson, Ph.D.
Staff Scientist, Environmental Micro. Group
Pacific Northwest National Laboratory
SIN P7-50
P.O. Box 999
Richland, WA 99352
Tel: 509-376-7063
E-mail: jim.fredrickson@pnl.gov

Lawrence P. Wackett, Ph.D.
Professor
Department of Biochemistry
Biological Process Technology
Gortner Laboratory
University of Minnesota
St. Paul, MN 551088
Tel: 612-625-3785
E-mail:wackett@biosci.cbs.umn.edu

Summary of Progress for NABIR Grant DE-FG02-04ER63918 (May 2003-July 2007): Final Report

In the last 3 years year, we have published twelve papers. The following is a summary of those publications and how they relate to our original NABIR goals. The papers published in this reporting period are listed at the end of this final report:

Ionizing Radiation (IR) Resistance in Bacteria

Until recently, there have been no clear physiologic predictors of a cell's ability to recover from ionizing radiation (IR) and other DOE-relevant oxidative stress conditions. In general, the most resistant bacteria have been Gram-positive (*e.g.*, *Deinococcus*, *Arthrobacter*, *Lactobacillus* & *Enterococcus* spp.) and the most sensitive have been Gram-negative (*e.g.*, *Pseudomonas*, *Shewanella* & *Neisseria* spp.). However, there are several reported exceptions to this paradigm, the Gram-negative cyanobacterium *Chroococcidiopsis* is extremely resistant to IR, whereas the Gram-positive *Micrococcus luteus* is sensitive. We have identified biomolecular signatures for radiation sensitivity and resistance which are independent of phylogeny, where very high and very low intracellular Mn/Fe concentration ratios correlated with very high and very low resistances, respectively; and restricting Mn(II) in the famously resistant *Deinococcus radiodurans* sensitized this bacterium to IR (http://cfyn.ifas.ufl.edu/radiation.pdf). For example, *D. radiodurans* (Mn/Fe ratio: 0.24) accumulates >300 times more Mn than the extremely IR sensitive *Pseudomonas putida* (Mn/Fe ratio: <0.0001), and *P. putida* accumulates 4.6 times more Fe than *D. radiodurans*; consistently, for the moderately resistant *Escherichia coli* and *Thermus thermophilus*, the intracellular Mn/Fe ratios are 0.007 and 0.04, respectively (see below, Daly *et al Science* **306**, 925-1084, 2004). However, the mechanism by which Mn(II) facilitates IR resistance was undefined. In summary, for eight phylogenetically distinct bacterial species, we have shown a strong relationship between oxidative stress resistance and intracellular Mn/Fe concentration ratios. These model bacteria were selected for investigation because they have been subjected to whole genome sequencing and annotation, revealing that they encode a similarly complex set of DNA repair and protection functions. Most recently, we showed that unlike radiation-induced DNA damaged *in vivo*, there is a strong correlation between bacterial IR resistance, Mn/Fe concentration ratios and radiation-induced oxidative protein damage.

A New Hypothesis

In one of the most cited textbooks on radiation biology, Clemens von Sonntag states 'In the hierarchy of targets for IR-induced reproductive cell death, DNA must surely be placed at the top.' The field of radiobiology is built on such assertions, found or inferred in virtually all publications that deal with this subject. Yet, the pathway connecting IR with endpoint biological damage is far from clear, largely because the identity of the first critical molecular targets is still not established. Much evidence has accumulated that is not readily explained by classical radiation toxicity models. Among these heretical results for prokaryotes are extreme radiation sensitivities observed in bacteria which encode and express a complement of DNA repair and protection systems

(Daly *et al.*, 2004); for eukaryotes, IR-induced bystander effects. Our current results support that protein is the main cellular target of the biological action of IR in bacteria.

We have reported four surprising and novel experimental results, which are framed within the context of a new view of IR resistance emerging for bacterial cells (Daly *et al*., *PLoS Biology*, 5(4), e92, 2007) : (i) Whereas a given dose of IR causes very similar DNA damage in different bacteria, this is not the case for proteins. We have shown a strong correlation between IR-induced protein damage and bacterial IR resistance; (ii) Under anaerobic conditions, we have identified and quantified a Mn(II)-dependent mechanism of IR-driven dioxygen (O_2) and hydrogen peroxide (H_2O_2) generation, and showed that radioresistant Mn-accumulating bacteria display the hallmarks of Mn(II,III) redox-cycling observed *in vitro*; (iii) Our findings support that Mn redox-cycling protects proteins from superoxide during *in vivo* irradiation, where inhibition of Mn-cycling leads to IR sensitivity and high levels of oxidative protein damage; and (iv) using a recently developed micro(spectro)scopic approach, we revealed an unusual distribution of Fe and Mn in *D. radiodurans* cells, which could forestall the generation of reactive oxygen species (ROS) during irradiation (Lai *et al*., Microscopy and Microanalysis, **13**, 1426-1427, 2007), and desiccation (Fredrickson *et al*., ISME *Journal*, submitted (2007)).

In summary, the possibility that DNA is not the first major class of molecules damaged by IR and other oxidative stress conditions warrants careful investigation, especially as it may come to affect estimates of risk, models of IR-induced toxicity, and approaches to modulating IR resistance in prokaryotes. A review discussing the possibility of modulating Mn and Fe homeostasis as a mechanism to increase the resistance of bacteria and eukaryotes has been published (Daly, *Clin Lab Med*, **26**(2), 491-504, 2006).

Transcriptome Analyses of Irradiated *D. radiodurans* and *Shewanella oneidensis*

We previously investigated the possibility that extreme IR resistance in *D. radiodurans* is determined by novel genes. At least 20 predicted genes of *D. radiodurans*, which were identified by transcriptional profiling following IR as the most highly induced, have been disrupted and the corresponding mutants have been characterized for IR resistance (http://www.usuhs.mil/pat/deinococcus/index_20.htm). Remarkably, the resistances of these novel mutants remained very high, indicating that survival of irradiated *D. radiodurans* might depend on a relatively conventional set of repair functions. Additionally, the transcriptome studies indicated that following irradiation, additional cellular damage might be prevented by attendant cellular responses that minimize the production of metabolism-induced ROS.

To identify cellular determinants of radiation sensitivity, we subjected *S. oneidensis* to transcriptome analyses following IR and compared the expression profiles to those of irradiated *D. radiodurans* (Qiu, *et al*., *J. Bacteriology*, 188(3), 1199-1204, 2006). In summary, approximately 80% of *S. oneidensis* cells were killed following exposure to just 40 Gy, which causes less than 1 DNA double stranded break (DSB) per genome (5.1 Mbp) and about 40 DNA single stranded (SSB) breaks per genome. In light of the strong induction of DNA repair and protection systems in irradiated *S. oneidensis*, the relatively minor DNA damage did not explain the high levels of cell-killing. As a respiratory generalist, *S. oneidensis* is rich in iron containing proteins, unlike *D.*

radiodurans. We concluded that a sudden increase of free iron due to protein damage could have proliferated ROS in *S. oneidensis* during and after irradiation, thereby predisposing *S. oneidensis* cells to a burst of oxidative stress at the onset of recovery. Furthermore, we showed the induction of genes for lytic phages in *S. oneidensis*, indicating that viral-induced cell death might have contributed to radiation toxicity. However, the analysis of two other *Shewanella* species, now shown not to encode prophages or other known viruses (unpublished data), were similarly sensitive to IR. Thus, virus-induction in *S. oneidensis* likely has only a small affect on survival following irradiation to IR or UV radiation.

The Role of Metal Reduction in Mn-Dependnet Deinococcal Species

D. radiodurans and *Deinococcus geothermalis* are able to reduce colloidal Mn(IV), presumably as a mechanism to acquire Mn(II) from the environment (Ghosal *et al.*, *FEMS Microbiology Reviews*, **29**, 361-375 2005). We are currently testing if activities involved in Mn reduction are also responsible for the reduction of U(VI), Tc(VII) and Cr(VI) by *D. radiodurans* and *D. geothermalis*. For. *D. geothermalis* we have published whole genome sequencing and comparative analysis (Makarova *et a*l., *PLoS ONE*, submitted). Genetic disruption of the predicted metal reduction (*e.g.*, cytochrome C-related protein, DR01936) and Mn transport systems (*e.g.*, Nramp, DR1709) of *D. radiodurans* is published (Makarova *et al*., 2007). However, with the exception of a putative Mn uptake regulator TroR (DR2539), gene knockouts have not yet yielded pure mutants, indicating that Mn-homeostasis genes are very important to *D. radiodurans*. As part of this work, we have completed a comprehensive annotation and analysis of the *D. geothermalis* genome (Makarova *et al*., 2007), which represents the forth major sequencing/annotation project supported by members of the USUHS group (*D. radiodurans*, *Clostridium acetobutylicum*, *Thermus thermophilus* (Omelchenko *et al*., BMC Evolutionary Biology, 5, 57-80, 2006) and *D. geothermalis* (Makarova *et al*., 2007)). Our whole genome comparisons between *D. radiodurans* and *D. geothermalis* have helped delineate the genes involved in metal reduction and Mn assimilation, with our findings summarized as follows:

Bacteria of the genus *Deinococcus* are extremely resistant to ionizing radiation (IR), ultraviolet light (UV) and desiccation. The mesophilic *Deinococcus radiodurans* was the first member of this group whose genome was completely sequenced. Extensive analysis of the genome sequence of *D. radiodurans* failed to identify unique DNA repair systems, supporting the existence of alternative resistance determinants. To further delineate the genes underlying the resistance phenotypes, we report the genome sequence of a second *Deinococcus* species, the thermophile *Deinococcus geothermalis*, which at its optimal growth temperature is as resistant to IR, UV and desiccation as *D. radiodurans*, and a comparative analysis of the two *Deinococcus* genomes. Many *D. radiodurans* genes previously implicated in resistance, but for which no sensitive phenotype was observed upon disruption, are absent in *D. geothermalis*. In contrast, most *D. radiodurans* genes whose mutants displayed a radiation-sensitive phenotype in *D. radiodurans* are conserved in *D. geothermalis*. Supporting the existence of a dedicated transcriptional regulator for a conserved set of resistance genes (the *Deinococcus* radiation response regulon), a sequence signature of a binding site was identified in both *Deinococci*. We

present the case that these two species evolved essentially the same diverse and complex set of gene families dedicated to generalized stress response, and to more specific aspects of radiation and desiccation resistance. Our reconstruction of the genomic evolution of the *Deinococcus-Thermus* phylum indicates that the corresponding set of enzymes proliferated, partly in a common ancestor of *Deinococcus* and *Thermus*, and partly at an early stage of evolution of the *Deinococcus* lineage.

Engineered *Deinococcus* Strains as Models for Bioremediation

Our collection of >110 aerobic heterotrophic bacteria isolated from beneath tank SX-108 (Fredrickson *et al*., *Appl. Environ. Microbiol.* **70**, 4230-4241, 2004) has expanded to include other interesting isolates from surface environments at the Hanford Site. To date, they include *D. radiodurans*, *Deinococcus murrayi* and *Deinococcus proteolyticus*, as well as numerous highly resistant non-deinococcal bacteria. Most interestingly, a strain of *Kocuria rosea* displays luxuriant growth on toluate (*m*-methylbenzoate) and related compounds as the sole carbon source under chronic IR (50 Gy/hour). As part of this project, we examined the metal-reducing and aromatic compound-degrading abilities of these Hanford Site strains. While no Hanford Site microorganism has yet been isolated which can couple metal-reduction with toxic organic compound degradation under radioactive conditions, we have succeeded in genetically engineering such *Deinococcus* strains, and others which reduce Hg(II) (Brim *et al.*, Microbiology, **152**(8), 2469-2477, 2006; Qin *et al*., Microbiology, **152**, 709-719, 2006). Importantly, these engineered strains are serving as models for comparison with native Hanford Site bacterial isolates now under investigation.

Papers Funded by Grant DE-FG02-04ER63918 (2004-2007):

1. J. K. Fredrickson, S. W. Li, E. K. Gaidamakova, V. Y. Matrosova, M. Zhai, H. M. Sulloway, J. C. Scholten, M. G. Brown, D. L. Balkwill, MICHAEL J. DALY (2007) Protein oxidation: Key to bacterial desiccation resistance. Nature Publishing Group: Submitted to The ISME Journal, 2, 393-403.
2. B. Lai, S. Vogt, J. Mase, B. Ravel, K. Kemner, MICHAEL J. DALY (2007) Applications and future prospects for x-ray fluorescence microprobe analysis. Microscopy and Microanalysis, 13, 1426-1427.
3. Makarova K. S., M.V. Omelchenko, E.K. Gaidamakova, V.Y. Matrosova, A. Vasilenko, M. Zhai, A. Lapidus, A. Copeland, E. Kim, M. Land, K. Mavrommatis, S. Pitluck, P. Richardson, J.C. Detter, T. Brettin, E. Saunders, B. Lai, B. Ravel, K.M. Kemner, Y. I. Wolf, A. Sorokin, A. V. Gerasimova, M. S. Gelfand, J.K. Fredrickson, E. V. Koonin, MICHAEL J. DALY (2007) *Deinococcus* geothermalis: The Pool of Extreme Radiation Resistance Genes Shrinks, PLoS ONE, submitted.
4. MICHAEL J. DALY, E.K. Gaidamakova, V.Y. Matrosova, A. Vasilenko, Min Zhai, R.D. Leapman, B. Lai, B. Ravel, Shu-Mei W. Li, K.M. Kemner, J.K. Fredrickson (2007) Protein oxidation implicated as the primary determinant of bacterial radioresistance. PLoS Biology, 5(4), e92.
5. J. Qin, L. Song, H. Brim, MICHAEL J. DALY, A. Summers (2006) Hg(II) sequestration and protection by the MerR metal-binding domain (MBD). Microbiology, 152, 709-719.

6. H. Brim, J.P. Osborne, H.M. Konstandarithes, J.K. Fredrickson, L.P. Wackett, MICHAEL J. DALY (2006) *Deinococcus radiodurans* engineered for complete toluene degradation facilitates Cr(VI) reduction. Microbiology, 152(8), 2469-2477.
7. MICHAEL J. DALY (2006) Modulating radiation resistance: Insights based on defenses against reactive oxygen species in the radioresistant bacterium *Deinococcus radiodurans*. Clin Lab Med, 26(2), 491-504.
8. X. Qiu, MICHAEL J. DALY, A. Vasilenko, M.V. Omelchenko, E.K. Gaidamakova, L. Wu, J. Zhou, G.W. Sundin, J.M. Tiedje (2006) Transcriptome analysis applied to survival of *Shewanella oneidensis* MR-1 exposed to ionizing radiation. J. Bacteriology, 188(3), 1199-1204.
9. M.V. Omelchenko, Yu.I. Wolf, E.K. Gaidamakova, V.Y. Matrosova, A. Vasilenko, Min Zhai, MICHAEL J. DALY, E.V. Koonin, K.S. Makarova. (2005) Comparative genomics of Thermus thermophilus and *Deinococcus radiodurans*: divergent routes of adaptation to thermophily and radiation resistance. BMC Evolutionary Biology, 5, 57-80.
10. D. Ghosal, M. V. Omelchenko, E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, A. Venkateswaran, H. M. Kostandarithes, H. Brim, K. S. Makarova, L. P. Wackett, J. K. Fredrickson, MICHAEL J. DALY (2005) How Radiation Kills Cells: Survival of *Deinococcus radiodurans* and *Shewanella oneidensis* Under Oxidative Stress. FEMS Microbiology Reviews, 29, 361-375.
11. MICHAEL J. DALY, E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai, A. Venkateswaran, M. Hess, M. V. Omelchenko, H. M. Kostandarithes, K. S. Makarova, L. P. Wackett, J. K. Fredrickson, D. Ghosal (2004) Accumulation of Mn(II) in *Deinococcus radiodurans* Facilitates Gamma-Radiation Resistance. Science 306, 925-1084.
12. J. K. Fredrickson, J. M. Zachara, D. L. Balkwill, D. Kennedy, S. W. Li, H. M. Kostandarithes, MICHAEL J. DALY, M. F. Romine, F. J. Brockman (2004) Geomicrobiology of high level nuclear waste contaminated vadose sediments at the Hanford Site, Washington state. Appl. Environ. Microbiol. 70, 4230-4241.

Patents: NONE

DOE Plant and Capital Equipment: NONE

We have submitted a pre-application to the ERSP program entitled "Protein Oxidation as a Specific Stress Indicator for Understanding the Metabolic Status and Physiology of Subsurface Microorganisms Catalyzing Contaminant Transformation."

If invited, a full ERSP proposal would be distinct from our NABIR-sponsored research, and would build on work showing that protein is the main cellular target of the biological action of radiation and other oxidative stress conditions in bacteria. Oxidative protein damage can be measured by conventional and high throughput proteomic approaches. Developing an approach, therefore, that can predict the activity of microorganisms involved in metal detoxification based on the level of oxidized proteins could help transform bioremediation from a largely empirical practice into an applied science.

Supplemental Final Technical Report for Grant DE-FG02-04ER63918 (May 2007-May 2008). 1-Year extension to NABIR **Grant DE-FG02-04ER63918.**

Since the first "Final" NABIR report we were granted a 1-year extension of NABIR grant DE-FG02-04ER63918. The NABIR name has changed to ERSP. Our 2007 ERSP application entitled "Protein Oxidation as an Indicator of Metabolic Status and Physiology of Subsurface Microorganisms Catalyzing Contaminant Transformation" was turned down in February 2007. However, Dr. Todd Anderson (Todd.Anderson@science.doe.gov) informed us he would add one funded year to our expired NABIR grant (to renamed ESRP).

The following is a summary of two publications (2007-2008) not "in print" at the time of our Final NABIR report. The two new papers are listed below under 'products delivered'. Please note, the Henry M. Jackson Foundation has submitted a request for a 1-year no-cost extension, which will allow us to address any experimental questions arising.

Background: Between 2004 and 2007, our work has utilized resources associated with the PNNL, as well as capabilities at the Advanced Photon Source whose development has been supported by BER and other offices within DOE. The research has fallen under our guiding rubric: Only with a clear understanding of bacterial resistance mechanisms will it be possible to optimize survival of microorganisms utilized for bioremediation. This ERSP (formerly NABIR) research represents important steps towards that goal. In general, we believe that an approach which could measure the fate of proteins within a targeted population of bacteria *in situ* could ultimately facilitate decision making for environmental remediation, providing a method to quantify the metabolic, physiologic and reproductive status of microorganisms catalyzing contaminant transformation. Additionally, whereas it was previously almost inconceivable to consider the possibility of substantially increasing the environmental stress resistance characteristics of bacteria, we believe that is no longer the case. Since publishing our work in *PLoS Biology*, we have positively identified a *D. radiodurans* Mn-complex, and have reconstituted the radioprotector *in vitro*. The complex scavenges superoxide and related reactive oxygen species (ROS), and protects proteins, but not DNA, from redox-related oxidation.

Overview: In 2003, we published an article in *PNAS* that reported the construction and utilization of a *D. radiodurans* whole-genome microarray. We reported transcriptome profiling of >3,000 genes in *D. radiodurans* recovering from 15,000 Gy. We concluded that the cellular transcriptional response to ionizing radiation in *D. radiodurans* was largely stochastic, and mutant analyses confirmed that most of the highly induced genes were unrelated to DNA repair. As a result, our focus shifted to cell-cleaning functions, and in 2004 we reported in *Science* magazine the identification of a widespread manganese(II)-dependent, nonenzymic mechanism required for extreme radiation resistance. Next, in a series of theoretical and whole-genome transcriptome papers, we formulated a hypothesis on the nature of targets protected by Mn(II) ions. In our article published in the April 2007 edition of *PLoS Biology* we validated our hypothesis, and resolved the paradox of how a relatively small set of structurally unremarkable DNA repair proteins in *Deinococcu*s species work with such great efficiency. We demonstrated

that Mn-complexes present in resistant bacteria prevent a specific form of iron-catalyzed protein oxidation, called carbonylation. Preventing intracellular carbonylation during irradiation allows enzyme systems involved in recovery to survive and function with far greater efficiency than in sensitive cells. Within this context, we have evaluated the stress resistance mechanisms of bacteria isolated from DOE's Hanford Site.

Previous studies on the microbiological characterization of core samples collected from high level waste (HLW) contaminated vadose sediment beneath leaking tank SX-108 in the Hanford Site resulted in the cultivation of viable microorganisms dominated by gram-positive organisms most closely related to *Deinococcus*, *Arthrobacter*, *Rhodococcus* and *Nocardia*. These isolates were highly resistant to IR, surviving acute does approaching 20 kGy. We hypothesized that *Deinococcus* was indigenous to Hanford soils and vadose sediments and that the extreme conditions established by the tank SX-108 contaminant plume led to the selective survival of this highly stress-resistant organism. Our present study is focused on the isolation of phylogenetically diverse strains of *Deinococcus* from irradiated shrub-steppe soils collected from DOE's Hanford Site located in southeast Washington. A total of 63 isolates were obtained from irradiated Hanford soils by plating soil dilutions on TGY agar and selecting pigmented colonies. Among these isolates, DNA was successfully extracted, PCR-amplified, and sequenced for 16S rRNA gene for phylogenetic analysis. The majority of the isolates were most closely affiliated with members of the genus *Deinococcus*. Representative *Deinococcus* strains exhibited significant resistance to ionizing radiation and desiccation as well as elevated intracellular Mn/Fe concentration ratios, suggesting that similar to other ionizing radiation and desiccation tolerant bacteria, the Hanford isolates maintain high intracellular concentrations of Mn relative to Fe to defend against the effects of oxidative stress.

Key Findings reported in two 'Products Delivered' (2007-2008):

Product 1) Fredrickson JK, Li SM, Gaidamakova EK, Matrosova VY, Zhai M, Sulloway HM, Scholten JC, Brown MG, Balkwill DL, Daly MJ. Protein oxidation: key to bacterial desiccation resistance? ISME J. 2008 Apr;2(4):393-403. doi: 10.1038/ismej.2007.116. Epub 2008 Feb 14.

1. Phylogenetically diverse *Deinococcus* sp. are endemic to the shrubsteppe soils of DOE's Hanford Site located in southeast Washington.

2. Similar to *D. radiodurans* R1, the Hanford soil *Deinococcus* isolates are resistant to extremes of IR and desiccation

3. These strains have high intracellular Mn/Fe ratios that have been implicated in the protection of cellular proteins in *D. radiodurans* and other IR-resistant bacteria against oxidative stress resulting from IR exposure and desiccation.

4. Desiccation results in extensive protein oxidation in sensitive, high Fe organisms such as *Shewanella oneidensis* but not in the *Deinococcus* strains. This indicates that

desiccation promotes protein oxidation and that high intracellular Mn concentrations provide protection against such damage.

5. High intracellular Mn/Fe ratios that provide resistance to protein oxidation are key features of *Deinococcus* that allows these organisms to proliferate in harsh environments including desiccated high-level waste contaminated vadose sediments at Hanford.

In collaboration with the Joint Genome Institute (JGI), we have completely sequenced, assembled, and annotated the whole genome of *Deinococcus geothermalis*. This work has provided even greater insight into environmental stress resistance mechanisms. In particular, it has validated our arguments that any comprehensive bioinformatics effort aimed at deciphering a complex, multi-gene phenotype using whole-genome, transcriptome and proteome approaches should aim to study at least two closely-related but distinct representatives. The manuscript presents the genome comparisons in combination with experimental analyses that test key predictions relating to the nature of environmental resistance observed in the lab as well as in the field.

Product 2) Makarova KS, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Lapidus A, Copeland A, Kim E, Land M, Mavrommatis K, Pitluck S, Richardson PM, Detter C, Brettin T, Saunders E, Lai B, Ravel B, Kemner KM, Wolf YI, Sorokin A, Gerasimova AV, Gelfand MS, Fredrickson JK, Koonin EV, Daly MJ. *Deinococcus geothermalis*: the pool of extreme radiation resistance genes shrinks. PLoS One. 2007 Sep 26;2(9):e955.

ABSTRACT: Bacteria of the genus *Deinococcus* are extremely resistant to ionizing radiation (IR), ultraviolet light (UV) and desiccation. The mesophile *Deinococcus radiodurans* was the first member of this group whose genome was completely sequenced. Analysis of the genome sequence of *D. radiodurans*, however, failed to identify unique DNA repair systems. To further delineate the genes underlying the resistance phenotypes, we report the whole-genome sequence of a second *Deinococcus* species, the thermophile *Deinococcus geothermalis*, which at its optimal growth temperature is as resistant to IR, UV and desiccation as *D. radiodurans*, and a comparative analysis of the two *Deinococcus* genomes. Many *D. radiodurans* genes previously implicated in resistance, but for which no sensitive phenotype was observed upon disruption, are absent in *D. geothermalis*. In contrast, most *D. radiodurans* genes whose mutants displayed a radiation-sensitive phenotype in *D. radiodurans* are conserved in *D. geothermalis*. Supporting the existence of a *Deinococcus* radiation response regulon, a common palindromic DNA motif was identified in a conserved set of genes associated with resistance, and a dedicated transcriptional regulator was predicted. We present the case that these two species evolved essentially the same diverse set of gene families, and that the extreme stress resistance phenotypes of the *Deinococcus* lineage emerged progressively by amassing cell-cleaning systems from different sources, but not by acquisition of novel DNA repair systems. Our reconstruction of the genomic evolution of the *Deinococcus- Thermus* phylum indicates that the corresponding set of enzymes proliferated mainly in the common ancestor of *Deinococcus*. Results of the comparative analysis weaken the arguments for a role of higher-order chromosome

alignment structures in resistance; more clearly define and substantially revise downward the number of uncharacterized genes that might participate in DNA repair and contribute to resistance; and strengthen the case for a role in survival of systems involved in manganese and iron homeostasis.

Although our 2007 pre-application to submit a full ERSP proposal was turned down in February, Jim Fredrickson, Ken Kemner and I intend to maintain close working relationships over the next year, with the aim of presenting a stronger case for a possible ERSP proposal in 2009.