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Manganese (II) and Protein Oxidation as Determinants of Bacterial Ionizing Radiation Resistance

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MANGANESE (II) AND PROTEIN OXIDATION AS DETERMINANTS OF BACTERIAL IONIZING RADIATION RESISTANCE

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ABSTRACT

The mechanisms that contribute to bacterial ionizing radiation resistance are not completely understood. The Daly model uses *Deinococcus radiodurans* to suggest that bacterial ionizing radiation resistance is primarily determined by the amount of protein oxidation produced in response to ionizing radiation, so protein oxidation will be limited in radioresistant microorganisms due to a high intracellular level of manganese (Mn). This thesis investigates the Daly model by first introducing oxidative damage to β-Galactosdiase using increasing doses of gamma irradiation. β-Galactosidase activity was measured by the production of *o*-nitrophenol (ONP) which is produced from the hydrolysis of o-nitrophenyl-β-D-galactoside (ONPG). β-Galactosidase activity decreased with increasing doses of gamma irradiation. β-Galactosidase activity did not decrease when the protein wasirradiated in the presence of 50 mM β-mercaptoethanol or 50 mM mannitol and 50 mM benzoic acid, β-Galactosidase. This demonstrated that free radicals present in the cell could protect proteins. To determine if proteins could also shield proteins from free radicals, β-Galactosidase was irradiated with 150 mg/ml BSA which was about half the concentration of proteins in *E. coli* cell. Surprisingly, we found that BSA protected β-Galactosidase from ionizing radiation induced damage. This suggested that proteins could protect other proteins in the cell and would less likely to be targets of free radicals, so this could occur in all microorganisms. Therefore, protein oxidation would not be the primary determinant of ionizing radiation resistance. To determine if Mn was essential for ionizing radiation resistance, we irradiated stationary phase *D. radiodurans* R1 in a rich medium and a minimal salts medium in which other compounds were added. When stationary phase R1 cells were irradiated in M9 minimal media containing Mn, cell survival

did not increase resistance and instead the cells showed an increase in sensitivity. When a carbon source was added to the M9 minimal media containing Mn, cell survival increased but not to the level of survival in rich medium. We concluded that Mn(II) was not essential for ionizing radiation resistance and appeared to be toxic to the cells.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Ionizing Radiation

Ionizing radiation is energy with adequate force to remove electrons from molecules (EPA, 2007). It occurs in the form of particles (particulate) or waves (electromagnetic) and both types are produced during radioactive decay (EPA, 2007; Cox and Battista, 2005). Alpha- (α) and β-particles are types of particulate radiation that are charged and interact with matter over short distances (von Sonntag, 1987; Cox and Battista, 2005; EPA, 2007). This encounter slows the particles down resulting in a loss of energy due to the interaction with electrons, which become excited and ions are produced (von Sonntag, 1987). X-rays and γ-rays are types of electromagnetic radiation, referred to as photons, and during their interaction with matter, a large amount of energy is lost (Spinks, 1964). The amount of energy photons possess is inversely related to their wavelength so that a photon with a longer wavelength has less energy while one with a shorter wavelength has more energy (Spinks, 1964; Cox and Battista, 2005). When a photon interacts with electrons in matter, it can deposit energy over long distances while the electrons can be ejected via the photoelectric effect and the Compton effect (von Sonntag, 1987; Cox and Battista, 2005). In the photoelectric effect, the energy of the photon is absorbed and transferred to the electron which is ejected from its orbital (Spinks, 1964). During the Compton effect the interaction of the photon and electron causes the electron to accelerate in a forward direction while the energy of the photon is reduced and can be scattered in a forward or backward direction (Spinks, 1964; von Sonntag, 1987). Once electrons become excited, ions are produced which can interact with molecules (Cox and Battista, 2005).

When considering bacteria, all biological macromolecules, including carbohydrates, lipids, proteins, and DNA are possible targets of ionizing radiation-induced damage (von Sonntag, 1987; Madigan and Brock, 2009). These molecules are affected in one of two ways by ionizing radiation (von Sonntag, 1987). Direct effect occurs when energy from radiation is deposited into the macromolecule, and among macromolecules, DNA, as the largest molecule in the cell, is arguably the most likely to suffer a direct hit (von Sonntag, 1987). DNA is also considered the most important target in the cell in that it stores all genetic information (von Sonntag, 1987). Direct energy deposition to DNA ionizes the molecule leading to chemical modifications that result in base damage and the introduction of single- and double-strand breaks into the phosphodiester backbone (von Sonntag, 1987; Balasubramanian et al., 1998). Macromolecules are also hit indirectly by free radicals produced by the radiolysis of water. Since water makes up over 70% of cell mass, it is the substance most affected by ionizing radiation (von Sonntag, 1987). Ejected electrons result in radiolysis of water, forming hydroxyl radicals, hydrogen peroxide, and superoxide (Daly et al., 2007; Daly et al., 2009). Intracellular ferrous iron (Fe^{2+}) reduces hydrogen peroxide (H_2O_2) forming hydroxyl radicals in a process known as the Fenton reaction. As long as the resulting Fe^{3+} can continue to interact with H_2O_2, this reaction will cycle, producing large quantities of hydroxyl radicals *in vivo* (Spinks, 1964; Jakubovics and Jenkinson, 2001; Friedberg, 2006). These oxygen radicals interact with and damage cellular macromolecules, including DNA. The production of free radicals is potentially harmful to the cell and depending on the target cell lethality could occur if damage is irreparable.

There are some microorganisms that are extremely resistant to ionizing radiation. Researchers have devoted much time investigating the mechanisms involved in determining this resistance. Even though there have been advancements in understanding processes of repair in

some radiation resistant organisms, there is still much more to be learned about their remarkable ability to tolerate ionizing radiation.

Ionizing radiation resistance is found in numerous genera of bacteria including *Deinococcus, Rubrobacter, Hymenobacter,* and *Chroococcidiopsis* (Makarova et al., 2007; Rainey et al., 2005). *Deinococcus radiodurans* has been studied most extensively.

Deinococcus radiodurans

Deinococcus radiodurans was discovered at a packing plant in Oregon in 1956 (Krabbenhoft et al., 1965). In that year A. W. Anderson isolated *D. radiodurans* (formerly called *Micrococcus radiodurans*) from cans of meat which had been exposed to a high dose of ionizing radiation (Krabbenhoft et al., 1965). *D. radiodurans* was initially designated *M. radiodurans* based on its superficial similarity to members of the family *Micrococcaceae*. However, latter phylogenetic studies indicated there was no relatedness between *M. radiodurans* and *Micrococcaceae* (Brooks et al., 1980), and the name was changed to *Deinococcus radiodurans* with the genus placed in a novel family known as the *Deinococcaceae*.

D. radiodurans is nonspore forming and non pathogenic and grows aerobically at 30°C in undefined rich medium (Battista, 1997; Daly et al., 2004; Makarova et al., 2001). It stains gram-positive but is more similar to gram-negative organisms due to its complex cell envelope. The colonies are coccus shaped and range from pink to red in color which upon cell division appear in pairs or tetrads in liquid culture (Battista, 1997). The genome size is 3.28 Mbp with two chromosomes, a megaplasmid, and a plasmid (JCVI CMR, 2000; Makarova et al., 2001; Cox and Battista, 2005). There are 4 – 10 genome copies per cell (Makarova et al., 2001; Cox and Battista, 2005).

The most remarkable characteristic about this microorganism is its resistance to ionizing radiation, a stress that introduces hundreds of DNA double strand breaks into each genome copy. The species has the ability to repair its shattered genome accurately, maintaining cell viability. In fact, *D. radiodurans* can survive exposure to 17,000 Gy of ionizing radiation (Daly, 2009). Such extreme tolerance of ionizing radiation has led to questions about why ionizing radiation resistance evolved. The highest naturally-occurring doses of ionizing radiation on the Earth are experienced in the monazite sands in Brazil. Here the dose rate is only 789 mGy per year according to the United Nations Scientific Committee on Effects of Atomic Radiation in 2000 (Ghiassi-nejad et al., 2002). There are also other areas on the Earth that are exposed to high doses of ionizing radiation (Iran, India, and Norway) but these doses are not as high as those reported in Brazil (Ghiassi-nejad et al., 2002).

D. radiodurans is also resistant to UV light, desiccation, and many other DNA damaging agents (Battista, 1997; Makarova et al., 2001; Makarova et al., 2007). Mattimore and Battista (1996) investigated the possibility that ionizing radiation resistance is a result of its resistance to desiccation. They found that desiccation introduces double strand breaks in the DNA, suggesting that ionizing radiation is incidental and a consequence of desiccation, since organisms could be naturally selected from arid environments (Mattimore and Battista, 1996). However, there are no environments with high levels of ionizing radiation where these microorganisms can be selected from. *D. radiodurans* and its relatives have been found in a variety of locations such as sewage, feces, dried food, and nuclear waste sites (Rainey et al., 2005). Relatives of *D. radiodurans* that also demonstrated radiation resistance were recovered from the Sonoran Desert in Arizona supporting the connection between ionizing radiation and desiccation (Rainey et al.,

2005). Locating the natural habitat of the organism would assist in understanding its ability to tolerate the DNA damaging effects of ionizing radiation.

D. radiodurans is not exempt from DNA damage during irradiation. In fact, cells in exponential phase experience over 250 double strand breaks per genome at the D_{37} dose (Cox and Battista, 2005). *D. radiodurans* survives radiation because of its remarkable repair capabilities. Based on the large amounts of damage to DNA and the necessity for its accurate repair of that damage, it has been assumed that processes that protect or repair DNA are most responsible for ionizing radiation resistance. However, there are suggestions that other previously ignored factors play a critical role in ionizing radiation resistance, and that these processes should be taken into consideration to help explain ionizing radiation resistance in *D. radiodurans* and other microorganisms.

Theories of Ionizing Radiation Resistance in *Deinococcus radiodurans*

Many hypotheses have been presented in an attempt to explain ionizing radiation resistance in *D. radiodurans*. Although there has not been definitive evidence supporting or refuting any single idea, most studies indicate that a combination of processes may be involved with different species relying on one mechanism to a greater or lesser extent.

More Effective Repair of Ionizing Radiation-Induced Damage to the Cell

An analysis of the genome of *D. radiodurans* revealed that the microorganism has almost all of the genes encoding DNA repair proteins found in more radiosensitive bacteria, however, it is possible that repair in *D. radiodurans* occurs more efficiently compared to other bacteria (Makarova et al., 2001; Daly et al., 2004). There is evidence of a unique and presumably highly efficient form of double strand break repair that has been recently described (Slade et al., 2009), but to understand this process one must consider observations made almost fifteen years ago.

Daly et al. (1994) investigated repair in the wild-type strain (*D. radiodurans* R1) following 17.5 kGy gamma irradiation. Following exposure to irradiation the genome was shattered into multiple fragments, yet R1 was able to repair more than 100 double strand breaks for each chromosome without any mutations or lethality (Battista, 1999; Daly et al., 1994). This repair required the RecA protein, a result which has been confirmed through the work of Slade et al. (2009). Mutations that inactivate RecA render the cell sensitive to irradiation (Daly et al., 1994). The *recA* mutant is unable to repair double strand breaks with the same efficiency as the wild-type, either repairing few breaks or none (Daly et al., 1994). When Daly and Minton (1996) further examined the repair process in *D. radiodurans*, they found that the initial repairs were not dependent on the RecA protein. In a *recA* mutant strain of *D. radiodurans*, a significant portion of the genome was reassembled after 1.5 hours indicating an alternative mechanism for repairing some DNA double strand breaks in this species that does not rely on homologous recombination (Daly and Minton, 1996). The identity of this alternative pathway remained unknown until the findings of Zahdraka et al. (2006).

D. radiodurans utilizes a series of steps including a process called extended synthesis dependent strand annealing (ESDSA) to accurately repair its shattered genome following ionizing radiation exposure. Once breaks occur in the DNA, the fragmented end is recessed so that 3' overhangs remain that are used to begin synthesis on overlapping fragments which act as templates using the RecA and RadA proteins (Slade et al., 2009). DNA synthesis is used to fill in any gaps, and two noncontiguous fragments are linked on another fragment through convergent elongations (Slade et al., 2009). The newly formed strands will then anneal to a complementary single-stranded extension which forms double-stranded DNA intermediates

(Slade et al., 2009). These intermediates are then made into circular chromosomes through homologous recombination (Slade et al., 2009).

The proteins that mediate ionizing radiation resistance and ESDSA are poorly described. Liu et al. (2003) examined gene expression in R1 after exposure to 15 kGy by comparing the changes in the levels of specific mRNA in unirradiated and irradiated cells. They found that 832 genes were induced at least twofold in response to irradiation with 23 genes exhibiting a pattern of expression that matched that of recA- a protein absolutely required for ionizing radiation resistance (Liu et al., 2003). Based on the overlapping pattern of expression, the authors postulated that the proteins encoded by these mRNAs were also involved in protecting the cell from ionizing radiation induced DNA damage. Many of these mRNAs encoded proteins of unknown function, suggesting that *D. radiodurans* employed novel mechanisms of protection.

Tanaka et al. (2004) also investigated gene expression in *D. radiodurans* following exposure to ionizing radiation. In this study, which examined cells in exponential phase growth, 72 genes were induced in response to irradiation which was divided into groups. Of the genes identified, the majority had no known function, and many of these overlapped with those identified by Liu et al. (2003). The Tanaka study also examined gene expression in R1 following two weeks of desiccation and found 73 genes were induced by this treatment. Ionizing radiation and desiccation introduce similar types of DNA damage, including double-strand breaks (Tanaka et al., 2004). A comparison of the pattern of gene expression following these stresses indicated that 32 of the mRNAs detected were the same, and 19 of the 32 genes encoded hypothetical proteins. It appears that these proteins play a central role in radioresistance, and determining their functions would aid in understanding the mechanisms utilized in DNA repair post-irradiation in *D. radiodurans*. Presently, it is unknown if they represent novel classes of repair

proteins or if they act in a manner that facilitates the conventional set of repair functions associated with all cells.

Does Nucleoid Compaction Facilitate Ionizing Radiation Resistance in *D. radiodurans*?

Levin-Zaidman et al. (2003) reported that the nucleoid of *D. radiodurans* exists as a ring shaped structure that they referred to as a toroid. This observation led to the suggestion that the shape may facilitate the species' ability to tolerate ionizing radiation in that the dense packing of a toroid would constrain diffusion of DNA fragments post-irradiation and aid in the repair process.

The idea that the ring shaped structure was responsible for ionizing radiation resistance was disproved. Zimmerman and Battista (2005) showed that other equally resistant members of the *Deinococceae* did not exhibit a ring-shaped nucleoid. Eltsov and Dubochet (2005) used cryoelectron microscopy to determine whether the structure found in *D. radiodurans* was a toroid. They found the nucleoids examined to be diffuse, and they found no evidence of an ordering of the DNA that is usually associated with toroidal structures. Zimmerman and Battista (2005) examined a number of ionizing radiation resistant species and found that not all had ring-like nucleoids. However, they did show that all species examined had a highly compact nucleoid. This suggested that this compaction, as opposed to the shape of the nucleoid, could contribute to ionizing radiation resistance. To date this has not been tested

Protecting Proteins from Oxidation May Be Responsible for Ionizing Radiation Resistance

In 2004, Daly et al. suggested that the ionizing radiation resistance of *D. radidodurans* was a function of the level of manganese present in the microorganism. Manganese (Mn) is one of many trace metals found in many bacterial cells and is essential for survival (Jakubovics and

Jenkinson, 2001). In fact, Mn can be involved in a number of cellular functions, including the cell's response to oxidative stress where it assists in detoxifying reactive oxygen species (Jakubovics and Jenkinson, 2001). Daly et al. (2004) showed *D. radiodurans* and its relative *D. geothermalis* have a higher intracellular concentration ratio of Mn relative to many other bacteria resulting in a higher Mn to iron (Fe) ratio intracellularly. *D. geothermalis*, like *D. radiodurans*, is also radiation resistant (Makarova et al., 2007). In contrast, microorganisms with a low intracellular ratio of Mn to Fe such as *Escherchia coli* and *Pseduomonas putida* are sensitive to ionizing radiation. Daly argues that Mn is protective by limiting oxygen radical formation. In this scenario a low Mn/Fe ratio is potentially dangerous because of the products of the Fenton reaction, which requires Fe, are more likely to form and cause damage to the cell.

For many years, DNA was believed to be the "target" of the oxygen radicals formed *in vivo* post-irradiation (von Sonntag, 1987). Genomic DNA is the largest molecule in the cell, and if it is destroyed, a cell cannot multiply resulting in the removal of that cell from the irradiated population. Recent suggestion has been made that proteins and not DNA are the primary target of ionizing radiation (Daly et al., 2007). Like DNA, proteins are damaged by ionizing radiation. Protein oxidation is the covalent modification of a protein that occurs through reactive oxygen species or by-products of oxidative stress (Shacter, 2000). Oxidized proteins may exhibit a total loss of function. The hypothesis that the inactivation of proteins is responsible for ionizing radiation sensitivity argues that the loss of specific proteins, for example proteins involved in DNA repair, prevent efficient recovery post-irradiation. Daly et al. (2007) reported a correlation between the level of protein oxidation observed post-irradiation and ionizing radiation resistance. They reported that while the level of ionizing radiation-induced damage to DNA is the same in all species regardless if they are ionizing radiation resistant or sensitive, those species that

exhibit a lower level of protein oxidation are more ionizing radiation resistant. On the other hand, those species that exhibit a high level of protein oxidation are more sensitive to ionizing radiation (Daly et al., 2007). Therefore, the proteins of the radioresistant bacteria *D. radiodurans* and *D. geothermalis* are less susceptible to protein damage following irradiation, whereas *E. coli* and *P. putida* experience much more extensive protein damage and are quite sensitive to irradiation. Daly et al. (2007) provided evidence that the high level of Mn(II) was responsible for protein protection.

In summary, this model (commonly referred to as the Daly model) postulates that a high intracellular concentration of Mn protects the cell from ionizing radiation-induced damage by limiting the oxidation of proteins post-irradiation. Some subset of these proteins is assumed to be necessary for the cell's ability to recover from damage. Ionizing radiation resistant species protect their proteins, and these cells retain the capacity to survive.

Challenges to the Daly Model

An analysis of the findings by Daly et al. (2004, 2007) discovered two results that stood out as potential setbacks to the Daly model.

First, the Daly model infers that microorganisms with a high intracellular concentration of Mn are protected from damage induced by ionizing radiation. However, *Neisseria gonorrhoeae* accumulates a high intracellular concentration of Mn (Tseng et al., 2001), yet this microorganism is sensitive to ionizing radiation (Mehr and Seifert, 1998). This contradicts the Daly model in that if a high level of Mn is present in a microorganism then the microorganism should survive ionizing radiation-induced damage since the high level of Mn would decrease the amount of protein oxidation to the cell. If such a correlation is made then it should apply to all

microorganisms and not just a select few in order to be used a determinant of bacterial ionizing radiation resistance.

Second, Daly et al. (2007) examined the ability of Mn to protect DNA and proteins during ionizing radiation. They found that Mn was able to protect the enzyme BamHI but was unable to protect plasmid DNA from damage. The Daly model is based on passive protection of proteins by Mn from free radicals generated during ionizing radiation. This passive protection by Mn indirectly affects DNA, but if Mn is essential for ionizing radiation resistance as the Daly model argues then it seems odd that Mn cannot protect DNA. Thus, this result weakens the argument by the Daly model and should not be ignored.

Study Objectives

The objectives of the studies described in this thesis are to test the hypothesis that protein oxidation is the primary determinant of bacterial ionizing radiation resistance and explore whether Mn facilitates ionizing radiation resistance by limiting protein oxidation. If protecting proteins from oxidation through a Mn-mediated process is the event most critical in cell survival post-irradiation then it seems reasonable to assume that it should be possible to reproduce protein protection *in vitro*. Increasing doses of gamma radiation were used to introduce oxidative damage to β-Galactosidase and changes in β-Galactosidase activity were used to monitor the effectiveness of the various agents in protecting the protein from inactivation. During the course of this study a surprising result was obtained, we found that bovine serum albumin (BSA) in concentrations similar to those found in *E. coli* shielded β-Galactosidase from oxidative damage. This result suggests that other proteins present in the cell can be protective regardless of whether Mn has been shown to protect proteins, and this protective role of proteins should not be ignored.

Then the role of Mn in bacterial ionizing radiation resistance was also examined. We attempted to determine if the exogenous addition of Mn to growth medium increases resistance in bacteria. Daly et al. (2004) reported stationary phase cultures of *D. radiodurans* became ionizing radiation sensitive when starved of Mn and that cell growth was dependent on Mn in the growth medium. These two facts seem to contradict each other. If a *D. radiodurans* cell cannot grow in the absence of Mn, it is difficult to imagine how one can accurately assess the effect of Mn on cell survival post-irradiation by removing Mn from the culture medium. In this study, we add Mn to the medium that the bacteria are suspended in during irradiation. These bacteria cannot grow because of nutrient limitation, but they presumably have all of the Mn needed to survive irradiation. Under these conditions, we find that *D. radiodurans* fails to fully recover from exposure to doses of ionizing radiation that have no effect on cultures grown in and irradiated in rich medium. The presence or absence of Mn had no effect on survival, suggesting that Mn is not the sole determinant of ionizing radiation resistance.

CHAPTER 2: MATERIALS AND METHODS

Bacterial Strain, Growth Conditions, and Treatment

Deinococcus radiodurans R1 ATCC13939 was used in all studies. All cultures of R1 were grown at 30°C in TGY broth (1% tryptone, 0.6% yeast extract, 0.2% glucose) or on TGY agar (1.5% agar). Cultures in exponential phase (OD_{600} of 0.05 – 0.15) and stationary phase (OD_{600} of 0.9 – 1.0) were evaluated for ionizing radiation survival (Harris et al., 2004; Daly et al., 2007). In some experiments cultures grown in TGY were harvested by centrifugation (5000 rev/min for 10 minutes at 4°C) and re-suspended in an equivalent volume of glucose-free M9 salts (0.04 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.01 M NaCl, 0.02 M NH_4Cl, 1M $MgSO_4$, and 0.01M $CaCl_2$) prior to irradiation. In some studies, the M9 salts were supplemented with sterile 0.2% glucose and/or 1 mM $MnCl_2$ prior to irradiation. Cultures were irradiated at 25°C. Gamma irradiation was applied using a Model 484R ^{60}Co (J. L. Shepherd and Associates, San Fernando, CA) at a rate of 7.5 Gy/min. Survival was determined through serial dilutions of irradiated cultures in triplicate on TGY plates and incubated at 30°C. Survivors were counted 3 days after incubation.

Enzymatic Preparation, Treatment, and Assay

β-Galactosidase isolated from *Escherichia coli* was purchased from Sigma-Aldrich (St. Louis, MO) in the form of lyophilized powder. β-Galactosidase (1000 units) was diluted in 1 ml of sterilized distilled water so that 1 unit = 1 µl and aliquoted in 1.5 ml sterile centrifuge tubes and held at 4°C for immediate use or stored at -20°C.

β-Galactosidase was irradiated in 1.5 ml centrifuge tubes. Depending on the study, aliquots of enzyme to be irradiated included 50 mM β-mercaptoethanol, 50 mM mannitol (Sigma-Aldrich) and 50 mM benzoic acid (Sigma-Aldrich), or 150 mg/ml Bovine serum albumin

(BSA) (Sigma-Aldrich). All samples were irradiated at 25°C. Gamma radiation was applied using a Model 484R ^{60}Co (J. L. Shepherd and Associates, San Fernando, CA) at a rate of 7.5 Gy/min.

Five units of β-Galactosidase were used in all assays. β-Galactosidase samples were added to the assay buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $Na_2HPO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, pH 7.0) and placed in 1.6 ml spectrophotometer cuvets. All assays were done in triplicate. The reaction was initiated with the addition of 4 mg/ml *o*-nitrophenyl-β-D-galactoside (ONPG) (Sigma Aldrich St. Louis, MO) dissolved in 0.1M phosphate buffer (pH 7.0). The reaction was allowed to proceed for 15 minutes at 25°C. A change in the solution from colorless to yellow indicates the production of *o*-nitrophenol from the hydrolysis of ONPG by β-Galactosidase. A solution that remains colorless indicates no production of *o*-nitrophenol. The reaction was stopped with the addition of 1 M Na_2CO_3. The absorbance was read at 420 and 550 nm (used for light scattering) using a SmartSpec Plus Spectrophotometer (Bio-Rad). The absorbance at 420 nm was used to calculate the specific activity of β-Galactosidase in μmol ml^{-1} min^{-1} using the formula in Table 1 (Miller, 1972).

Table 1. Equation for the specific activity of β-Galactosidase.

Activity (μmol ml^{-1} min^{-1}) = A_{420} (V_{Tot}) / ε ℓ T (V_{Enz})
Where: A_{420} = the absorbance at 420 nm V_{Tot} = Total volume of the stopped reaction in ml ε = extinction coefficient, 4.5 ml μmol^{-1} cm^{-1} (value for O-nitrophenyl-β-D-galactoside) ℓ = light path length in cm, 1 cm (spectrophotometer cuvette) T = time V_{Enz} = Volume of enzyme added in ml

Statistical Analyses

Samples were compared using a two-sample, unpaired Student's t-test (SigmaPlot, version 9.0). In other words, for each report of significance made herein we are testing the null hypothesis that the means of two normally distributed populations are equal. A confidence interval of 95% ($P<0.05$) was considered significant.

CHAPTER 3: RESULTS

The Reduction of β-Galactosidase Activity by Ionizing Radiation

To determine how ionizing radiation affects protein activity, β-Galactosidase was exposed to increasing doses of gamma irradiation over a range from 1000 Gy to 10,000 Gy (Fig. 1). Following the hydrolysis of ONPG, β-Galactosidase activity was calculated based on the absorbance of ONP. Thus, the effect of ionizing radiation on this protein's activity can be measured by determining how much ONP is generated per unit time.

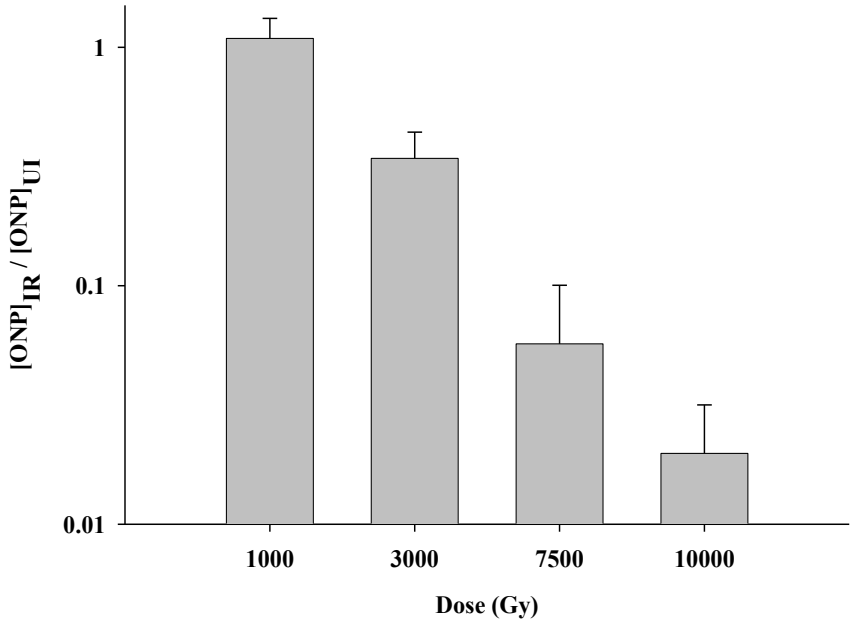

Fig. 1. Specific activity of β-Galactosidase based on the production of *o*-nitrophenol (ONP) in irradiated (IR) β-Galactosidase. Bars in the graph represent the ratio of the concentration of ONP formed by the irradiated (IR) protein divided by the concentration of ONP formed by the unirradiated (UI) protein following a 15 minute reaction with ONPG. Values are the mean +/- the standard deviation of at least eight independent trials, n = 24.

Not unexpectedly, as the dose of ionizing radiation increased β-Galactosidase activity decreased. At 1000 Gy, the specific activity of the protein did not change from that of the unirradiated protein yielding a ratio of one. At 3000 Gy, the activity of the irradiated protein decreased 3-

fold, and at 7500 and 10,000 Gy activity was reduced 20-fold and 100-fold, respectively. Unirradiated controls were held at room temperature throughout the irradiation to ensure that enzyme activity did not degrade non-specifically during the course of irradiation. The results obtained indicate that ionizing radiation will inactivate β-Galactosidase if a sufficiently high dose is applied.

Protecting β-Galactosidase Activity During Exposure to Ionizing Radiation

It is reasonable to assume that β-Galactosidase activity decreases through the indirect effect of oxygen radicals formed when ionizing radiation interacts with the water in which the protein is suspended. To provide experimental evidence of this assumption, we irradiated the protein in the presence of free-radical scavengers that should shield the protein from oxidative damage. Two treatments were employed: a) proteins were suspended in buffer containing β-mercaptoethanol, a hydroxyl radical scavenger that is readily oxidized (von Sonntag, 1987) (Fig. 2), and b) a combination of mannitol and benzoic acid were added to the protein (Fig. 3). Mannitol and benzoic acid are routinely added to protect protein preparations from oxidation; this combination has proven to serve as an excellent scavenger of hydroxyl radicals (Eichler et al., 1987).

β-Mercaptoethanol and the combination of mannitol and benzoic acid effectively suppressed ionizing radiation-induced loss of activity in irradiated samples of β-Galactosidase. The results in Figures 2 and 3 indicate that oxygen radicals are responsible for ionizing-radiation induced damage to the protein in these studies.

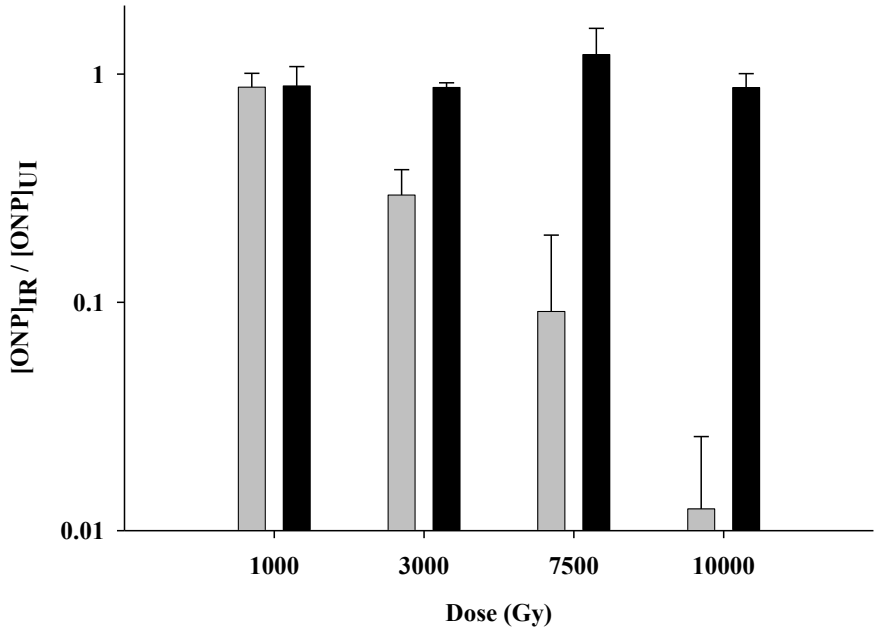

Fig. 2 Specific activity of β-galactosidase based on the production of *o*-nitrophenol (ONP) in irradiated (IR) β-Galactosidase. β-Galactosidase irradiated without treatment (gray bars) and in the presence of 50 mM β-mercaptoethanol (black bars). Bars in the graph represent the ratio of the concentration of ONP formed by the irradiated (IR) sample divided by the concentration of ONP formed by the unirradiated (UI) sample following a 15 minute reaction with ONPG. Values are the mean +/- the standard deviation of at least three independednt trials, n=9.

Even at doses as high as 10,000 Gy – a dose that will eliminate all cultures except the most ionizing radiation resistant species – these concentrations of β-mercaptoethanol or mannitol and benzoic acid completely protect the protein. This indicates that the basic premise of the Daly model is sound. If a free radical scavenger is present in sufficient concentration *in vivo*, proteins can be protected from the damaging effects of ionizing radiation, and they will be available to function in any physiological processes as needed.

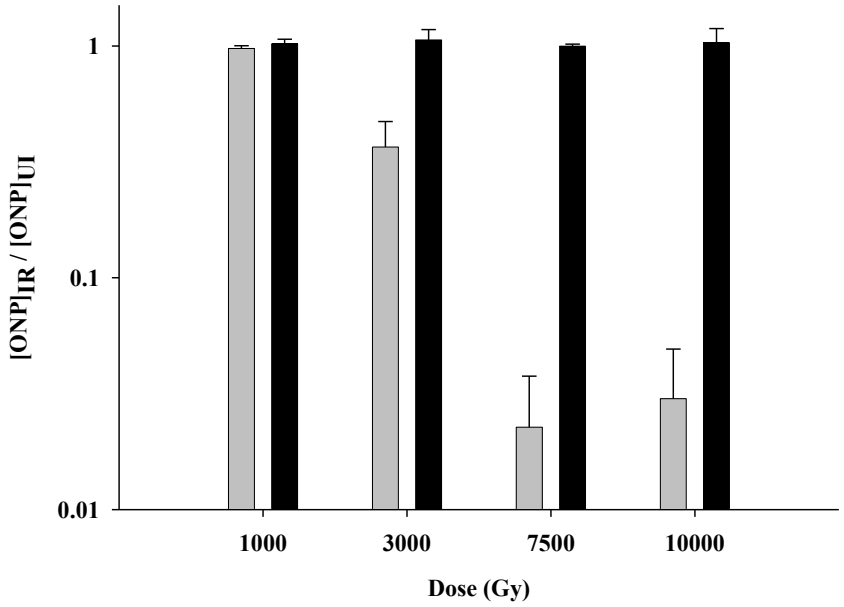

Fig. 3. Specific activity of β-Galactosidase irradiated without treatment (gray bars) and in the presence of 50 mM mannitol and 50 mM benzoic acid (black bars). Bars in the graph represent the ratio of the concentration of ONP formed by the irradiated sample divided by concentration of ONP formed by the unirradiated (IR) sample following a 15 minute reaction with ONPG. Values are the mean +/- the standard deviation of at least three independednt trials, n=9.

The Effect of Bovine Serum Albumin (BSA) on β-Galactosidase Activity

In considering the Daly model and the associated evidence presented in favor of a role for Mn (II) in the ionizing radiation resistance of *D. radiodurans*, we were struck by the fact that the presence of other intracellular macromolecules did not seem to influence the outcome. When ionizing radiation interacts with matter that energy is deposited randomly throughout an irradiated cell, and there is a probability associated with the inactivation of any molecule within the cell at a given dose. The cytosol of an *E. coli* cell contains 2,600,000 protein molecules (Cyber Cell Database, 2008) and cultures of this species are quite sensitive to ionizing radiation (Whitkin, 1946). Why doesn't the presence of these additional proteins eliminate the possibility

of all copies of a critical protein from being inactivated in vivo? To examine this question, we studied the activity of β-Galactosidase in the presence of bovine serum albumin (BSA).

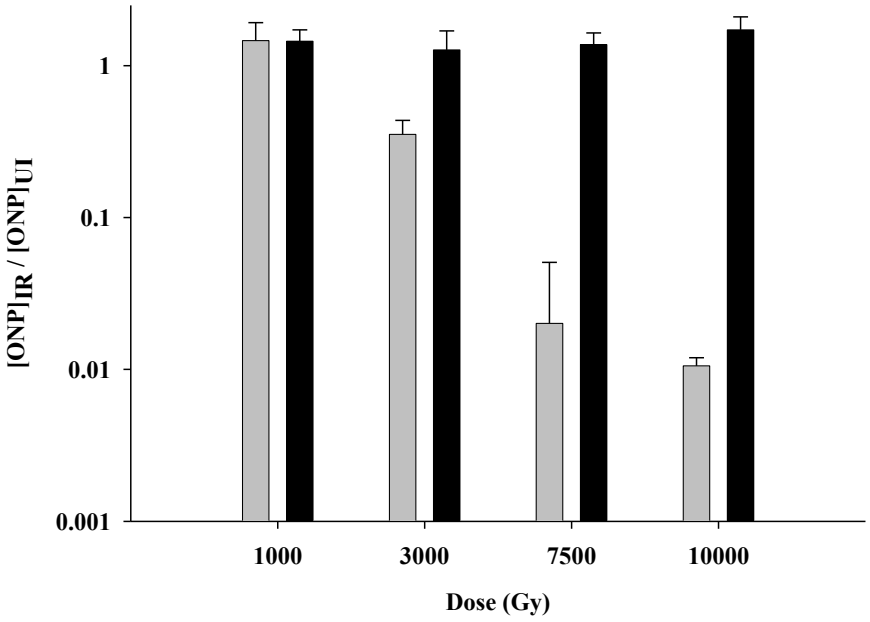

Fig. 4. Specific activity of β-Galactosidase irradiated without treatment (gray bars) and with 150 mg/ml BSA (black bars). Bars in the graph represent the ratio of the concentration of ONP formed by the irradiated (IR) sample divided by concentration of ONP formed by the unirradiated (UI) sample following a 15 minute reaction with ONPG. Values are the mean +/- the standard deviation of at least three independednt trials, n=9.

β-Galactosidase and BSA were combined and exposed to the same dose of ionizing radiation used in the studies described above for free radical scavengers (Fig. 4). The intracellular concentration of protein in *E. coli* is estimated to be between 200 – 320 mg/ml (Cyber Cell Database, 2008). The concentration of BSA added to β-Galactosidase in Fig. 4 is 150 mg/ml. In the presence of BSA, β-Galactosidase activity was not affected by increasing doses of ionizing radiation. Without BSA, activity dropped by as much as 170 fold at the highest dose.

The results in Figure 4 were unexpected in that a concentration of BSA less than what is ordinarily found in *E. coli* completely preserves β-Galactosidase activity. This result suggests that intracellular proteins (and possibly other macromolecules) can indirectly serve to shield critical proteins from ionizing radiation-induced damage and inactivation. Given this observation, it is difficult to argue that the inactivation of proteins by ionizing radiation is responsible for sensitivity in response to ionizing radiation observed in most species.

Ionizing Radiation Cell Survival in *D. radiodurans* R1

Once the effect of ionizing radiation on protein activity was determined, the next study investigated the survival of bacterial cells post-irradiation. This study would compare cell survival in rich medium and minimal salts medium, and like with the protein study, additional compounds would be added to the growth medium to examine their effect on cell survival.

The Effect of Age on the Survival of *D. radiodurans* R1 Following Exposure to ionizing Radiation

Most of the work of Daly and colleagues appears to have been done using R1 cultures in stationary phase, and interpreted with the assumption that the results are applicable to this species in any stage of growth. Recently Sukhi *et al*. (2009) suggested that *D. radiodurans* displayed differential survival in different growth phases despite the fact that the levels of protein oxidation and Mn concentration did not change as cells grew. Figure 5 compares cell survival following exposures at 8000 and 10,000 Gy between cultures treated during the exponential and stationary phases of growth. As indicated by the black bars in Fig. 5, exponential phase cultures that were grown and irradiated in TGY broth had an average survival of 83% at 8000 Gy and 74% at 10,000 Gy. Cells irradiated in stationary phase (white bars) exhibited a significantly lower viability with an average survival of 29% at 8000 Gy (p=0.0143, df=14) and 16% at

10,000 Gy (p=0.0112, df=14). These results indicate that stationary phase cells are slightly more susceptible (between 3 and 4 fold) relative to cultures in exponential phase growth.

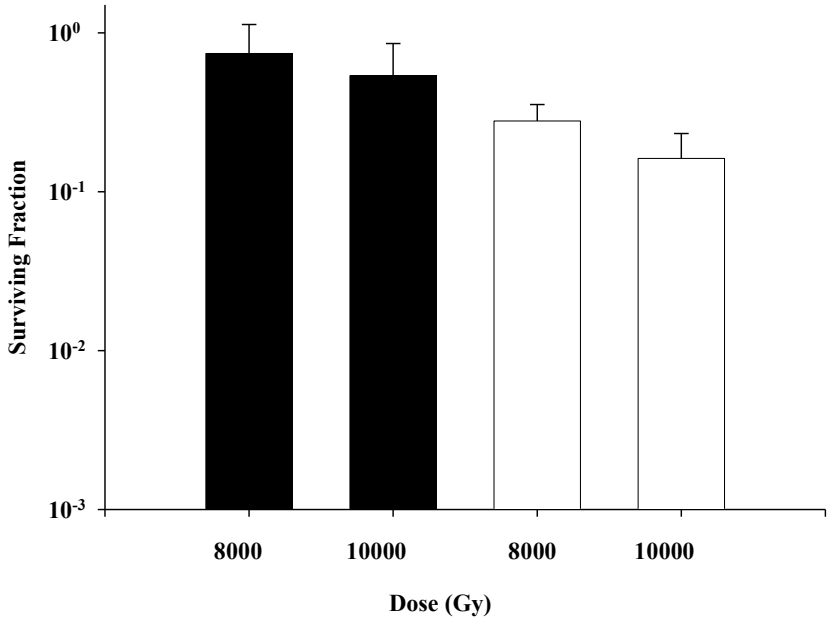

Fig. 5 A comparison of the survival of *D. radiodurans* R1 after growth in TGY broth in exponential phase (black bars) after growth in TGY broth in stationary phase (white bars) following exposure to ionizing radiation Bars in the graph represent the fraction of the culture that survives the dose applied. Each population is titered before and after irradiation, and the fraction of survivors determined by calculating the ratio of the number of colony forming units that survive over the number of colony forming units that were irradiated. Values are the mean +/- the standard deviation of at least five independent trials, n=15.

The Effect of Growth Medium on the Ionizing Radiation Resistance of *D. radiodurans* R1

If the concentration of Mn and the levels of protein oxidation introduced by ionizing radiation do not change during growth, we must assume that some other physiological function has been altered in stationary phase and that this factor affects survival. Changes are occurring during stationary phase, and many of these changes are the consequence of either nutrient deprivation or the accumulation of toxic waste materials that inhibit cell growth and metabolism.

To explore the possible influences on reductions in cell viability in stationary phase, we grew *D. radiodurans* to stationary phase and then re-suspended the cells in a defined medium with and without a carbon source. Survival was measured at 8000 and 10,000 Gy and compared with cells grown and irradiated in TGY broth. As illustrated in Fig. 6, re-suspension and irradiation in M9 salts without a carbon source did not significantly change cell survival relative to irradiation in spent TGY at 8000 Gy (27% survival in TGY and 30% survival in M9 salts), whereas there was an eight fold reduction in viability when cultures re-suspended in M9 salts were irradiated at 10,000 Gy relative to cells irradiated in TGY.

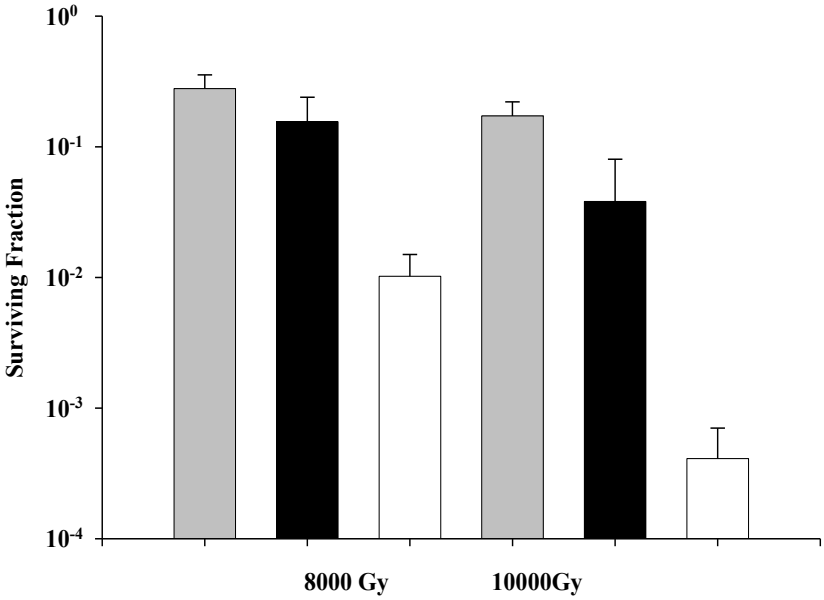

Fig. 6 A comparison of the survival of *D. radiodurans* R1 after growth in TGY broth in stationary phase and then irradiated in either spent TGY (grey bars), M9 salts (black bars), or M9 salts + 1mM $MnCl_2$ (white bars). Bars in the graph represent the fraction of the culture that survives the dose applied. Each population is titered before and after irradiation, and the fraction of survivors determined by calculating the ratio of the number of colony forming units that survive over the number of colony forming units that were irradiated. Values are the mean +/- the standard deviation of at least five independent trials, n=15.

Because of the importance attributed to the intracellular Mn concentration, we wondered if the reduction in viability at 10,000 Gy was related to changes in Mn and studies were conducted in which we included Mn in the M9 salts. The result was surprising – the addition of 1mM $MnCl_2$ to M9 salts dramatically reduced the viability of irradiated cultures. The presence of Mn in the medium resulted in a 30-fold drop in viability at both doses of ionizing radiation [at 8000 Gy (p=0.005, df=15) and at 10,000 Gy (p=0.005, df=16)] relative to cultures irradiated in M9 salts alone. The results oppose the proposed role for Mn in ionizing radiation resistance in this species. The simplest interpretation argues that Mn is toxic to irradiated cells at this concentration.

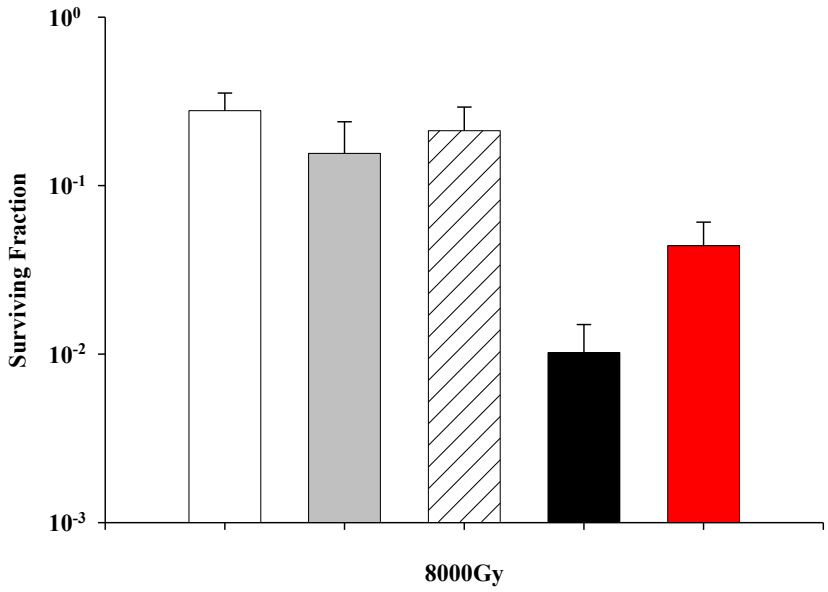

Fig. 7 A comparison of the survival of *D. radiodurans* R1 after growth in TGY broth in stationary phase and then irradiated at 8000 Gy in either spent TGY (white bar), M9 salts (grey bar), M9 salts + 0.2% glucose (striped bar), M9 salts + 1mM $MnCl_2$ (black bar), and M9 salts + 0.2% glucose + 1mM $MnCl_2$ (red bar). Bars in the graph represent the fraction of the culture that survives the dose applied. Each population is titered before and after irradiation, and the fraction of survivors determined by calculating the ratio of the number of colony forming units that survive over the number of colony forming units that were irradiated. Values are the mean +/- the standard deviation of at least five independent trials, n=7.

The Role of a Carbon Source in Cell Survival Following the Exposure of *D. radiodurans* R1 to Ionizing Radiation

Stationary phase R1 cells were irradiated at either 8000 (Fig. 7) or 10,000 Gy (Fig. 8) in the presence of TGY broth or M9 media supplemented with combinations of either 0.2% glucose or 1mM $MnCl_2$ or both treatments. Cells irradiated in spent TGY exhibited the best survival and as indicated in Fig. 6, irradiating in M9 salts or M9 salts with $MnCl_2$ increased sensitivity to ionizing radiation. It is possible that cells in stationary phase require access to a carbon source in order to maintain internal Mn concentrations and that calls cannot take advantage of the Mn added to the M9 salts in the absence of a carbon source. The addition of glucose to M9 salts did not significantly improved cell survival post-irradiation, but it did significantly increase survival in cultures in which Mn was present, increasing survival at 8000 Gy four-fold (p=0.007, df=10) and twelve-fold at 10000 Gy (p=0.006, df=10). At no exposure did the level of resistance return to that observed when cells were irradiated in spent TGY or in M9 salts, indicating that glucose only partially alleviated the lethal effect of Mn addition.

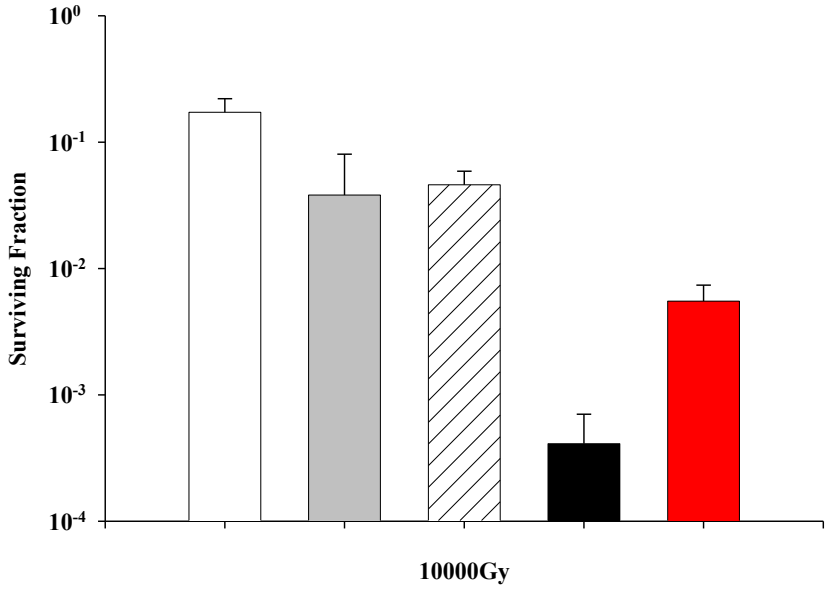

Fig. 8 A comparison of the survival of *D. radiodurans* R1 after growth in TGY broth in stationary phase and then irradiated at 10000 Gy in either spent TGY (white bar), M9 salts (grey bar), M9 salts + 0.2% glucose (striped bar), M9 salts + 1mM $MnCl_2$ (black bar), and M9 salts + 0.2% glucose + 1mM $MnCl_2$ (red bar). Bars in the graph represent the fraction of the culture that survives the dose applied. Each population is titered before and after irradiation, and the fraction of survivors determined by calculating the ratio of the number of colony forming units that survive over the number of colony forming units that were irradiated. Values are the mean +/- the standard deviation of at least five independent trials, n=7.

CHAPTER 4: DISCUSSION

The Daly model for ionizing radiation resistance was evaluated in two studies. First we asked if a protein needs Mn to be protected. We irradiated a purified protein in vitro and determined that it could be protected by other proteins if they are present in concentrations relative to those found in vivo. Second, we attempted to determine if the exogenous addition of Mn would protect cells during irradiation and found that the addition of a relatively high concentration of Mn is detrimental to cell survival, reducing a culture's viability when irradiated relative to cultures that did not contain Mn. The results of both studies were unexpected and, although they do not definitively rule out a role for protein oxidation and Mn concentration in ionizing radiation resistance, they force us to ask whether the Daly model is too simplistic. Each result argues that something more than preventing protein oxidation is responsible for ionizing radiation resistance

Bovine Serum Albumin Protects β-Galactosidase from Oxidative Damage

Ionizing radiation was used to introduce oxidative damage in an attempt inactivate β-galactosidase activity. As the applied dose of irradiation increased, the activity of the protein decreased in response to oxidative damage, suggesting that higher doses would completely inactivate the protein. In the presence of the antioxidants, β-mercaptoethanol or mannitol and benzoic acid, β-galactosidase activity was restored presumably because these antioxidants act as a "sink" for oxygen free radicals formed during irradiation,allowing β-Galactosidase to avoid oxidative damage.

When another protein, BSA (in about half the concentration of proteins in an *E. coli*) was irradiated with β-galactosidase; β-galactosidase activity did not decrease as the dose increased. BSA apparently shielded β-galactosidase from oxidative damage by acting as a alternative target

for free radicals. This result indicates that intracellular proteins have the potential to protect other proteins from inactivation by ionizing radiation. The proteins do not need to specialized molecules, intended specifically for that purpose. All proteins can be modified by ionizing radiation, but the sheer number of proteins found *in vivo* all but guarantees that not every copy of a specific protein (or its function) will be lost post-irradiation. This conclusion forces us to question the Daly model. In this model, its authors postulate that the prevention of protein oxidation is critical to cell survival and that in species such as *D. radiodurans* specific mechanisms are in place to limit protein oxidation. We ask why such as system would need to be in place given our results. Clearly, Daly and colleagues have demonstrated a correlation between lower protein oxidation in radioresistant species and radiosensitive species, but this correlation has never been linked to a specific function. The identity of the most important proteins affected by ionizing radiation-induced oxidation is unknown, and until they are known it will not be possible to assess how partial inactivation of proteins affects the cell's ability to survive exposure to ionizing radiation.

Ruhl et al. (2011) recently published work that supports the conclusions made by these studies. They investigated the use of gamma irradiation as a means of sterilizing human saliva so that proteins present in saliva could be studied independent of the mouth's microflora. The goal of this study was to find a dose that would not damage the proteins, but eliminate the bacteria. They found that all bacteria were destroyed at 3500 Gy, but the proteins of interest remained functional. In fact, there was no visible protein damage at 5000 Gy using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As with the data we have presented, it does not appear that the dose of ionizing radiation needed to inactivate a bacterial cell corresponds with that needed to inactivate a protein. Therefore, it seems unlikely that

protecting proteins for oxidative damage is the principle determinant of ionizing radiation resistance.

Addition of Mn(II) Does Not Increase Ionizing Radiation Resistance in Stationary Phase *D. radiodurans*

Exponential and stationary phase cultures of *D. radiodurans* R1 were exposed to 8000 and 10,000 Gy gamma irradiation in spent TGY. Stationary phase cells were more sensitive at these doses presumably due to the scarcity or depletion of nutrients and the amount of cells present at this growth phase compared to exponential phase cells. Also, the cells were irradiated for 18 and 22 hours to achieve doses of 8000 and 10,000 Gy, respectively. It was possible that this lengthy time of exposure could have been more problematic for stationary phase cells. To determine how the cells would fare irradiation in a minimal salts medium without a carbon source, stationary phase cells were grown in TGY but re-suspended in M9 minimal salts without a carbon source prior to irradiation. Cell survival did not decrease at 8000 Gy, but there was a reduction at 10,000 Gy. Since the Daly model argues that Mn is necessary for ionizing radiation resistance, we tested this by adding Mn to the M9 minimal medium and irradiating the cells in this medium with the intent of seeing an increase in viability. Unfortunately, the addition of Mn reduced cell viability at both 8000 and 10,000 Gy with the most reduction seen at 10,000 Gy. In fact, cells were more sensitivity with the addition of Mn compared to survival in TGY and M9 without a carbon source. It appeared that the exogenous addition of Mn harmed the cells. However, when cells were irradiated in M9 with a carbon source and Mn, survival increased compared to survival in M9 with Mn alone. This suggested that the presence of the carbon source could have assisted in the uptake of the Mn by the cells. Even with the addition of the carbon source, cell survival was not restored to the level of survival in TGY suggesting that the nutrients provided in TGY could contribute to resistance.

Based on these results, we determined that Mn is not essential for ionizing radiation resistance in *D. radiodurans*. If Mn was necessary then cell survival should have increased with the addition of Mn, and an energy source was needed to transport Mn into the cell then once the energy source was provided in the medium, cell survival would have increased.

The conclusions of this experiment are supported by the results reported by Chou and Tan (1990). They examined the effect Mn had on cell survival of exponential and stationary phase *D. radiodurans* following gamma irradiation exposure, and their results showed that stationary phase cell tolerance decreased. Also changes in growth patterns following the addition of Mn showed increased cell growth but increased cell viability. Although *D. radiodurans* cells contain Mn, the intracellular level of Mn present in the cell must be regulated to avoid problems for the cell because changes in these levels can be toxic to the cell (Jakubovics). Therefore, the Mn experiments in this thesis and Chou and Tan (1990) argues that the addition Mn is not essential for radioresistance, instead it harms stationary phase *D. radiodurans* cells leading to a decrease in radioresistance.

It is possible that Mn contributes to ionizing radiation resistance but under certain circumstances. If the concentration of Mn is high in the cell then it can play a role in reducing the amount of oxidized proteins. However, to imply that Mn is a determinant of ionizing radiation resistance is misleading. Instead, this thesis suggests that the factors of the Daly model only contribute to bacterial ionizing radiation resistance and are not the sole determinants. Bacterial ionizing radiation resistance may not depend on a single factor or correlation but a combination of factors which may be known and have not been pieced together or yet to be uncovered.

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VITA

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