THE EFFECTS OF IONIZING RADIATION AND OXIDIZING SPECIES ON STRAINS OF DEINOCOCCUS RADIODURANS LACKING ENDOGENOUS OXIDATIVE PROTECTION METHODS

THESIS

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AFIT-ENP-MS-16-J-017

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Dylan L. Klawuhn, BS

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Abstract

Multiple strains of *Deinococcus radiodurans* were transformed, creating knockout mutations in genes responsible for manganese ion transport, manganese and copper/zinc super-oxide dismutase, and bacillithiol synthesis. These mutated strains were then irradiated with ~20,000 Gys. The results showed that the mutated strains had a higher sensitivity to ionizing radiation, those responsible for bacillithiol synthesis having an increase in sensitivity 3000 times more than wild type *Deinococcus radiodurans*. In addition to radiation the mutated strains were also exposed to paraquat, an oxidizing herbicide. Strains missing manganese super-oxide dismutase showed increased sensitivity.

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Dylan L. Klawuhn

"You can observe a lot by watching."

Yogi Berra

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I. Introduction

Research Statement

The primary objective of this research is to investigate if the removals of genes responsible for producing reactive oxygen species (ROS) scavengers has any effect on the resistance to ionizing radiation possessed by *Deinococcus radiodurans*.

D. radiodurans is a species of bacteria capable of withstanding ionizing radiation 1000 times greater than human cells and 30 times greater than *E. coli* [59]. It has been established that the main target of ionizing radiation that causes cell death is DNA. Recent investigation of this has shown that the destruction of DNA repair enzymes may prove to be the main cause of cell death rather than the destruction of the DNA itself [24,42]. Removal of the genes that are responsible for producing enzymes and small particles that protect the cell from reactive oxygen specs may show a decrease in DR's resistance to ionizing radiation. The metrics for measuring survivability after irradiation of DR are colony forming units while for paraquat experiments optical density of the cultures was used.

Problem Statement

The purpose of this research is to measure the effectiveness of DR's radiation resistance after having manganese and copper/zinc scavenger genes removed, both separately and in double and triple gene knockouts and then exposed to gamma (γ) irradiation using a cesium-137 source and beta radiation from the Texas A&M LINAC.

The metrics used to quantify the radioprotection efficiency were comparative ratios of colony-forming units (CFUs) between treated and untreated strains. Studying the effects of ROS scavengers, DR's DNA repair mechanisms, and other redundancies that possibly help with radiation protection were instrumental in understanding the possible mechanisms. Investigating the resistance enzymes gave a clearer understanding of why repair mechanisms may not be fully responsible for DR's robustness and how ROS scavengers may be providing vital protection.

Research has been conducted concerning protective enzymes and particles against oxidation. Recent studies at the Air Force Institute of Technology (AFIT) investigated the mitigation of oxidative damage by small molecules and manganese [70]. The addition of Mn^{+} scavengers to bacterial cells has shown an increase in radiation resistance and suggests that related dismutase scavengers, that act to reduce oxidative reactions, may be providing similar protection in DR [23]. With the genes responsible for ROS scavenging in *Deinococcus radiodurans* knocked out, the protection enzymes they produce against ionizing radiation and the oxidizing agents can be measured and possibly open up several opportunities to learn more of how it is able to survive such hazards and how this ability can possibly be augmented in other bacterial species [22].

Deinococcus radiodurans has been researched over the past 60 years. However, the mechanisms that provide its protection from radiation and desiccation are not completely known, though several hypotheses have been offered. The growth conditions and their effects on DR's resistances, DR's genetic makeup and genome, its methods of DNA repair, as well as the limits of its resistances have all been studied but there has been no consensus of what mechanisms are responsible.

The rate of growth for DR is fairly slow, usually taking two to three days for a culture to grow to saturation. This allows for an ease of observing the outgrowth after irradiation. Also, the cesium-137 source used in this research produces approximately 357 Gys per hour which means that obtaining doses that will reduce the viability of DR will take a deal of time but will still be able to overcome DR's resistances. Irradiating the cultures will cause damage in the form of breaks in the DNA which will halt cell reproduction and thus outgrowth. Delay in outgrowth is assumed to reflect the damage of DNA and enzymes within the cell.

The effects from the knockout of a single, double, and triple set of ROS scavengers will provide insight into the effectiveness of their protective mechanisms. Results after the irradiation will show the susceptibility of the mutant cells at differing radiation doses. Experiment data sets of the knockouts and the consequences of their removal from *Deinococcus radiodurans* were created. The results gained from this experiment will then be used to measure these scavengers' capabilities as radioprotectants.

Motivation

Cellular damage induced by ionizing radiation is quite thoroughly researched as well as the occurring oxidation in the cell. However the mechanisms by which *Deinococcus radiodurans* is able to resist radiation damage is still in question. The direct introduction of manganese species and other ROS scavengers such as sulfur have shown to increase the resistance of cells before irradiation but this has only been done by directly introducing the species into cells [22,24]. The reactive oxygen species

scavengers produced by *Deinococcus radiodurans* however, have not been fully investigated. Further applications of this research may be in radiation medicine such as in cancer treatments and acute radiation syndrome. The research could also further fields in occupational and combat studies. Another possibility is to use this research to find ways of counteracting radiation resistance in bacteria for the purposes of sterilization.

For the purpose of finding ways to increase or decrease radiation resistance, *Deinococcus radiodurans* will be studied. By finding ways to manipulate its radiation resistance we may learn to do so in other species and further our understanding of how radiation resistance is achieved. The dismutase genes and their proteins are the focus the research and how they impact DR's radiation resistance.

Research Focus

Current research at AFRL's Human Effectiveness directorate is focused on exploring the factors relating to cell death by way of radiation and the effects of radiation on DNA and enzymes. *Deinococcus radiodurans* has been the primary focus of this research. *Deinococcus radiodurans'* ability to withstand over 17,000 Gray has raised many questions as to how it is capable of doing so and studying its cell structure and repair mechanisms is of great importance to this work. Possible mechanisms that have been suggested are small protective molecules, extremely efficient repair enzymes, or robust repair mechanisms [17,22,23,60].

Ionizing radiation, which is produced by gamma rays and x-rays, and its effects on DR, has been studied since DR's discovery by Arthur W. Anderson to gain a greater understanding to its limits against radiation. Studies have also been conducted to

investigate its resistance to desiccation, UV irradiation, and thermal effects [7,59]. While other studies have looked at the effects of desiccation and UV effects, the focus of this study will be on ionization and the oxidative effects as this resistance is quite unique in DR and the study of knocking out its oxidative scavenger by way of gene transformation is novel.

The Air Force Institute of Technology (AFIT) has performed previous work concerning cell death by way of radiation; including pulsed UV inactivation, continuous UV inactivation, and gamma inactivation of *Bacillus anthracis*. Research has also been conducted concerning the effects of certain scavengers protecting enzymes during irradiation.

Of particular interest are *Deinococcus radioduran*'s super-oxide dismutase enzymes that are suspected to provide protection from the effects of ionizing radiation. These enzymes come in two types; Mn super-oxide dismutase and Cu/Zn super-oxide dismutase. Both of these enzymes act as oxidative scavengers that reduce oxidizing reactions [1]. Their removal may indicate their importance in DR's radiation resistance.

An additional focus of this research is the exposure of DR to paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) which acts as an electron acceptor in redox and radical actions. Paraquat has the effect of increasing the rate of oxidation reactions inside of the bacterial cell and causing further oxidative damage. It acts as an electron acceptor and then transfers the electron to molecular oxygen which produces oxidative species. Also, the knocking out of manganese transport genes will be conducted much in the same way as the knocking out of the manganese superoxide dismutases themselves, to see if this too has any effect is reducing DR's resistance to radiation. Bacillithiol is a thiol

compound first recognized in *Bacillus anthracis* and later identified in *Deinococcus radiodurans*. Its full function is unknown but it is believed to serve in sensing peroxides. It may also be a replacement for glutathione, which reduces hydroxyl radical by using its sulfide bonds.

Approach

This research will focus on the resistance *Deinococcus radiodurans* demonstrated against the effects of ionizing radiation and oxidation. This will involve growing *Deinococcus radiodurans* cultures and then by way of engineered gene deletions, knocking out the genes which are thought to contribute to ROS neutralization including Mn and Cu/Zn superoxide dismutases and Mn transporters. To test whether or not the genes do contribute, the transformed strains will be irradiated.

First, *E. coli* cultures will be grown and transformed with a vector that cannot replicate in DR and serves as the backbone for all constructs. A selection marker will be assembled with DNA fragments flanking the gene to be deleted to enable selection of the gene replacement upon transformation of *Deinococcus* with the assembled vector and selection on the appropriate antibiotic. To check that the intended gene deletion was achieved, PCR will be used to amplify the region spanning the gene of interest and gel electrophoresis will be used to check the base pair length corresponding to the gene replacement. Post irradiation colony growth will be used as the metric to determine any changes in the cells' resistances.

Assumptions

Assumptions made throughout this research include: cell death was considered to be the inability to outgrow a viable colony of daughter cells; the measured optical density of cultures is assumed to be colony-forming units.

Document Structure

This document is partitioned into 5 chapters, each with respective sections and subsections. Chapter I discusses the purpose of the research and the general outline of the processes taken to resolve the research objective. Chapter II covers the science and theory behind the topic of the research. This chapter serves as the analytical backbone of the project and includes: *Deinococcus radiodurans* background; DR damage repair mechanisms; background of the scavenger species; transformation and gel electrophoresis; and oxidative damage as produced by ionizing radiation. Chapter III outlines the preparation and subsequent irradiation of the bacterial cultures to provide empirical data for measuring the test metrics. Chapter IV introduces and analyzes the data. The results of the data will reveal implications on the efficacy of the scavenger species. Chapter V will provide an overview of research accomplishments and suggested future work. The appendix contains additional content that supports the methods of the project but is not directly related to the primary research focus.

II. Background & Theory

Chapter Overview

The purpose of this chapter is to provide information about *Deinococcus radiodurans*, radiation damage, and radiation protection. Topics covered in this chapter include: DR background; gene knockout; IR damage and repair mechanisms; manganese transporters; and oxidative damage. There is also discussion about the theoretical approach to the experiment, including: gel electrophoresis; PCR; and bacterial transformation.

Deinococcus radiodurans

Deinococcus radiodurans is a gram-positive, red-pigmented, nonsporulating, nonpathogenic bacterium that forms diads and tetrads with an average cell diameter of 1 µm. DR contains two chromosomes, one of 2,648,638 and one of 412,348 base pairs. It also contains two plasmids measuring 177,466 and 45,704 base pairs. One of the most impressive aspects of this species is its ability to survive and mitigate the effects of oxidative damage, especially damage caused by ionizing radiation, being able to resist up to 15,000 Gy, over 1000 times more radiation than human cells. DR was originally isolated from gamma-irradiated canned meat in Oregon. This bacterium can be found in a variety of habitats including animal gut, hot springs, and Antarctica. It has mesophilic, thermophilic, and psychrophilic strains. DR is naturally transformable and this accomplished with ease if transformed with nonmethylated donor DNA passed along through *E. coli* [7,59].

The extensive ability of DR to withstand oxidative damage has been attributed to exceptional DNA repair mechanisms in the past [57,59]. Other mechanisms have also been suggested such as protective small molecules or a structural form that eases DNA repair. The DNA repair mechanisms available to DR include direct damage reversal, base and nucleotide excision repair, mismatch repair, and recombinational repair. However, current research shows that DR's repair systems are less complex than that of *E. coli* yet DR is still 30 times more resistant to oxidative damage than *E. coli* [22,24,60]. There is a possibility that DR's exceptional resistance is from resistance enzymes or a combination of enzymes, both protective and for transport of Mn and that is what is to be investigated in this research.

Deinococcus radiodurans **Damage**

Many studies have examined inactivation of DR by different methods. Researchers have focused on inactivation by radiation, including ionizing and UV radiation, and desiccation. A great deal of research has been conducted to quantify how much oxidation DR is capable of withstanding, exploring the differences in oxidation source, the impact of nutritional and growth media differences, as well as the effects of adding and exposing DR to varying scavenger species [7,59].

Structural Mechanisms.

The physical structure of DR commands some note. Even though DR is a gram-positive bacterium, it has a multilayered cell envelope which is unusual and is more common in gram-negative bacteria. There are at least 5 layers. There is a cytoplasmic membrane, the peptidoglycan-containing holey layer, the compartmentalized layer, the

interior layer, and a fragile soft layer. The holey layer contains a diamino acid L-ornithine which is rare in bacteria. Approximately 43 percent of the lipids found in the membrane are unique to DR. These lipids are both straight- and branch-chained, monounsaturated and saturated lipids [6]. The cell envelope is approximately 150 nm across. The total cell diameter is 1 µm across on average [59].

Oxidative stress produced by ROS species can be mitigated by some of the metabolic processes utilized by DR. Proteolysis, the breakdown of protein by enzymes, is DR's main form of energy production. By absorbing degraded proteins, DR is able to import peptides and amino acids that help reduce biosynthetic demands and helps boosts antioxidant complexes of amino acids and peptides with manganese. DR's glucose metabolism also helps DNA-damage recovery by converting glucose into DNA building blocks, dNTPs precursors, and possibly manganese complexes. ROS production inside of DR is also thought to be reduced by the lack of iron-sulfur cluster enzymes that may release free iron that furthers oxidative stress [1,7,59].

DNA

DNA stands for deoxyribonucleic acid. This molecule contains the genetic information of an organism. The DNA molecule is a double helical structure composed of two anti-parallel strands. The two strands of DNA have a backbone comprised of phosphorous and ribose sugar molecules. The DNA molecule is a polynucleotide made up of four bases: cytosine, guanine, thymine, and adenine. The base cytosine will only pair with guanine while only thymine will pair with adenine. This is due to their structure and the hydrogen bonding potential of the particular bases. Adenine and guanine are purines with double ring structures while thymine and cytosine are

pyrimidines that have a single ring structure. The bonds between adenine bases and thymine bases are held by 2 hydrogen bonds while the bonds between cytosine and guanine are made of 3 hydrogen bonds.

DNA can normally be found within a cell's nucleus, or for bacterial cells within an area of cell known as the nucleoid. In eukaryotic cells, DNA strands form chromosomes which are wound tightly in a coil-like structure which protects the molecule. These coils are wrapped tightly around groups of proteins, called histones. Only when the cell requires the DNA to produce proteins is the DNA coil released by unwinding enzymes. Histones are found in eukaryotes but a current study by Ghosh and Grove have identified a protein, DrHU, that allows for compaction of DR's DNA much the same way as would be for an eukaryotic cell. This coiling of the DNA could further enhance DR's protection against oxidation by keeping the ROS from reacting with a large amount of the DNA molecule [33,39].

Ionizing Radiation.

Ionizing radiation is defined as radiation that causes an atom or molecule to lose electrons by depositing energy into the molecule. Gamma and X-rays are common examples of ionizing radiation. Ionization can occur by way of several mechanisms: photoelectric absorption, Compton scattering, and pair production. Photoelectric absorption predominates at energy levels below 0.2 MeV and when a photon has enough energy to overcome the binding energy of an atom's electron, freeing an outer orbital electron. Compton scattering occurs by way of the photon being redirected, or scattered, by a particle. The photon deposits some of its energy and energy and momentum must be conserved. The angle of scatter depends on the energy of the photon and the mass of the

atom they reflect off of. The heavier the atom and the less energy, the greater the angle of scatter [38].

Ionizing Radiation Damage Mechanisms.

Ionizing radiation reacts with DNA in two ways. The first way is through direct effects. Direct effects occur when the ionizing radiation ionizes or excites the DNA molecule directly. Ionization occurs when the absorbed radiation removes an orbital electron from the molecule it comes into contact with. Ionization is usually perpetrated by X rays, γ rays, alpha radiation, and beta radiation. Ionizing radiation is best defined as radiation whose energy is greater than the ionization potential and frees electrons from atoms or molecules. Excitation occurs with the radiation coming into contact with a molecule and raising one of its electrons to a higher energy level. This is done without going past the ionization potential and is commonly produced by UV radiation and ion-electron recombination [39].

The main effect of direct ionizing radiation is that it can cause bond breaking in DNA, producing radical effects like photoproducts, photolysis, and other radical reactions. The effect of direct ionizing radiation on DNA can cause the sugar-phosphate backbone to break which can result in a strand break, where the backbone of the DNA essentially “snaps”. Alternatively it can cause deamination, which is the removal of an amine group from a molecule. Deamination of the base pair changes cytosine to uracil, guanine to xanthine, and adenine to hypoxanthine. These changes can lead to DNA mutagenesis, where base pairings are changed [5].

The second way that ionizing radiation interacts with DNA is through indirect effects. Indirect effects generate free radicals by interacting with water which makes up a

majority of the cells. The free radicals that are produced by the radiolysis of water can go on to interact with the target molecule of DNA and cause damage. When water becomes ionized, an ion pair is formed consisting of an electron and ion radical of water. The ion radical of water is formed in a high vibrational state which goes on to produce a hydroxyl radical while the ionization electron will go on to form a solvate electron which acts as a reactive reducing agent. In bimolecular reactions, the excited electron can also go on to produce a hydrogen free radical while the hydroxyl radical can produce hydrogen peroxide [5].

For example, a radical produced by water radiolysis, like hydroxyl or a hydrogen free radical, can interact with the DNA and abstract hydrogen and cause the bond to break which can cause a strand break. Further damage that can be caused by the indirect effects is fixation. When exposed to oxygen, the DNA can form a DNA hydroperoxy radical which can further combine with a hydrogen free radical to form a DNA hydroperoxide. These organic peroxyl radicals cannot be repaired easily.

From the indirect effects, addition of the hydroxyl radicals and hydrogen free radicals are the most common pathway to damaging DNA. The DNA can then suffer damage to its functional groups resulting in an incorrectly coded nucleotide, damage to purines or pyrimidines, or a radical can come into contact the sugar-phosphate backbone and cause a strand break. A double strand break can occur if two strand breaks are close together, approximately within 10 base pairs. Additionally, radicals interacting with the backbone can cause crosslinking and scission. Scission is where the radical causes reactions that cause a break in the chain of sugar links while crosslinking is where the radical prevents new bonds between the bases. The presence of oxygen can inhibit

scission. Ionizing radiation causes damage to the DNA which can then prevent the cell from reproducing. The cell could possibly repair the damage or the damage itself could lead to a mutation. This damage could possibly lead the cell to commit apoptosis.

IR Damage Repair.

Single-strand breaks (SSBs) are often repaired rapidly in DNA. Double-strand breaks (DSBs) occur at random sites on the chromosome and thus take more time as a homologous donor molecule is needed for homologous recombinational repair. Single strand breaks are easily repaired within the cell and contribute very little to any mutations that may occur. So out of the majority of reactions that occur, most will be repaired and those that are not repaired are likely to cause the cell to commit apoptosis [29]. For single strand breaks there are two main methods of repair; the first being base-excision repair. This repair mechanism is used predominantly in DNA molecules that are replicating. Excision repair removes damaged base pairs that could possibly cause mutations through mispairing or breaks by replacing a single nucleotide or by synthesizing a new chain of nucleotides. The other repair method is error-prone repair. This occurs when there is a large amount of DNA fragments and RecA is utilized. This has a high error rate and is usually a last line of defense for a cell [58].

In DR, there are two main forms of recombinational repair, extended synthesis-dependent strand annealing (ESDSA) and homologous recombination by crossovers. ESDSA allows for homologous repair and is a vital capability. This allows damaged DNA to be repaired by moving correct segments of DNA from an undamaged chromosome to the damaged section. Double strand breaks are much more difficult to repair. Homologous recombination repair is accomplished by taking a sister chromatid,

DR has 2 chromosomes, and using it to synthesize a matching segment of DNA which serves as a patch that can be placed over the two broken ends of the damaged strands. A second repair method is non-homologous end joining. This repair method does not take from a sister chromatid and instead simply joins the ends of double strand breaks [11]. During the fast reaction portion of the repair, which occurs within the first 10 minutes after damage, the ends are joined and a small number of base pairs, approximately 10, are deleted to allow for joining. The slow reaction occurs after this 10 minute period and takes several hours to repair and is used only if the fast reaction is unsuccessful. It utilizes the same repair mechanisms as the fast reaction but is RecA dependent [11,59,60].

Extended synthesis-dependent strand annealing (ESDSA) is the initial step that allows homologous repair. When there is a double-strand break on the DNA, a protein complex will bind to each end of the break, and with the help of the nucleases, create 3' overhangs of single-stranded DNA. These strands are then formed into a nucleoprotein filament that can then be inserted into another chromosome. The 3' strand is then extended on the homologous DNA to form a strand of DNA that can be annealed onto the original break. RecA recombinase is required for these processes as it primes DNA repair synthesis as templates after the DSBs are turned into 3' single-stranded DNA substrates. RecA must find homologous DNA sites and the method of how it does this is unknown. In DR, four models are suggested: genome condensation, ring-like nucleoid morphology, and DNA-membrane association, and chromosome alignment [11].

After irradiation, DR is dependent on ESDSA and recombination for DNA repair. The fragmented DNA is recessed into 5'-3' direction. The 3' single strand overhangs are

then freed by way of RecA- and RadA strand invasion and then prime DNA synthesis on overlapping fragments. The enzymes Pol III and Pol I then initiate DNA synthesis and newly synthesized single-strands anneal to complementary single-strands to form a double-strand that go on to be formed into circular chromosomes by RecA [11].

What causes the formation of the 3' overhangs that result in RecA and RadA strand invasion is unknown. Bentchikou et. al states that the model bacteria of *E. coli* has several enzymatic activities needed for the processing of double stranded DNA: a helicase, 5'-3' exonuclease, and a mediator function for RecA filament formation. This is carried out by RecBCD complex "which is the major component for initiation of recombinational repair of DNA double-strand breaks… However, if RecBCD is inactivated, an alternate pathway, the RecF pathway, promotes recombinational DSB repair," [11]. *Deinococcus radiodurans* does not have RecBCD complexes and is also missing any AddAB, another common repair complex in many bacteria.

Deinococcus radiodurans does have homologs for the components of the RecF pathway which are RecJ, RecQ, RecF, RecO, and RecR. This is why the RecF is considered to be the main recombinational repair pathway. The RecF, RecO, and RecR proteins are also thought to be responsible for the loading of RecA onto DNA substrates which further repairs DSBs by way of ESDSA and recombinational repair. RecA is also thought to regulate double strand ends by controlling DNA degradation and synthesis as well as expression of nuclease-activity that responds to radiation damage. The protein RecJ also seems to be essential in the repair of DNA as Bentchikou et. al saw that inactivation of RecJ resulted in fully lethal phenotype [11].

Another possible protective mechanism is that the genomic DNA of DR is observed to be more condensed than radiation-sensitive species of bacteria. Genomic condensation is thought to protect against radiation damage by protecting DNA from radicals made in ionized water, restricting diffusion of DNA fragments, and preventing interaction of degradation enzymes. Stationary-phase DR cells have a ring-like structure where DNA is wrapped about a proteinaceous core. This structure is thought to prevent diffusion as well as providing a greater deal of DNA end-joining capacity (nonhomologous end-joining has not been observed in DR). Levin-Zaidman et. al posited that the toroidal shape of the DNA allowed for a maintained rigid matrix of DNA even after multiple strand breaks. DR also has complimentary DNA in each of the four compartments that make up its tetrad morphology. Levin-Zaidman viewed that after irradiation, toroidal DNA unstructured itself into an open S-like morphology [44]. This DNA was then spread to another compartment through the membrane and then resulted in two nucleoids. These nucleoids would then provide a basis for template-dependent recombination. However, Gao et. al supports the idea that due to other species of *Deinococcus* having non-toroidal DNA structures and that are just as radioresistant shows that there is no added benefit for the DNA to be toroidal. DNA-membrane association is when DNA is attached to the membrane and supports correct recombination and may bind RecA to the cell membrane [32].

Dose Rate of Ionizing Irradiation

The dose of ionizing radiation that a cell absorbs has greater effect with increased rate. Increasing the dose rate increases the concentration of ROS species which in turn increases the amount of oxidative damage. When exposed to radiation the signaling

pathways are activated by the presence of oxidative reaction products which trigger mechanisms that protect against ROS. It is possible that if the dose rate is high enough the protective mechanisms can be overwhelmed and unable to mitigate the increasing damage. These higher dose rates deposit energy at high rates. This causes a cascade of oxidative damage which may become too much to be repaired. Lower dose rates could allow the protective mechanisms and enzymes time to provide full protection as they will not be overwhelmed.

Manganese in *Deinococcus radiodurans*

Deinococcus radiodurans is known to actively collect and transport Mn into its cell body from its surrounding environment. It has been observed that DR contained approximately 100 times more Mn than *E. coli*, ~0.29E-18 mol Mn/cell when grown in defined minimal medium with trace Mn. X-ray fluorescence has been used to show that Mn is also taken up by DR while being grown in tryptone-yeast-glucose media (TGY). Contrarily, Fe is less prevalent and is only found outside the cytosol of the cell. Bacteria with high Mn to Fe ratios have been shown to be ionizing radiation resistant, the Mn defending against oxidation damage but not the amount of DSBs [4,17,24].

Daly conducted a study where the outgrowth of DR was measured after being grown in differing amounts of Mn. It was observed that DR grown in a greater concentration of Mn had a higher degree of radiation resistance, while DR grown in a low amount of Mn had a much lower rate of survival. When grown with a lower Mn to Fe ratio, DR is more sensitive to ionizing radiation and this can possibly be attributed to the production of free iron which further increases oxidative stress. [24]

The active uptake of Mn^{+2} by DR influences homeostasis and oxidative stress response. *Deinococcus radiodurans* has 2 of 3 known Mn transporters, including natural resistance-associated macrophage (Nramp) family and a transporter from the ATP dependent ABC-type transporter family. *Deinococcus radiodurans* is lacking the P-type ATPase. Manganese transport is thought to be regulated by a transcriptional regulator, the manganese transport regulator-diphtheria toxin repressor (MntR-DxtR) family that has a Mn^{+2} configuration. It has been observed that DR grown in high Mn^{+2} content has 5.6 times more Mn content than low Mn^{+2} [24].

Mn and Cu/Zn Super-oxide Dismutase

Resistance enzymes in DR include Mn and Cu/Zn super-oxide dismutases which act as oxidative scavengers. It has been reported that Mn SOD is capable of eliminating high concentrations of O_2^- by way of a rapid protonation mechanism off of the bound peroxide from the oxidized metal. There is also evidence of DR having two pathways to dismutate superoxide, a slow and a fast protonation of superoxide. These enzymes act to reduce the oxidation species and the method by which this is done is shown below. It can also be seen as Mn SOD interacts with ROS it regenerates [2,8].

$$M^{n+}SOD + O_2^- \rightarrow M^{(n-1)+}SOD + O_2$$

$$M^{(n-1)+}SOD + O_2^-(+2H^+) \rightarrow M^{n+}SOD + H_2O_2$$

The structure of Mn SOD is shown in Figure 2. The structure comes in two forms. The first is composed of a single homodimer with two Mn^{3+} ions and 366 solvent molecules while the second form is composed of two homodimers with four Mn^{3+} ions and 567 solvent molecules. Both of these forms have a metal-ion coordination sphere that has a water molecule and amino-acid residues arranged in trigonal bipyramidal

geometry around the metal ion. It has been noted that the activity of Mn SOD is lower of that than the Mn SOD found in *E. coli.* However, they both perform the same way [2,28]. Mn SOD also has a reaction rate of 2.0 x 10^9 $M^{-1}S^{-1}$ while the diffusion limit is 2.2 x 10^9 $M^{-1}S^{-1}$ [47] and the total concentration is thought to be 200 µM [64] giving an estimated rate of 4 x 10^5 S^{-1}.

An interesting detail of Mn SOD is that DNA binds in a positively semi-circular groove formed at the monomer-monomer interface of the Mn SOD homodimer with amino acid side-chains forming two loops on the surface of each monomer that allow anchoring of the DNA. In this way the Mn SOD is often found in close proximity to DNA which may provide a protection mechanism against oxidative damage [28].

The other SOD type of DR is of Cu/Zn SOD. This is expressed in two different genes. Its method of reducing superoxides is shown below and regenerates in the same way as Mn SOD. It is unclear if this has the same binding as Mn SOD. However, Cu/Zn SOD in humans bind to the cell membrane [8].

$$Cu^{2+}Zn^{2+}SOD + O_2^- \rightarrow Cu^{+}Zn^{2+}SOD + O_2$$

$$Cu^{+}Zn^{2+}SOD + O_2^-(+2H^+) \rightarrow Cu^{2+}Zn^{2+}SOD + H_2O_2$$

Target Genes

The genes targeted in this experiment are DR_1279, DR_1546, and DR_A0202. The first is a manganese family superoxide dismutase (MnSOD) gene while the last two are copper-zinc family superoxide dismutase (Cu/ZnSOD). These genes are responsible for producing their corresponding enzymes that catalyze the disproportionation of superoxide anion (O_2^-) radicals to hydrogen peroxide and molecular oxygen. This

prevents oxidative damage and keeps the concentration of superoxide low. The superoxide is reduced to O_2^- which dismutes to O_2 and hydrogen peroxide. The hydrogen peroxide will most likely dismute to either hydroxyl radical or water. The other genes that are being investigated are manganese transporter genes. It is hypothesized that blocking these genes should lead to a higher degree of oxidation sensitivity as well as radiation sensitivity as the transformed DR will have a reduced ability to absorb Mn. The genes themselves are DR_1709 and DR_2283-Dr_2284. Finally, the gene BshA will be targeted which is responsible for bacillithiol synthesis.

Bacillithiol

Bacillithiol is a thiol compound first recognized but unidentified in *Bacillus anthracis*. It was later found in identified in *Deinococcus radiodurans*. Its full function is unknown but it is believe to serve in sensing peroxides, and thus helping in defending against, peroxide species. However, it may also be a replacement for glutathione, which reduces disulfide bonds oxidizing agents form with cysteines. If bacillithiol serves this function, it could be key in DR's defense against oxidation as well as against ionizing radiation [44,53].

Paraquat

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) is a non-selective bipyridinium herbicide classed as a viologen. It is toxic to humans and animals. It is made up of a cation formed by two pyridine rings. The rings each have a quarternary amine which keeps it in an initial dication state. Paraquat acts as a redox cycler, having a

large negative reduction potential. This negative reduction potential prevents it from reacting with strong reductant compounds. If the dication of paraquat accepts an electron from a reductant, the resulting monocation of paraquat will then begin to react with oxygen which will then produce superoxide radical and then move on to produce the other ROS species. Paraquat was used in our experiments to compare the resistance of DR to oxidative stress produced by radiation to what was produced from growing a culture in the presence of paraquat. The cultures of DR were started at lag phase and then grown in the presence of varying amounts. [43]

III. Methodology

Chapter Overview

This chapter details the methods developed and adapted to develop selected mutant strains of DR. The DR was transformed to knock out super-oxide dismutase genes, manganese transporter genes, and bacillithiol synthesizer genes. The strains were subsequently exposed to ionizing radiation from a cesium-137 source. The strains were also exposed to paraquat. The survival of the transformed strains was compared to controls of unirradiated DR as well as untransformed and unirradiated wild type DR. The transformed strains grown in paraquat had survival curves measured using their culture's optical density.

Microbial Technique

Initial samples of *Deinococcus radiodurans* and the knockout plasmids were provided by Dr. Thomas Lamkin and his research group at the 711th Human Performance Wing.

Plasmid Construction

The plasmids that are used to perform the knockouts in both *E. coli* and DR were first created in NEBuilder. The plasmids were constructed to contain an upstream and downstream homology region as well as a resistance marker. These components were delivered separately and were then combined during PCR, which amplified the amount of plasmids. The correct plasmid construct was verified by using gel electrophoresis and the plasmids were extracted.

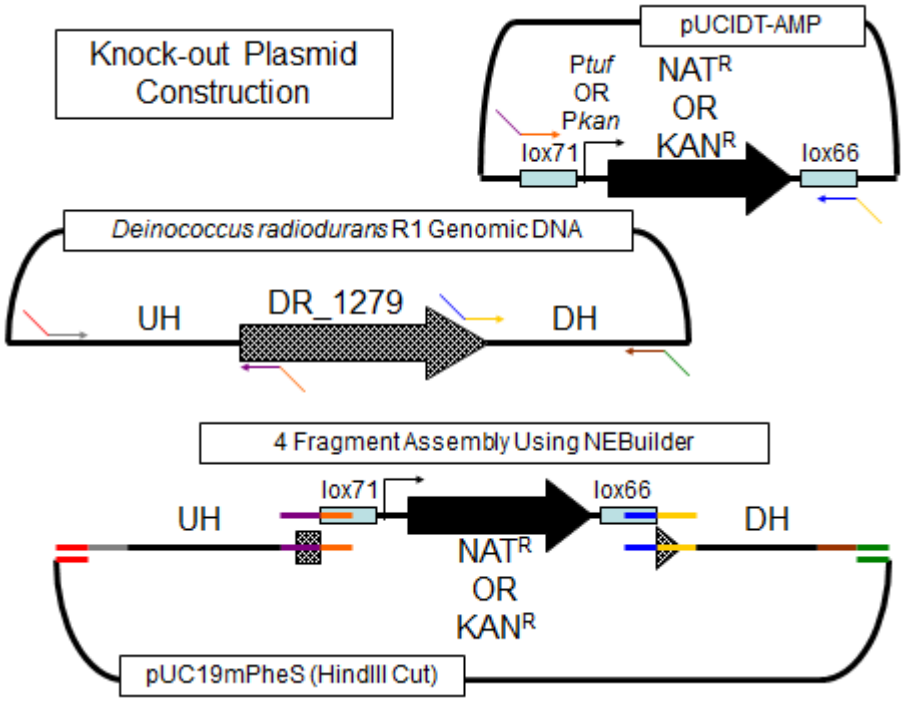


Figure 2. The knockout plasmids made with NEBuilder to transform DR. The antibiotic marker used to select for integration of the plasmid into the *Deinococcus* chromosome is flanked by lox sites and so can be removed upon expression of the Cre protein introduced in a subsequent step. This allows marker recycling and construction of double and triple knockouts [50].

Gel extraction was performed using the QIAquick Spin Kit. The process included excising the proper plasmid band, melting the gel band into a buffer mixture, and then performing a series of washes and an elution (Appendix A).

E. coli Transformation

Transformation was first performed on samples of *E. coli* to amplify the knockout plasmids. The DAM-/DCM- cells came pre-measured from New England Biolabs (NEB). The cells were thawed and gently mixed. The DAM-/DCM- cells were then added to a 2 mL micro-centrifuge tube that was put on ice. The knockout plasmids were then each placed into a transformation tube and the tube was flicked by hand so that the DNA and cells would mix. The mixture was placed on ice for a half hour, heat shocked at 42° C for

30 seconds and then placed on ice for another 5 minutes. This mixture was then incubated at 37° C for an hour, with shaking of 150-200 rpm. The mixture was then applied to selection plates consisting of LB media with 50 µg/mL of either nourseothricin (NAT) or kanamycin (KAN). Any *E. coli* cells that had been transformed to contain the proper resistance markers would grow on the corresponding selection plates while untransformed cells would be unable to survive and grow out on the selection plate. Colonies were observed to have grown on the selective media so it was evident that transformation had occurred. Isolated colonies were selected and inoculated into an overnight culture with 50 µg/mL of either NAT or KAN correspondingly. Plasmids were then isolated using the overnight cultures of *E. coli* using the QIAquick Spin kit (Appendix A).

***Transformation of* Deinococcus radiodurans**

The transformation of DR involves first making a streak of DR. A colony from this streak is then inoculated into 2.5 mL of TGY media and incubated at 32 C. After overnight growth, a 1:10 dilution is made by adding the culture to fresh TGY media and grown for 2 hours. 100 mM $CaCl_2$ is then added and incubated for 2 more hours. Aliquots of the culture are then prepared and the DNA to be transformed is added. The culture is placed on ice for an hour and then fresh TGY media is added. The culture is then allowed to grow overnight, incubated at 32 C and plated on selective media. Candidates were grown in selective media and the presence of the intended deletion was verified by PCR.

PCR

Polymerase chain reaction, or PCR, is a process to amplify DNA where the DNA is cyclically heated and cooled. Taq polymerase is placed in the presence of short segments of single-strand DNA known as primers designed to hybridize at a specific locus in the DNA target as well as the needed dNTPs and additional reaction components. Primers are defined as short single-stranded DNA fragments of a specific sequence which will hybridize to homologous spots in the genome or the DNA target in general. Heating the DNA causes the strands to separate and the DNA is then cooled. The primers hybridize to specific sites to the single strands of DNA and the Taq polymerase uses the supplied dNTPs to then synthesize a specific portion of DNA between the designed primers. This allows the rapid amplification of the desired segment of DNA. The shorter primers are in great molar excess and are far more likely to attach to the strand fragments than the fragments are to re-anneal. In our tests the PCR mixes were typically heated to ~ 90 degrees Celsius, cooled to ~ 60 degrees, and then raised to 70-90 degrees. This was done in approximately 30-35 cycles with cycles and temperatures based on the primers configuration. See Appendix C for further information on specific primers.

Gel Electrophoresis

Gel electrophoresis is used to confirm that the fragments being amplified are in fact the desired DNA segments. This is done by running an electric field through an agarose gel that has been implanted with the DNA in question. The DNA is negatively charged and the DNA will move with the field towards the positive charge. The rate of

migration is dependent on the size of the DNA fragment. A DNA ladder, fragments of a known size, is included with the gel to allow size comparison of amplified DNA fragments that have been amplified are of the proper size.

Ionizing Irradiation Experiments

The ionizing irradiation experiments were performed at Cincinnati Children's Hospital and Medical Center (CCHMC) and at Texas A&M. The samples were irradiated in these different locations for purposes of convenience and to see the changes in using the different dose rates from the 2 sources that were available. At CCHMC, the source used was a 1449.29 Cs 137 source that was a part of a J.L. Shepherd & Associates Mark I Model 68A Irradiator and had a dose rate of ~6 Gy per minute. The Texas source was a was a linear accelerator (LINAC) that had a dose rate of ~250 Gy per second. The cell cultures were prepared the day of the irradiation. For the irradiations performed by CCHMC the cell cultures were transported by car over a ~45 minute drive. The Texas samples were grown and irradiated on site.

Ionizing Irradiation Setup

The samples for gamma irradiation were prepared according to the protocol found in Appendix A. The cell cultures were diluted from overnight grown stocks into 0.1 x TGY. Originally the cell cultures had been placed into 1x TGY but it was noticed that TGY media provides a buffer against radiation. The samples were placed in a carousel inside of the irradiator. Figure 3 shows a model of the carousel.

The samples were retrieved after the appropriate irradiation times, ~56 hours for the CCHMC and ~85 s for the LINAC, and were immediately put in a refrigerator at 4 °C to slow radiolysis reactions and any culture growth. Unirradiated control samples were

left outside of the irradiator and were refrigerated at the same time the irradiated samples were refrigerated. The treated and untreated samples were both once again transported by ground vehicle back to the lab at USAFSAM for further analysis. The analysis was conducted immediately upon returning to the lab, the samples being unrefrigerated for about an hour.

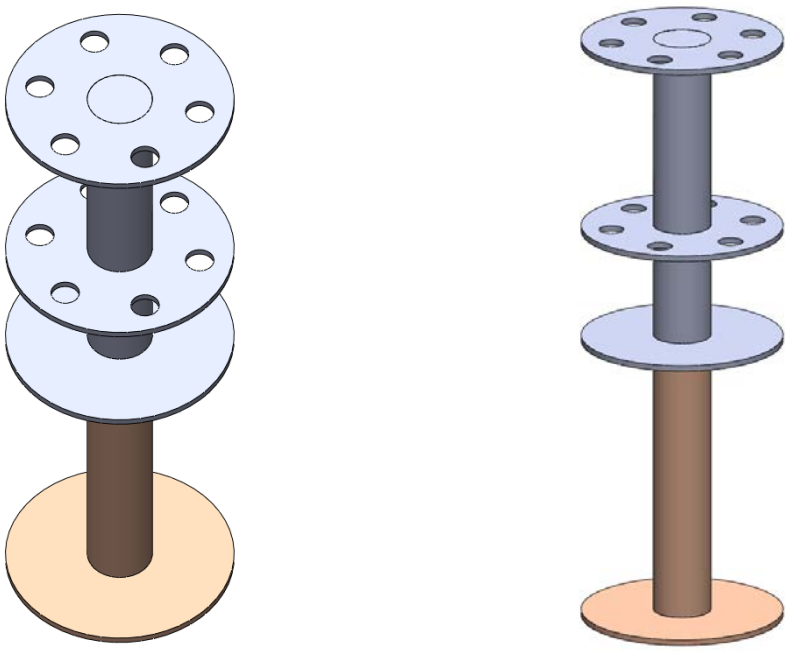


Figure 3. The carousel positioned within the radiation chamber held the 12 pairs of Eppendorf tubes and constantly rotated during irradiations. The two views are an isometric bird's-eye view (left) and a profile (right) of the carousel. The model was created using SolidWorks software [36].

The cell cultures irradiated in Texas were prepared at the Texas A&M labs there using procedures previously described except that these were all done 1x TGY media. These samples were irradiated to a total dose of 21,400 Gy over 85 seconds. A control sample was treated identically except for radiation exposure. The samples were then frozen and then sent back to USAFSAM.

Survivability Measurements

The irradiated samples were frozen when they were received. The Texas LINAC samples had to be shipped but were frozen in transit, which was overnight. After samples were received the samples were stored at 4 °C and dilution series were performed for both irradiated and untreated samples starting with an initial amount of 200 µl from the samples, transferring 20 µl for each dilution. After the dilution series were completed, the dilutions were spotted on plates. The samples were grown for two days so that initial CFUs could be counted. The counts obtained from the plate spotting were used to determine the optimal dilutions to prepare for spread plating and achieve a concentration of 30-300 CFU when 100 µl are plated. Appropriate dilutions were prepared, plated, incubated, and counted. To ensure no colonies that were recovering slowly from irradiation were missed, plates were checked on day 2, 3, and 4. The CFUs were then counted and recorded. A ratio was then calculated between the irradiated and untreated samples. This ratio was then used to infer what, if any, sensitivities occurred from the knockouts

Paraquat Experiments

The transformed strains were also exposed to paraquat to test how they reacted to oxidative damage produced by a nonradioactive source. The experiment itself was conducted by taking various knockout strains and growing them up into a culture overnight at 32 degrees C with shaking. The cultures were then diluted to an optical density (OD) of ~0.1 at 600 nm. A dilution series of paraquat was added to the cell

cultures, a Biotek Synergy 2, was used to measure the optical density of the dilutions over time as the cell cultures grew in density every 20 minutes for 24 hours at a temperature of 32 °C. From the accumulated data growth curves could be constructed over 24 hour periods.

The experiment was performed three different times. The first experiment used Paraquat in a dilution series of 125 μM, 62.5 μM, 32.25 μM, 15.62 μM, 7.81 μM, and 3.9 μM. The second experiment was performed in much of the same way, except that the strain DR_1709+2283-84 was included. The second experiment was also broken up into two sets, one where the bacteria were grown in media with 2 μM $MnCl_2$ 4 hours prior to the dilution series and one set that was not. This was done to see if a large amount of Mn in the culture would impact survival, as the DR_1709+2283-84 knockout removed Mn transporters from DR and Mn was seen to be required for growth. The third experiment was done in the same manner as the second with larger concentrations of paraquat; using 12.5 μm, 25 μm, 50 μm, 100 μm, 200 μm, 400 μm, and 800 μm.

IV. Analysis and Results

Chapter Overview

This chapter discusses the results in context of the research objectives stated in the research statement. The data collected from the methods described in the previous chapter are analyzed and the relevance to the hypothesis is reviewed. Discussion of error is included and the integrity of the research results is examined. A review of these results is covered in the final chapter.

DR Gene Knockouts

All target genes were successfully knocked out by transformation. Each deletion was confirmed by using PCR and comparing band sizes to that of wild type and the ladder. Mutant strains were grown on selective media under the presumption that those transformed would be the only ones to grow. These strains were verified by PCR. Double knockouts were constructed either simultaneously by using two antibiotic resistant markers with flanking homology directing each marker to independent genes or serially where the first marker was recombined through the action of Cre recombinase and the second desired mutation introduced through another recombinational gene replacement step. Finally, triple knockouts were completed and then confirmed in an analogous manner. The triple knockouts had both resistance markers.

Ionizing Radiation Outgrowth

Ten-fold serial dilutions of the irradiated cells were performed and 5 ul spots of each dilution were spotted to generate a preliminary estimate of viable cells. The counts obtained from the plate spotting were used to determine the optimal dilutions to prepare for spread plating and achieve a concentration of 30-300 CFUs per 100ul plated.

Appropriate dilutions were prepared, plated, incubated and counted. To ensure no colonies that were recovering slowly from irradiation were missed, plates were checked on day 2, 3 and 4. Using the counts, the total CFU/ml was calculated by multiplying the count by a dilution factor. Ratios of sensitivity were created between the treated and the untreated samples by dividing the untreated amount by the irradiated amount.

From the Cincinnati Children's Hospital irraditions it appeared that the media used for the cultures was providing shielding from the ionizing radiation. The media was then diluted and the exposure repeated in 0.1x TGY. From Table 1 it was seen that the gene ΔDR_1279 (Mn SOD) had the highest ratio of cell death as a single knockout, ~2 to 4 more times than wild type. There was also an increase in double and triple knockouts that were missing DR_1279. ΔDR_1279+ΔDR_1546, ΔDR_1279+ΔDR_A0202, and ΔDR_1279+ΔDR_BshA all had increases in sensitivity of approximately 1.5 to 4 times greater than the controls. The triple knockout ratio was 19 times more sensitive than the ratios seen in the wild type samples for the Nov. 13 irradiation. This provides evidence that DR_1279 provides some protection. DR_1279 is of interest as the Cu/Zn SOD knockouts had sensitivity ratios close to that of the wild type samples. The Cu/Zn SOD DR_A0202 however, showed the least amount of change in ionizing radiation resistance, both exposures' sensitivities being lower than wild type, 0.9 and 0.7 less respectively.

Another set of irradiation experiments were performed at Texas A&M using their Linear Accelerator (LINAC). The LINAC has a much more powerful radiation source. The Cincinnati source had an output of ~6 Gy/min while the LINAC has an output of ~250 Gy/second. The LINAC is also not a gamma source but an e^- source of radiation. All but two samples showed an increased sensitivity (Table 2). Of particular note was the

BshA2 strain which had a 2000-4000 fold drop in resistance. This sensitivity was ~265 times more than that seen in the wild type samples irradiated in the LINAC. The double knockout DR_1279+BshA (Mn SOD+ bacillithiol synthesis) had 10,000 less colony forming units. Several of the knockouts showed differing kill ratios, such as BshA1. Almost all knockouts were at least an order of magnitude greater in sensitivity than wild type.

The difference between the dose rates of the cesium-137 and the Texas LINAC may have resulted in a change in generation of ROS, the LINAC having more due to a faster reaction rate. The G value listed is the number of molecules of interest created per 100 eV of absorbed energy. The ROS generated were calculated based on the dose rate and the calculation used is shown below:

$$Dose\ Rate\left[\frac{Gy}{min}\right] \rightarrow DoseRate\left[\frac{J/kg}{min}\right] \rightarrow DoseRate[\frac{J}{kg\ min}]$$

$$Dose\ Rate\left[\frac{J}{kg\ min}\right] xMass\ of\ water[kg]xG\ val\left[\frac{mol}{J}\right] = [\frac{mol}{min}]$$

The values are calculated are shown below. It can be seen that the LINAC has a higher amount of ROS generation when compared to the CCHMC generation.

ROS Concentration Generation

ROS Concentration Generation per Minute (Product Yield)							
Species							
	$-H_2O$	H_2	H_2O_2	e^-_{aq}	$^{\bullet}H$	$^{\bullet}OH$	$^{\bullet}HO_2$
G value	0.43	0.047	0.073	0.28	0.062	0.28	0.0027
CCHMC	2.56E-09	2.80E-10	4.34E-10	1.67E-09	3.69E-10	1.67E-09	1.61E-11
LINAC	6.45E-06	7.05E-07	1.10E-06	4.20E-06	9.30E-07	4.20E-06	4.05E-08
Total ROS Concentration Generation [mol/(1 mL H2O)]							
CCHMC	5.18E+18	5.66E+17	8.79E+17	3.37E+18	7.46E+17	3.37E+18	3.25E+16
LINAC	5.54E+18	6.06E+17	9.41E+17	3.61E+18	7.99E+17	3.61E+18	3.48E+16

Table 1: Calculated generation of ROS species (mol/(1 mL H_2O)). The calculations were done for both sources for each dose even though CCHMC only gave an exposure dose of 20,000 Gy while the LINAC was only used to give exposures of 21,400 Gy.

CCHMC Irradiation Data

Strain ` Treated	Media	Ratio 11/13/15	Ratio 12 /01/15
Wild Type	1x TGY	3 ± 0.4	4*
Wild Type	0.1x TGY	11 ± 0.1	21 ± 2
ΔDR1279 (Mn SOD)	0.1x TGY	26 ± 3	77 ± 5
ΔDR1546 (Cu/Zn SOD)	0.1x TGY	10*	8 ± 1
ΔDRA0202 (Cu/Zn SOD)	0.1x TGY	3 ± 0.1	4 ± 0.4
ΔBshA1 (Bacillithiol Synthesis)	0.1x TGY	12 ± 0.1	5 ± 0.5
ΔBshA2 (Bacillithiol Synthesis)	0.1x TGY	4 ± 0.4	22 ± 2
ΔDR1709 (MntH)	0.1x TGY	Not Tested	4 ± 0.5
ΔDR2283-2284 (Mn ABC Transporter)	0.1x TGY	Not Tested	10 ± 1
ΔDR1279+ΔDR1546	0.1x TGY	40 ± 3	26 ± 3
ΔDR1279+ΔDRA0202	0.1x TGY	16 ± 2	34 ± 3
ΔDR1279+ΔDRBshA(2)	0.1x TGY	40 ± 4	31 ± 5
ΔDR1546+ΔDRA0202	0.1x TGY	17 ± 2	21 ± 2
ΔDR1279+ΔDR1546+ΔDRA0202	0.1x TGY	68 ± 0.4	16 ± 2

Table 2: Radiation sensitivity test data of *Deinococcus radiodurans* at Cincinnati Children's Hospital using a Cs^{137} source. The irradiation was approximately 56 hours giving samples a dose of ~20,000 Gy. Those highlighted yellow showed a large increase in sensitivity. ΔDR_A0202 had no change. * These values only had one available set of counts.

Texas L&M LINAC Irradiation Data

Strain Treated	Media	A Ratio 12/14/15	B Ratio 12 /14/15
Wild Type	1x TGY	20 ± 2	15 ± 2
ΔDR1279 (Mn SOD)	1x TGY	160 ± 20	190 ± 2
ΔDR1546 (Cu/Zn SOD)	1x TGY	110 ± 1	130 ± 1
ΔDRA0202 (Cu/Zn SOD)	1x TGY	130 ± 16	8 ± 1
ΔBshA1 (Bacillithiol Synthesis)	1x TGY	1400 ± 1	550 ± 1
ΔBshA2 (Bacillithiol Synthesis)	1x TGY	2300 ± 3	3900 ± 4
ΔDR1279+ΔDR1546	1x TGY	92 ± 1	125 ± 1
ΔDR1279+ΔDRA0202	1x TGY	29 ± 3	400 ± 4
ΔDR1279+ΔDRBshA(2)	1x TGY	>1700 ± 124	250 ± 323
ΔDR1546+ΔDRA0202	1x TGY	170 ± 1	64 ± 1
ΔDR1279+ΔDR1546+ΔDRA0202 (Strains 5, 3.1)	1x TGY	320 ± 3	340 ± 1
ΔDR1279+ΔDR1546+ΔDRA0202 (Strains 14, 8.2)	1x TGY	20 ± 2	150 ± 1

Table 3: Radiation sensitivity test data of *Deinococcus radiodurans* at Texas A&M using LINAC. The irradiation was approximately 85.6 seconds giving samples a dose of ~21,400 Gy. The cells highlighted yellow showed a large increase in sensitivity while those highlighted in red have little difference compared to wild type.

Average Comparison Between CCHMC and Texas LINAC

Strain Treated	CCHMC Average	Texas LINAC Average
Wild Type	**16 (4 at 1x)**	**18 (73)**
ΔDR1279 (Mn SOD)	**51**	**180 (740)**
ΔDR1546 (Cu/Zn SOD)	**9**	**120 (500)**
ΔDRA0202 (Cu/Zn SOD)	**3**	**70 (290)**
ΔBshA1 (Bacillithiol Synthesis)	**8**	**980 (4,100)**
ΔBshA2 (Bacillithiol Synthesis)	**13**	**3080 (12,800)**
ΔDR1279+ΔDR1546	**33**	**110 (450)**
ΔDR1279+ΔDRA0202	**25**	**210 (890)**
ΔDR1279+ΔDRBshA(2)	**36**	**990 (4,120)**
ΔDR1546+ΔDRA0202	**19**	**120 (490)**
ΔDR1279+ΔDR1546+ΔDRA0202 (Strains 5, 3.1)	**41**	**330 (1,400)**

Table 4: An average was taken for the values of Table 1 and Table 2 and presented here for ease of side-by-side comparison. ΔDR_1279 showed the greatest sensitivity for the CCHMC exposures while ΔBshA2 was most sensitivity for the Texas A&M exposures. A value in parenthesis is presented in the LINAC column entries to show a 4.16 times increase in kill due to the Texas samples being in 1x TGY which provides shielding. ~4.16 is the difference in sensitivity compared between 1x TGY and 0.1 TGY of wild type from CCHMC.

Of the Texas LINAC exposures, the Mn SOD and Cu/Zn SOD knockouts were close in value to each other. ΔDR_1279 was still more sensitive than the Cu/Zn SOD, being ~12 to 13 times more sensitive than wild type. The double knockout ΔDR_1279+ΔDR_A0202 was ~4 times more sensitive in the B set but the A set was only slightly more sensitive than wild type. The double knockout ΔDR_1279+ΔDR_BshA had almost a complete kill in set A while it was only ~20 times more sensitive in set B. The first triple knockout set, the same strains used for the CCHMC exposures, was about 30 times more sensitive but the second set using different strains had no change in set A but was 12 times more sensitive in set B. It should also be noted that the Texas irradiations were carried out in 1x TGY media, so there was still shielding from the media. Table 3 addresses this with the second number in parentheses under the Texas LINAC column that shows an approximate increase in sensitivity if the media had been diluted.

The ratios were then averaged and compared between the CCHMC and Texas LINAC exposures (Table 3). The Texas wild type ratio was only 5 times more sensitive than the CCHMC sample. The other sets had differences from 3 to 238 times more sensitive, ΔBshA2 having the largest difference in sensitivity. Oddly, the lowest difference was the double knockout ΔDR_1279+ΔDR_1546.

Paraquat Outgrowth

In addition to radiation a set of experiments were conducted to view how the transformed strains grew in the presence of the oxidizing herbicide paraquat and to further view if the transformed strains behaved in accordance to the irradiation data. Strains were grown to lag phase then diluted to an optical density of 600 nm. The strains

were then placed in a row and paraquat was added in a dilution series from a 200 µl starting culture to 20 µl in 180 µl of TGY. Figure 4 shows data of when this experiment was first performed. It is observed that those strains with DR_1279 knocked out do not display the same outgrowth as wild type at 62.5 µm until the paraquat concentration is 7.81 µm.

A second set of experiments was completed which includes all knockouts and also included strains incubated in $MnCl_2$ for 4 hours prior to exposure. This was included to investigate if Mn contributes to the survival of strains missing their Mn transporters. Figure 5 shows these reactions. Of note, it can be seen that after some time, cultures in 31.25 µm of paraquat or less will grow out, begin to die off, and then begin to regrow. A third experiment was conducted. This experiment was identical to the second except it doubled the concentration of paraquat in the dilution series (Figure 6). The increase in paraquat concentration was to investigate if the amount of bacterial cells present was providing a barrier, essentially causing an amount of cells to be shielded and unexposed to the paraquat and leading to regular culture growth.

Similar results were seen between the second and third experiments but a higher sensitivity was viewed in the ΔDR_1279 and ΔDR_2283-84+ΔDR_1709 knockouts. At 62.5 µm of paraquat, ΔDR_1279 and ΔDR_1709+2283-84 were observed to be the most sensitive being grown with $MnCl_2$. At 31.25 µm of paraquat, ΔDR_1279 is the most sensitive in both the second and the third experiment, for growth with and without $MnCl_2$ prior to exposure. Cultures grown without $MnCl_2$ showed more resilience, having growth at 62.5 µm in the second experiment (Figure 5) and even showing some growth at 100/125 µm in the third experiment (Figure 6).

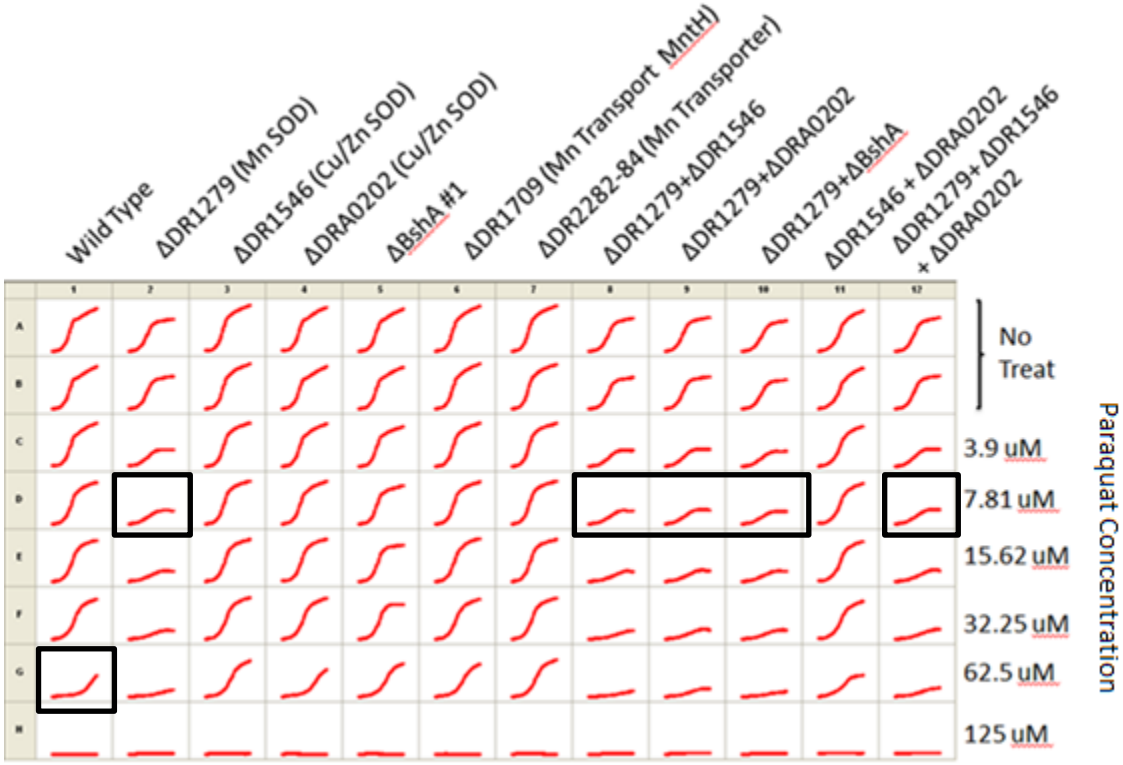


Figure 4. Transformed strains were grown in paraquat over the course of 24 hours and the optical density was measured. Wild type is highlighted at the highest concentration of paraquat it grows in. The knockouts composed of ΔDR_1279 are highlighted at the concentration where they have same outgrowth as wild type.

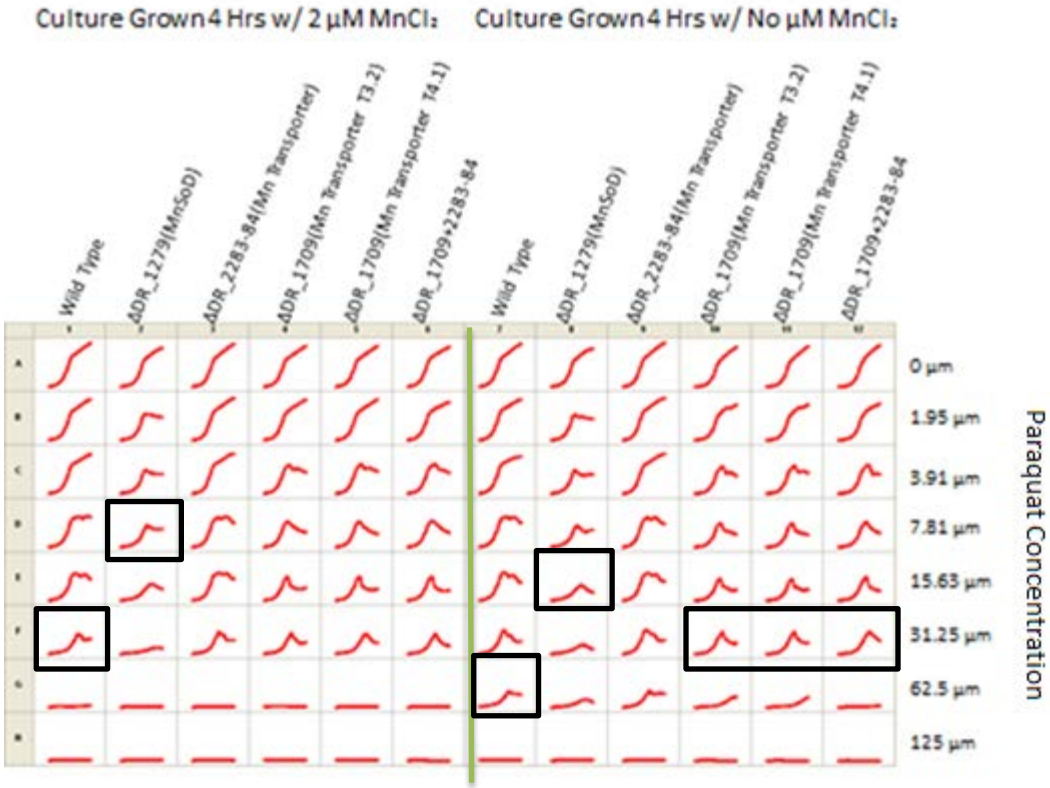


Figure 5. Transformed strains were grown in paraquat over 24 hours. The left 6 columns represent cultures grown 4 hours prior in media with $MnCl_2$. The cells highlighted show wild type growth compared equal knockout strain growth at much lower concentrations of paraquat.

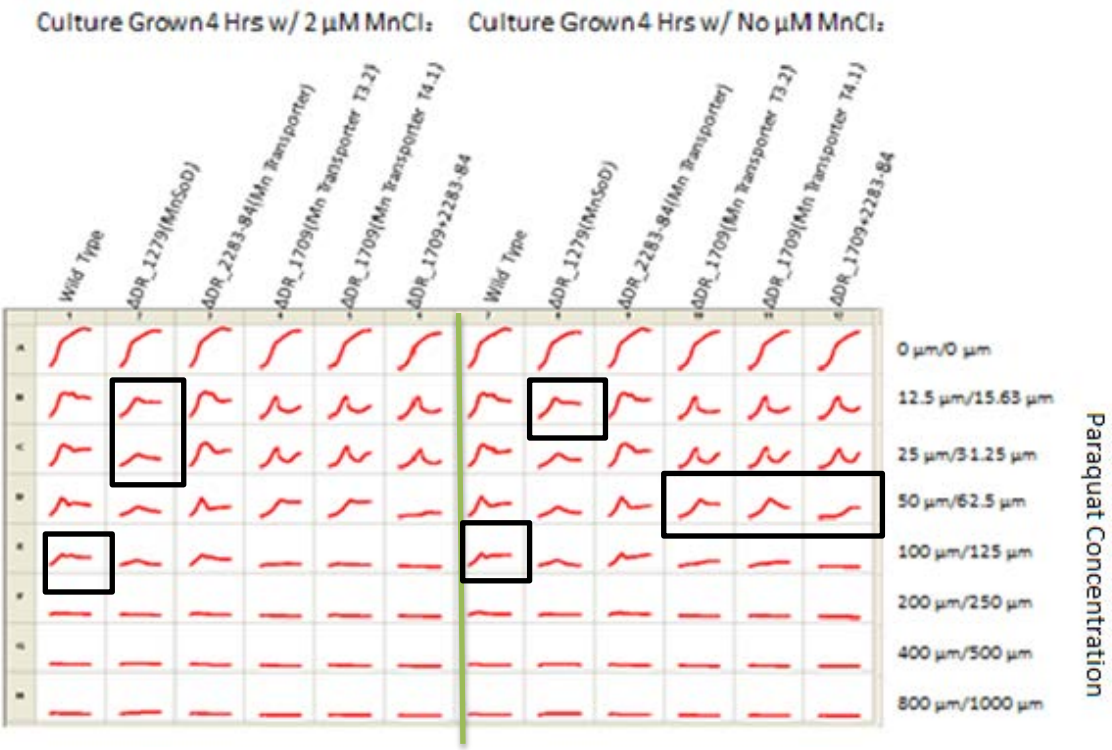


Figure 6. Transformed strains were grown in paraquat over 24 hours. The left 6 columns represent cultures grown 4 hours prior in media with $MnCl_2$. The cells highlighted show wild type growth compared equal knockout strain growth at much lower concentrations of paraquat.

V. Conclusions and Recommendations

Chapter Overview

This chapter contains an overview of the previous chapters and the research as a whole. The research objectives are evaluated and reviewed given the collected data. This chapter also contains a summary of the research and future research recommendations.

Conclusions of Irradiation Research

In strains of *Deinococcus radiodurans,* genes thought to be important for mitigating oxidative damage were knocked out. The genes targeted were Mn and Cu/Zn super-oxide dismutases, Mn transporters, and bacillithiol synthesizers. These transformed strains were then irradiated by two different sources of ionizing radiation to a total dose of ~20,000Gy. The CCHMC samples showed the greatest increase in sensitivity among those knockouts that were missing DR_1279, Mn SOD. This supports the hypothesis that Mn SOD helps mitigate radiation damage.

The Cu/Zn SOD DR_A0202 however, showed the least amount of change in ionizing radiation resistance and it can be speculated that this has little function in protecting from ionizing radiation. This could be a result of Mn SOD's ability to bind to the DNA and give immediate protection while it is unknown if Cu/Zn SOD performs in the same way. Cu/Zn SOD may not be able to bind to the DNA and provide as much protection as Mn SOD and may not serve as large a role in irradiation resistance. It may be that Cu/Zn SOD has bindings elsewhere, like the cell membrane as can be seen in humans, and is not largely involved in DNA protection [19].

The Texas results imply that the removal of bacillithiol synthesis causes a vast decrease, at least 500 times more so, in radiation resistance and that bacillithiol may be of some importance which is different from what was seen in CCHMC. There was some variance of the bacillithiol knockout strains though. BshA2 was seen to have the highest kill ratio, 2300, compared to BshA1 which had a kill ratio of ~1400. However, in the second set of irradiations the kill ratios were 3900 to 550 respectively. It is unknown why BshA1's sensitivity decreased so much in the second run or why BshA2's sensitivity increased. These values do show a massive increase to radiation sensitivity though, and are more than any of the other knockouts. Of those, the triple knockout ΔDR_1279+ΔDR_1546+ΔDR_A0202 was the highest. The double knockout ΔDR_1279+ΔDR_BshA had a complete kill in the first set but the second set only had a kill ratio of 250. It is observed that DR_1279 is still important in fighting off the oxidative damage from ionizing radiation. However, it seems to be less important than bacillithiol. One possible reason is that bacillithiol is capable of reacting quickly to the oxidative damage, the LINAC possibly causing a greater oxidation reaction, and that DR_1279 may have a slower reaction than that of bacillithiol.

The variance seen between several of the strains from the LINAC exposures is confusing. There is a possibility of the strain itself mutating or a colony that remained untransformed but resistant to the antibiotics, though these are unlikely as the controls set in place should have kept such occurrences low. However, a culture that had wild type DR alongside the transformed strain may show some resistance as wild type colonies would be far more resistant than any of the mutants have shown. The Texas irradiation configuration is unknown so it is possible that the positioning within the irradiator may

have also had an effect as a sample could have been possibly been shielded by an obstacle or was simply positioned in a way to keep its exposure low. However, the results show that all of the cultures experienced an increase in sensitivity much greater than that of wild type in at least one of the exposures.

The Texas samples can be seen to have a much greater sensitivity than those in the CCHMC samples, even though both were to have absorbed approximately the same dose of radiation. Gamma radiation from the CCHMC source and beta radiation (e^-) from the LINAC have the same relative biological effectiveness (RBE) of 1. The Texas A&M LINAC system has a much greater dose rate than that of CCHMC, 250 Gy/s compared to 6 Gy/min, and it is speculated that its quicker delivery of radiation produces a greater rate of oxidative species generation which lead to a greater amount of cell death in the irradiated cultures. This contrasts with the CCHMC samples which were exposed to radiation over a much greater amount of time, giving damaged cells the chance to repair and recover from any ionizing radiation damage that occurs. The Texas LINAC irradiations were also done in 1x TGY compared to 0.1x TGY at CCHMC. Therefore the Texas samples may have experienced shielding from the ionizing radiation. It is also possible DR could possibly be producing Mn SOD during low rate exposures that helps keep the damage low.

Whether bacillithiol functions in some way to signal Mn scavengers to mitigate oxidation is unknown but is a possibility. There can be seen some fluctuation in sensitivity among the two sets of the mutant strain ΔBshA1. The second run showed a greater sensitivity than wild type but much less than the first Texas irradiation. It is unknown why this is, but possibilities could be from a change in placement in the

irradiator or some unforeseen mutation of the strain. It is thought that perhaps from the experiments done by Daly, that bacillithiol may be a critical particle for *Deinococcus radiodurans* resistance [24].

Paraquat Research Conclusions

Paraquat experiments were then performed, placing mutants in a dilution series of paraquat, a herbicide that causes oxidative damage intracellularly. In the first experiment it was seen that the knockout of DR_1279, a manganese family super-oxide dismutase, showed a decrease in resistance to oxidative damage compared to wild type. Double and triple knockouts involving DR_1279 also showed a greater decrease in resistance. This can be linked back to what was seen in the irradiation experiments and that DR_1279 does seem to play a important role in mitigating oxidative damage. Given that Mn SOD reduces ROS in the cytoplasm while Cu/Zn SOD reduces extracellular ROS. It can be observed that there was almost no change in resistance comparing wild type to ΔBshA, a simple thiol that reduces hydroxyl radical and not superoxide which is the main reactive oxygen species produced by paraquat. The knockout was capable of growing within 62.5 µm of paraquat. One possible reason is that paraquat may not activate bacillithiol. It may be that bacillithiol is located somewhere within the cell that did not change the cell's response to the oxidative damage. It could also be just the paraquat interacts with a cell in that other defense mechanisms are readily available to fight it off.

The second experiment included a double knockout of both Mn transporter genes and also implemented growing the cultures in $MnCl_2$ before exposure to paraquat. The $MnCl_2$ was included to allow certain strains that required it to grow. It could be seen that those grown without $MnCl_2$ before had slightly higher optical densities before beginning

to die off. It can also be seen when comparing the first experiment to the second that those grown with $MnCl_2$ could not grow in 62.5 µm of paraquat while some like DR_2283-83 could. It is thought that the amount of Mn present and DR's propensity towards readily absorbing it from its environment may have led to a slightly toxic effect for DR. It could possibly be that those grown without the $MnCl_2$ were not as actively scavenging their surroundings for Mn as those grown in the $MnCl_2$, leading them to have slightly better survival in higher concentrations of paraquat. The overall reason is still unknown. After the die-off, some samples gained optical density (Wild Type and DR_2283-84), suggesting culture regrowth. It is possible that the paraquat stopped causing oxidation and the culture was able to recover. This could be due to a high amount of bacterial cells in the cultures that act as a sacrificial shield for the rest of culture. In this scenario a large amount of the cells die off but there are enough surviving cells to eventually grow out.

The third test repeated the second but with double the amount of paraquat. The paraquat was doubled to test if some cells were in fact acting as a shield for the culture. The increase ensured enough paraquat was available in the culture so that it could potentially react with every bacterial cell. Similar results were seen in the third experiment as that of the second, where some cultures began to grow back after exposure. It is possible that DR has some form of oxidation mitigation that allows it to sustain damage and halt cell growth, somehow remove or scavenge oxidation, repair the damage, and then resume normal cellular function. This could possibly explain the decrease in culture growth, where damaged cells halt growth, repair the damage, and then reproduce.

Overall Conclusions

Overall consistence would appear that the genes DR_1279 and Bsha have some importance in resistance against oxidation and ionizing radiation damage. The two sources tested include ionizing radiation and paraquat, work in different ways. Ionizing radiation is able to penetrate through the cell and interact directly within it while paraquat must first get past the cell barrier. However, DR_1279 seems to provide a great deal of resistance when dealing with oxidation from both sources whereas bacillithiol seems to be majorly important in resisting oxidative damage within the cell. However, its removal only causes moderate sensitivity to ionizing irradiation. Bsha seems to have a much larger role at higher rates. It could be that bacillithiol provides an essential step in signaling scavengers to protect cells from oxidizing radicals. Once signaled, it could be viewed that DR_1279 acts as the lead scavenger.

Some alternate mechanisms to consider in radiation resistance for DR include that it carries multiple copies of its DNA. This makes it easier for DR to undergo homologous recombination which is the best way to repair DSBs. Also, DR has a multitude of unique lipids that make up its cell membrane. Lipids themselves can act as oxidative reducers and these lipids may be providing additional protection. Bacillithiol also can act as a reducing agent as a thiol and it may be that there is a large concentration of this small molecule that provides a constant protective presence inside of the cell. Mn SOD and bacillithiol act as protectors against ionizing radiation but they are most likely only part of a whole system that works in concert to provide DR its exceptional resistance.

Some limitations with this work involved a lack of experimentation. While irradiations were performed, a greater amount of data sets are needed to properly perform a statistical analysis that could show significant values. Also, among the irradiation sets some values have a large variance and this is so far unexplainable. Other sources of oxidation could be investigated as well to see just how important, in general, these knockouts are.

Future Recommendations

This research is a starting point for further investigation into the mechanics responsible for *Deinococcus radioduran's* resistance to ionizing radiation and oxidation. The research could lead on to applications such as therapeutics to protect against radiation exposure and can be used in medical fields like tissue implantation and treating infections. However, this research still requires more work and more study is needed to understand the mechanics at work. Some further research suggestions are suggested below.

Further research is needed to gain a clearer picture of just how important the genes of note, namely DR_1279 and Bsha are to radiation resistance. While some evidence has been gathered indicating their importance, only a few irradiations have been performed and more are needed to gain a better statistical analysis. It can also be seen that some strains have large differences in sensitivity between irradiations that needs to be investigated. Also, exposure to differing sources of oxidation, such as growing the cultures up in hydroxyl radical, was planned but we were unable to complete them.

The next step to further understanding *Deinococcus radiodurans* is to investigate the apparent importance of Bsha and bacillithiol. While Bsha strains had some variance in sensitivity, it can be seen that lacking Bsha in the Texas A&M tests led to high increase in sensitivity. Currently, further investigation into Bsha is being done similar to Daly's work. Plans are to take filtrate extracts of bacillithiol from *Deinococcus radiodurans* and see if it confers any protection to other bacterium.

Appendix A: Experimental Protocols

TGY Media Recipe

The following recipe prepares 1 L of 1X growth media. Add the following ingredients and fill to 1 L with dH_2O. Filter sterilization is recommended.

1X Growth Media:

5 grams of Tryptone
3 g Yeast Extract
1 g Glucose

HiFi DNA Assembly® Protocol

Optimal Quantities

NEB recommends a total of 0.03–0.2 pmols of DNA fragments when 1 or 2 fragments are being assembled into a vector and 0.2–0.5 pmoles of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmols of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons)

50 ng of 5000 bp dsDNA is about 0.015 pmols.

50 ng of 500 bp dsDNA is about 0.15 pmols.

The mass of each fragment can be measured using the NanoDrop instrument, absorbance at 260 nm or estimated from agarose gel electrophoresis followed by ethidium bromide staining.

Assembly Protocol

Set up the following reaction on ice:

	Recommended Amount of Fragments Used for Assembly		
	2-3 Fragment Assembly*	4-6 Fragment Assembly**	Positive Control†
Recommended DNA Ratio	vector:insert = 1:2	vector:insert = 1:1	
Total Amount of Fragments	0.03–0.2 pmols* X µl	0.2–0.5 pmols* X µl	10 µl
Assembly Master Mix (2X)	10 µl	10 µl	10 µl
Deionized H_2O	10-X µl	10-X µl	0
Total Volume	20 µl***	20 µl***	20 µl

1. * Optimized cloning efficiency is 50–100 ng of vectors with 2 fold excess of inserts. Use 5 times more inserts if size is less than 200 bps. Total volume of unpurified PCR fragments in the assembly reaction should not exceed 20%.
   ** To achieve optimal assembly efficiency, it is recommended to design ≥ 20 bp overlap regions between each fragment with equimolarity (suggested: 0.05 pmol each).
   † Control reagents are provided for 5 experiments.
   ‡ If greater numbers of fragments are assembled, increase the volume of the reaction, and use additional Assembly Master Mix.
2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled or 60 minutes when 4-6 fragments are being assembled. Following incubation, store samples on ice or at –20°C for subsequent transformation.

   Note: Reaction times less than 15 minutes are generally not recommended. Extended incubation times (up to 4 hours) have been shown to improve assembly efficiencies in some cases. Do not incubate the assembly reaction overnight.

3. Transform NEB 5-alpha Competent E. *coli* cells (provided in the cloning kit or purchased separately from NEB) with 2 µl of the assembled product, following the appropriate transformation protocol.

Transformation Protocols

Transformation with chemically competent cells.

1. Thaw chemically competent cells on ice.

2. Transfer 50 µl of competent cells to a 1.5 ml microcentrifuge tube (if necessary).

3. If the chemically competent cells are from New England Biolabs, add 2 µl of assembled product to NEB competent cells and go to step 4 directly. If competent cells are purchased from other manufacture, dilute assembled products 4-fold with H_2O prior transformation. This can be achieved by mixing 5 µl of assembled products with 15 µl of H_2O. Add 2 µl of the diluted assembled product to competent cells.

4. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix.

5. Heat shock at 42°C for 30 seconds.* Do not mix.

6. Transfer tubes on ice for 2 minutes.

7. Add 950 µl of room temperature SOC media* to tubes.

8. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.

9. Warm selection plates to 37°C.

10. Spread 100 µl of the cells onto the plates with appropriate antibiotics. Use Amp plates for positive control sample.

11. Incubate plates overnight at 37°C.

    * Please note: Follow the manufacturer's protocols for the duration and temperature of the heat shock step, as well as the optimal medium for recovery. Typically, transformation of our positive control assembly product will yield more than 100 colonies on an Amp plate with greater than 80% colonies containing inserts.

    NEB recommends NEB 5-alpha Competent *E. coli* (NEB #C2987) for transformation of Gibson Assembly products. It is also possible to use other NEB competent *E. coli* strains, with the exception of BL21, BL21(DE3), Lemo21(DE3) and Nico21(DE3). For example, Shuffle T7 Express Competent *E. coli* can be used for the expression of a difficult to express protein. When using competent *E. coli* from a vendor other than NEB, we have seen decreased robustness of transformation with the Gibson Assembly reaction.

Transformation with electrocompetent cells.

1. Thaw electrocompetent cells on ice.
2. Transfer 50 µl of electrocompetent cells to a pre-chilled electroporation cuvette with 1 mM gap.
3. Dilute assembled products 3-fold with H_2O prior electroporation. This can be achieved by mixing 5 µl of assembled products with 10 µl of H_2O. Add 1 µl of the diluted assembly product to electrocompetent cells.
4. Mix gently by pipetting up.
5. Once DNA is added to the cells, electroporation can be carried out immediately. It is not necessary to incubate DNA with cells.
6. Add 950 µl of room temperature SOC media to the cuvette immediately after electroporation.
7. Place the tube at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.

9. Spread 100 µl of the cells onto the plates.
10. Incubate overnight at 37°C.

QIAquick PCR Purification Kit Protocol using a microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

The yellow color of Buffer PBI indicates a pH of 7.5. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume). All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.

Procedure

1. Add 5 volumes of Buffer PBI to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene. For example, add 500 µl of Buffer PBI to 100 µl PCR sample (not including oil).
2. Check that the color of the mixture is yellow (similar to Buffer PBI without the PCR sample). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min. IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge. IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

QIAquick Gel Extraction Kit Protocol using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit. The yellow color of Buffer QG indicates a pH of 7.5. Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume). All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.

Procedure

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl). For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation. IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH of7.5. Buffer QG contains a pH indicator which is yellow at pH !7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix. For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA

fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. Discard flow-through and place QIAquick column back in the same collection tube. Collection tubes are reused to reduce plastic waste.
9. Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min. Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x g (13,000 rpm). IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel. Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Appendix B: Raw Data

Texas A&M Data

Included below are the colony counts of the irradiated and untreated samples from Texas A&M LINAC. Appendix B Table 1 details the untreated (blue) samples and Table 2 details the irradiated (red) samples. Table 3 acts as a key and also records survivability ratios.

BLUE A	Dilution	Factor	Count	CFU/ml	BLUE A	Number Used	BLUE B	Dilution	Factor	Count	CFU/ml	BLUE B	Number Used
1	1.00E-04	1.00E+04	188	1.88E+07	1	1.88E+07	1	1.00E-04	1.00E+04	183	1.83E+07	1	1.83E+07
1	1.00E-05	1.00E+05	16	1.60E+07			1	1.00E-05	1.00E+05	25	2.50E+07		
2	1.00E-04	1.00E+04	229	2.29E+07	2	2.29E+07	2	1.00E-04	1.00E+04	267	2.67E+07	2	2.67E+07
2	1.00E-05	1.00E+05	31	3.10E+07			2	1.00E-05	1.00E+05	28	2.80E+07		
3	1.00E-04	1.00E+04	232	2.32E+07	3	2.32E+07	3	1.00E-04	1.00E+04	246	2.46E+07	3	2.46E+07
3	1.00E-05	1.00E+05	33	3.30E+07			3	1.00E-05	1.00E+05	26	2.60E+07		
4	1.00E-04	1.00E+04	238	2.38E+07	4	2.38E+07	4	1.00E-04	1.00E+04	280	2.80E+07	4	2.80E+07
4	1.00E-05	1.00E+05	30	3.00E+07			4	1.00E-05	1.00E+05	24	2.40E+07		
5	1.00E-04	1.00E+04	278	2.78E+07	5	2.78E+07	5	1.00E-04	1.00E+04	297	2.97E+07	5	2.97E+07
5	1.00E-05	1.00E+05	30	3.00E+07			5	1.00E-05	1.00E+05	26	2.60E+07		
6	1.00E-04	1.00E+04	386	3.86E+07	6	3.86E+07	6	1.00E-04	1.00E+04	cont.	#VALUE!	6	4.10E+07
6	1.00E-05	1.00E+05	27	2.70E+07			6	1.00E-05	1.00E+05	41	4.10E+07		
7	1.00E-04	1.00E+04	291	2.91E+07	7	2.91E+07	7	1.00E-04	1.00E+04	207	2.07E+07	7	2.07E+07
7	1.00E-05	1.00E+05	31	3.10E+07			7	1.00E-05	1.00E+05	38	3.80E+07		
8	1.00E-04	1.00E+04	131	1.31E+07	8	1.31E+07	8	1.00E-04	1.00E+04	132	1.32E+07	8	1.32E+07
8	1.00E-05	1.00E+05	9	9.00E+06			8	1.00E-05	1.00E+05	11	1.10E+07		
9	1.00E-04	1.00E+04	334	3.34E+07	9	3.34E+07	9	1.00E-04	1.00E+04	379	3.79E+07	9	3.79E+07
9	1.00E-05	1.00E+05	30	3.00E+07			9	1.00E-05	1.00E+05	30	3.00E+07		
10	1.00E+00	1.00E+00	173	1.73E+03	10	1.73E+03	10	1.00E+00	1.00E+00	250	2.50E+03	10	2.50E+03
10	1.00E-01	1.00E+01	22	2.20E+03			10	1.00E-01	1.00E+01	26	2.60E+03		
11	1.00E-04	1.00E+04	123	1.23E+07	11	1.23E+07	11	1.00E-04	1.00E+04	158	1.58E+07	11	1.58E+07
11	1.00E-05	1.00E+05	11	1.10E+07			11	1.00E-05	1.00E+05	17	1.70E+07		
12	1.00E-04	1.00E+04	184	1.84E+07	12	1.84E+07	12	1.00E-04	1.00E+04	174	1.74E+07	12	1.74E+07
12	1.00E-05	1.00E+05	20	2.00E+07			12	1.00E-05	1.00E+05	14	1.40E+07		

Table 1: The colony counts and estimated CFU/ml for untreated samples from Texas A&M. The sample sets sent to Texas A&M were done in two sets.

RED A	Dilution	Factor	Count	CFU/ml	RED A	Number Used	RED B	Dilution	Factor	Count	CFU/ml	RED B	Number Used
1	1.00E-03	1.00E+03	91	9.10E+05	1	9.10E+05	1	1.00E-03	1.00E+03	125	1.25E+06	1	1.25E+06
1	1.00E-04	1.00E+04	6	6.00E+05			1	1.00E-04	1.00E+04	10	1.00E+06		
2	1.00E-02	1.00E+02	202	2.02E+05	2	2.02E+05	2	1.00E-02	1.00E+02	210	2.10E+05	2	2.10E+05
2	1.00E-03	1.00E+03	35	3.50E+05			2	1.00E-03	1.00E+03	32	3.20E+05		
3	1.00E-02	1.00E+02	251	2.51E+05	3	2.51E+05	3	1.00E-02	1.00E+02	197	1.97E+05	3	1.97E+05
3	1.00E-03	1.00E+03	30	3.00E+05			3	1.00E-03	1.00E+03	19	1.90E+05		
4	1.00E-03	1.00E+03	82	8.20E+05	4	8.20E+05	4	1.00E-02	1.00E+02	70	7.00E+04	4	7.00E+04
4	1.00E-04	1.00E+04	10	1.00E+06			4	1.00E-03	1.00E+03	7	7.00E+04		
5	1.00E-03	1.00E+03	17	1.70E+05	5	1.70E+05	5	1.00E-02	1.00E+02	155	1.55E+05	5	1.55E+05
5	1.00E-04	1.00E+04	1	1.00E+05			5	1.00E-03	1.00E+03	17	1.70E+05		
6	1.00E-02	1.00E+02	122	1.22E+05	6	1.22E+05	6	1.00E-02	1.00E+02	120	1.20E+05	6	1.20E+05
6	1.00E-03	1.00E+03	11	1.10E+05			6	1.00E-03	1.00E+03	11	1.10E+05		
7	1.00E-03	1.00E+03	22	2.20E+05	7	2.20E+05	7	1.00E-03	1.00E+03	257	2.57E+06	7	2.57E+06
7	1.00E-04	1.00E+04	1	1.00E+05			7	1.00E-04	1.00E+04	26	2.60E+06		
8	1.00E-02	1.00E+02	76	7.60E+04	8	7.60E+04	8	1.00E-02	1.00E+02	206	2.06E+05	8	2.06E+05
8	1.00E-03	1.00E+03	10	1.00E+05			8	1.00E-03	1.00E+03	26	2.60E+05		
9	1.00E-03	1.00E+03	170	1.70E+06	9	1.70E+06	9	1.00E-02	1.00E+02	246	2.46E+05	9	2.46E+05
9	1.00E-04	1.00E+04	10	1.00E+06			9	1.00E-03	1.00E+03	39	3.90E+05		
10	1.00E+00	1.00E+00	0	0.00E+00	10	0.00E+00	10	1.00E+00	1.00E+00	1	1.00E+01	10	1.00E+01
10	1.00E-01	1.00E+01	0	0.00E+00			10	1.00E-01	1.00E+01	0	0.00E+00		
11	1.00E-01	1.00E+01	88	8.80E+03	11	8.80E+03	11	1.00E-01	1.00E+01	285	2.85E+04	11	2.85E+04
11	1.00E-02	1.00E+02	8	8.00E+03			11	1.00E-02	1.00E+02	29	2.90E+04		
12	1.00E-01	1.00E+01	80	8.00E+03	12	8.00E+03	12	1.00E-01	1.00E+01	45	4.50E+03	12	4.50E+03
12	1.00E-02	1.00E+02	6	6.00E+03			12	1.00E-02	1.00E+02	2	2.00E+03		

Table 2: The colony counts and estimated CFU/ml for irradiated samples from Texas A&M. The sample sets sent to Texas A&M were done in two sets.

Cincinnati Children's Hospital Data

Appendix B Table 3 and 4 display the raw data of the Cincinnati Children's Hospital irradiations. It shows the counted CFUs and the survivability ratios.

1.00E-05	Statistically Significant Numbers	Statistically Significant Numbers	Total Culture CFU plated	Untreated BLUE	1.00E-04	1.00E-05	1.00E-06	Statistically Significant Numbers	Statistically Significant Numbers	Total Culture CFU plated	Number	Strain	Ratio Untreated: Irradiated
	234		234000	1		26	3		260	2600000	1	WT	11.1
		260	260000	2		25	0		250	2500000	2	1546	9.6
	67		67000	3	271	18		271		2710000	3	1279'1546	40.4
	137		137000	4	224	32		224		2240000	4	1279'A0202	16.4
	92		92000	5	243	24		243		2430000	5	1279	26.4
	38		38000	6	257	39		257		2570000	6	Triple	67.6
11		960	960000	7		24	4		240	2400000	7	A0202	2.5
	134		134000	8	227	25		227		2270000	8	1546'A0202	16.9
1		340	340000	9		31	4		310	3100000	9	Triple	9.1
	38		38000	10	152	9		152		1520000	10	1279'BshA	40.0
	161		161000	11		20	2		200	2000000	11	BshA1	12.4
4		650	650000	12		24	0		240	2400000	12	BshA2	3.7
12		1350	1350000	13		46	4		460	4600000	13	WT-TGY	3.4

Table 3: Irradiation data from Cincinnati Children's Hospital. Red designates irradiated samples while blue is untreated. Run date 11/13/2015

			Total				Total			Ratio
Irradiated			Culture	Untreated			Culture			Untreated:
RED	1.00E-03	1.00E-04	CFU plated	BLUE	1.00E-04	1.00E-05	CFU plated	Number	Strain	Irradiated
1	111	12	111000	1	233	20	2330000	1	WT	20.990991
2	21	4	21000	2	161	18	1610000	2	1279	76.666667
3	215	15	215000	3	180	20	1800000	3	1546	8.372093
4	469	59	590000	4	214	19	2140000	4	A0202	3.6271186
5	427	42	420000	5	187	20	1870000	5	BshA1	4.452381
6	67	9	67000	6	149	16	1490000	6	BshA2	22.238806
7	362	45	450000	7	189	21	1890000	7	1709	4.2
8	237	17	237000	8	227	23	2270000	8	2283-84	9.5780591
9	65	5	65000	9	167	18	1670000	9	1279'1546	25.692308
10	49	9	49000	10	166	25	1660000	10	1279'A0202	33.877551
11	19	1	19000	11	59	9	590000	11	1279'BshA	31.052632
12	74	5	74000	12	153	17	1530000	12	1546'A0202	20.675676
13	114	9	114000	13	180	21	1800000	13	Triple	15.789474
14	TNTC	104	1040000	14	TNTC	45	4500000	14	WT TGY	4.3269231
15	0	0	0	15	1	1	10000	15	WT 3mM Pep	#DIV/0!
16	7	0	7000	16	TNTC	41	4100000	16	WT <100uM MnCl2	585.71429
17	TNTC	55	550000	17	TNTC	35	3500000	17	Triple TGY	6.3636364
18	0	0	0	18	1	1	10000	18	Triple 3mM Pep	#DIV/0!
19	3	2	3000	19	114	27	1140000	19	Triple <100uM MnCl2	380
20	TNTC	66	660000	20	210	14	2100000	20	1279'BshA TGY	3.1818182
21	0	0	0	21	5	1	50000	21	1279'BshA 3mM Pep	#DIV/0!
22	22	2	22000	22	230	23	2300000	22	1279'BshA <100uM MnCl2	104.54545

Table 4: Irradiation data from Cincinnati Children's Hospital. Red designates irradiated samples while blue is untreated. Run date 12/01/2015

Deinococcus radiodurans CFU vs OD_{600}

One concern while experimenting was that the optical density of cultures may not be accurate and that there could be an amount of dead cells that are distorting the actual values of live cells. A small experiment was done to see at what amount of initial CFUs in a culture would an accurate optical density be measured at. This was done by growing up several cultures with varying initial CFUs and then comparing the optical density of undiluted cultures to a comparative 1:10 dilution.

				CFU/ml			OD600 1:10	Linear
Count A	Count B	CFU/ml A	CFU/ml B	Average	OD600 UN	OD600 1:10	X 10 Dil. Fact.	OD600 Read
1.7E+06	1.3E+06	3.4E+08	2.6E+08	3.0E+08	3.105	0.812	8.12	8.12
1.1E+06	1.8E+06	2.2E+08	3.6E+08	2.9E+08	2.682	0.439	4.39	4.39
5.0E+05	7.0E+05	1.0E+08	1.4E+08	1.2E+08	1.87	0.25	2.5	2.5
2.9E+05	2.2E+05	5.8E+07	4.4E+07	5.1E+07	1.057	0.133	1.33	1.33
1.5E+05	1.4E+05	3.0E+07	2.8E+07	2.9E+07	0.572	0.063	0.63	0.572
6.0E+04	8.0E+04	1.2E+07	1.6E+07	1.4E+07	0.3	0.034	0.34	0.3
3.4E+04	3.0E+04	6.8E+06	6.0E+06	6.4E+06	0.156	0.017	0.17	0.156
1.0E+04	2.4E+04	2.0E+06	4.8E+06	3.4E+06	0.079	0.009	0.09	0.079
9.0E+03	9.0E+03	1.8E+06	1.8E+06	1.8E+06	0.042	0.006	0.06	0.042
7.0E+03	3.2E+03	1.4E+06	6.4E+05	1.0E+06	0.022	0.003	0.03	0.022
2.3E+03	1.7E+03	4.6E+05	3.4E+05	4.0E+05	0.012	0.003	0.03	0.012
1.5E+03	1.0E+03	3.0E+05	2.0E+05	2.5E+05	0.007	0.002	0.02	0.007
5.0E+02	8.0E+02	1.0E+05	1.6E+05	1.3E+05	0.005	0.003	0.03	0.005

Table 5: CFU vs OD_{600} data. Each set was initially grown with a comparative amount of CFUs. Then the optical density was measured for an undiluted sample and 1:10 dilution.

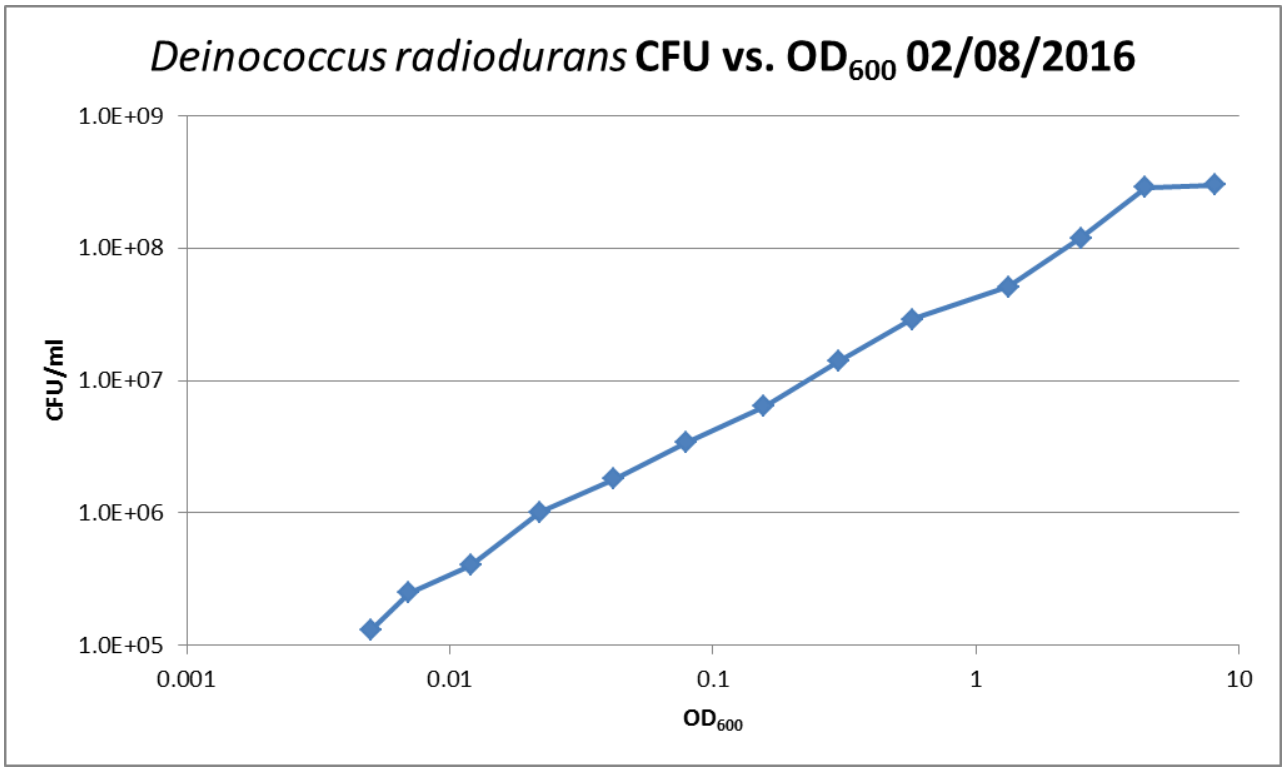


Figure 1: A graphical representation of Table 5's data. The linear region from 1.0E+04 to ~3E+05 is the acceptable region of initial CFU inoculation for accurate OD_{600} readings.

Appendix C: Vendor Provided Fact Sheets

Plasmid Fact Sheet

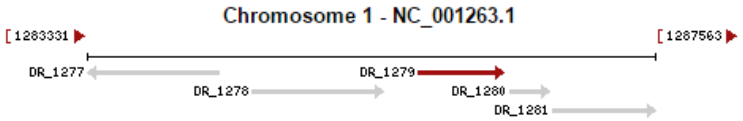
Listed are the parameters for the plasmids constructed in NEBuilder.

ΔDR_1279

DR_1279 Chromosomal location (NCBI)

Location: chromosome: 1

Sequence: Chromosome: 1; NC_001263.1 (1285792..1286427)



Notes:
261bp separate DR_1278 stop codon and DR_1279 ATG which suggests that DR_1279 has a promoter in this region. A promoter here may drive downstream genes and the intergenic region should be left intact.
58bp separate DR_1279 stop codon from DR_1280 ATG. This is not much room for a promoter. If a promoter exists it may partially lie in the DR_1279 ORF. This will require the DR_1279 deletion to leave a portion of the ORF just in case a promoter for the downstream genes exists.

Procedure:
Primers were designed to amplify ~1kb of genomic DNA upstream (5') and downstream (3') of the DR_1279 ORF (which may contain minimal portions of the ORF) to allow for deletion of a significant portion of the ORF after double crossover homologous recombination. These primers also contain extensions with homology to the pUC19 MCS (multiple cloning site) or an antibiotic resistance cassette flanked by mutated lox sites for latter removal using Cre/lox recombination. These primer extension homologies will allow for linking of 4 DNA fragments simultaneously using the NEBuilder Cloning system. 3 different antibiotic resistance cassettes will be cloned to test which ones work well in *D. radiodurans*. All 3 resistance cassettes are flanked by the same lox sites which will allow for use of the same primer sets.
Primers were designed to check the status (wildtype or deletion) of the gene following the knockout attempt.

Primers highlighted on sequence from *D. radiodurans* genomic DNA:

DR1279 5' GenomeCheck	>>>	misc_feature	603..622
DR_1279_UHFP	>>>	misc_feature	910..927
DR_1279 ORF	>>>	misc_feature	1793..2428
DR_1279_UHRP	>>>	misc_feature	1860..1878
DR_1279_DHFP	>>>	misc_feature	2387..2404
DR_1279_DHRP	>>>	misc_feature	3218..3235
DR1279 3' GenomeCheck	>>>	misc_feature	3429..3446

Primer list for DR_1279 knockouts:

Primer List for Deinococcus radiodurans DR_1279 MnSOD KO Cassettes					
#	Name	5'	Sequence	3'	Purpose
	pUC19MCS_DR1279UHFP	5'	tctagagtcgacctgcaggcatgcACTCTACTACGAGGCTTCG	3'	Amplify DR1279 upstream crossover homology with cloning pUC19MCS homology
	lox71_DR1279UHRP	5'	atgtatgctatacgaacggtaGTGTGGTGAATTTCCATCG	3'	Amplify DR1279 upstream crossover homology with cloning lox71 homology
	lox66_DR1279DHFP	5'	gcatacattatacgaacggtaAACTGGGACGAAGTCTCG	3'	Amplify DR1279 downstream crossover homology with cloning lox66 homology
	pUC19_HindIII_DR1279DHRP	5'	aacagctatgaccatgattacgccAagcttAGTCCACGAAGACGGTGC	3'	Amplify DR1279 downstream crossover homology with HindIII and cloning pUC19 homology
	DR1279 5' GenomeCheck	5'	GAATGTCTTGCAACTGATCG	3'	Amplify DR1279 genomic region to check KO status
	DR1279 3' GenomeCheck	5'	GTCAGGAAGCGTTGCAGC	3'	Amplify DR1279 genomic region to check KO status

ΔDR_A0202

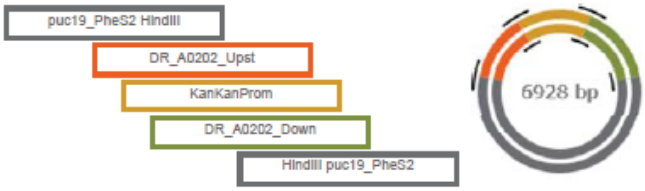
User Selected Settings

Product Version	E5520 - NEBuilder High-Fidelity DNA Assembly Cloning Kit
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	25 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Vector Digestion

Vector backbone opened with HindIII

Fragment Arrangement



Required Primers

Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta *	6-Frame
puc19_PheS2	tctagagtcgacctgcaggcatgcaGGCTACCTCTACGCCTCC	DR_A0202_Upst	Fwd	65.1°C	68.1°C	view
KanKanProm	tatacgaacggtaCGAACTCGTGGACGTGCA	DR_A0202_Upst	Rev	65.9°C	68.1°C	view
DR_A0202_Upst	gtccacgagttcgTACCGTTCGTATAGCATAC	KanKanProm	Fwd	55.6°C	58.6°C	view
DR_A0202_Down	cacgaggtcgttcTACCGTTCGTATAATGTATGC	KanKanProm	Rev	56.9°C	58.6°C	view
KanKanProm	tatacgaacggtaGAACGACCTCGTGGTGAC	DR_A0202_Down	Fwd	63.4°C	66.4°C	view
puc19_PheS2	aacagctatgaccatgattacgccaTTCACCTCGCACGGCAAG	DR_A0202_Down	Rev	66.4°C	66.4°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.
- Any element of a construction that includes the 5' overhang of a restriction site will be altered upon assembly. For example, the essential cysteine codon at the N-terminus of the intein segment of IMPACT vectors is present in the 5' overhang of the SapI site in those vectors. The bases removed in the assembly reaction can be added back by including them in the PCR primers for the corresponding insert.

ΔDR_1546

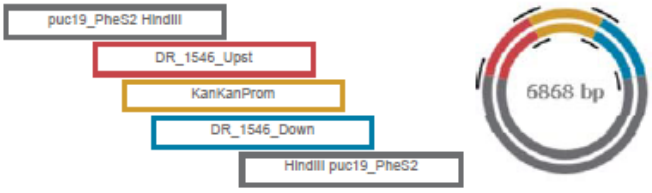
User Selected Settings

Product Version	E5520 - NEBuilder High-Fidelity DNA Assembly Cloning Kit
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	25 bp
PCR Polymerase	Q5 High-Fidelity DNA Polymerase
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Vector Digestion

Vector backbone opened with HindIII

Fragment Arrangement



Required Primers

Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta *	6-Frame
puc19_PheS2	tctagagtcgacctgcaggcatgcaGTAGTGCCCGTAGCTGCT	DR_1546_Upst	Fwd	65.1°C	67.2°C	view
KanKanProm	tatacgaacggtaGTCACTTTGCCGGTCTGG	DR_1546_Upst	Rev	64.2°C	67.2°C	view
DR_1546_Upst	ccggcaaagtgacTACCGTTCGTATAGCATAC	KanKanProm	Fwd	55.6°C	58.6°C	view
DR_1546_Down	tgatagtcgtcggTACCGTTCGTATAATGTATGC	KanKanProm	Rev	56.9°C	58.6°C	view
KanKanProm	tatacgaacggtaCCGACGACTATCAGACCAAC	DR_1546_Down	Fwd	62.8°C	65.8°C	view
puc19_PheS2	aacagctatgaccatgattacgccaGAACATTGCCTTCCGCCT	DR_1546_Down	Rev	63.6°C	65.8°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

Notes

- The NEBuilder Assembly short protocol (15 min incubation) works best with 1-2 insert fragments and a vector backbone. Assembly efficiency drops by 5-10 fold when 3 or more insert fragments are assembled into a cloning vector compared to 1 insert fragment. We recommend using the longer protocol (up to 60 min incubation) for the assembly of 3 or more inserts into a vector in 1 reaction.
- Any element of a construction that includes the 5' overhang of a restriction site will be altered upon assembly. For example, the essential cysteine codon at the N-terminus of the intein segment of IMPACT vectors is present in the 5' overhang of the SapI site in those vectors. The bases removed in the assembly reaction can be added back by including them in the PCR primers for the corresponding insert.

ΔDR_1709

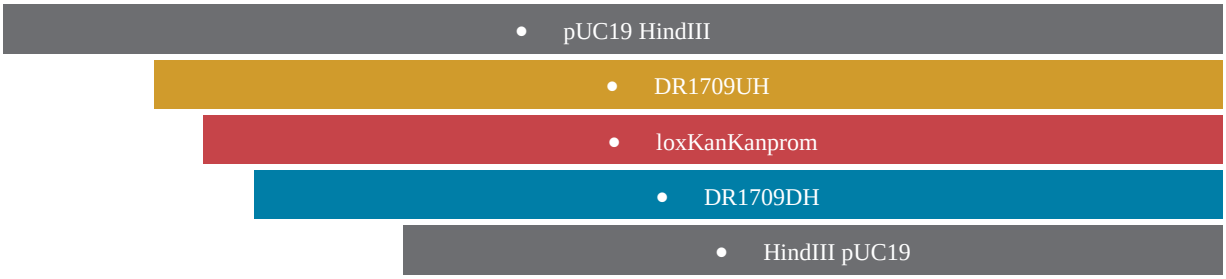
User Selected Settings

Product Version	E5520 - NEBuilder High-Fidelity DNA Assembly Cloning Kit
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	25 bp
PCR Polymerase	Phusion High-Fidelity PCR Kit (GC Buffer)
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Vector Digestion

Vector backbone opened with HindIII

Fragment Arrangement



Required Primers

pUC19	tctagagtcgacctgcaggcatgcaCGTGACCGTCAATCAGCA	DR1709UH	Fwd	64.9°C	64.9°C	view
loxKanKanprom	tatacgaacggtaGCGTCGAGATGCTGTTCTG	DR1709UH	Rev	64.9°C	64.9°C	view
DR1709UH	agcatctcgacgcTACCGTTCGTATAGCATACATTATAC	loxKanKanprom	Fwd	58.9°C	59.6°C	view
DR1709DH	cactgtgaaggagTACCGTTCGTATAATGTATGC	loxKanKanprom	Rev	56.6°C	59.6°C	view
loxKanKanprom	tatacgaacggtaCTCCTTCACAGTGATTGGCTG	DR1709DH	Fwd	64.7°C	64.7°C	view
pUC19	aacagctatgaccatgattacgccaTGGTCAGCGTGACCCAGA	DR1709DH	Rev	67.1°C	64.7°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

ΔDR_2283-83

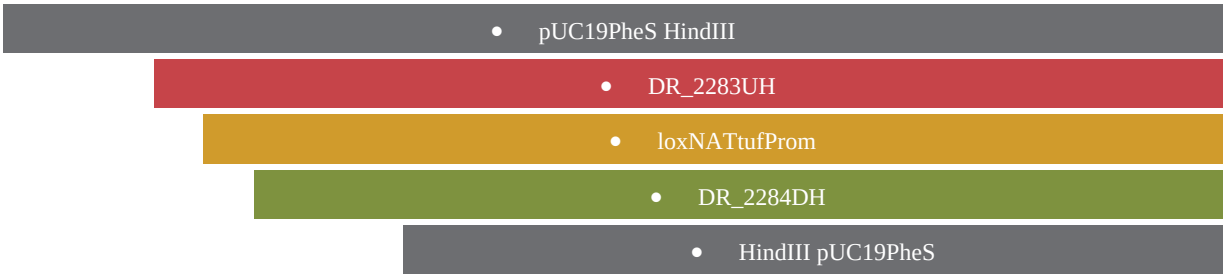
User Selected Settings

Product Version	E5520 - NEBuilder High-Fidelity DNA Assembly Cloning Kit
No. of Fragments	4-6 fragments (including vector)
Min. Overlap	25 bp
PCR Polymerase	Phusion High-Fidelity PCR Kit (GC Buffer)
PCR Primer Conc.	500 nM
Min. Primer Length	18 nt

Vector Digestion

Vector backbone opened with HindIII

Fragment Arrangement



Required Primers

pUC19PheS	tctagagtcgacctgcaggcatgcaCATGAATGCCAAAGGCGA	DR_2283UH	Fwd	65.9°C	62.2°C	view
loxNATtufProm	tatacgaacggtaGCCAGTTATTACCTCGACACC	DR_2283UH	Rev	62.2°C	62.2°C	view
DR_2283UH	ggtaataactggcTACCGTTCGTATAGCATACATTATAC	loxNATtufProm	Fwd	58.9°C	59.6°C	view
DR_2284DH	tcactgccgtgatTACCGTTCGTATAATGTATGC	loxNATtufProm	Rev	56.6°C	59.6°C	view
loxNATtufProm	tatacgaacggtaATCACGGCAGTGAACTGC	DR_2284DH	Fwd	62.6°C	61.1°C	view
pUC19PheS	aacagctatgaccatgattacgccaGGAAGGTTGTGGCTACTACG	DR_2284DH	Rev	61.1°C	61.1°C	view

* 3' Ta (recommended annealing temperature for PCR) is calculated for the gene-specific portion of the primer for use with the selected PCR polymerase.

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14. ABSTRACT
Multiple strains of Deinococcus radiodurans were transformed, creating knockout mutations in genes responsible for manganese ion transport, manganese and copper/zinc super-oxide dismutase, and bacillithiol synthesis. These mutated strains were then irradiated with ~20,000 Gys. The results showed that the mutated strains had a higher sensitivity to ionizing radiation, some having an increase in sensitivity 3000 times more than wild type Deinococcus radiodurans. In addition to radiation the mutated strains were also exposed to paraquat, an oxidizing herbicide. Strains missing manganese super-oxide dismutase and bacillithiol synthesis showed increased sensitivity.

15. SUBJECT TERMS
Deinococcus radiodurans, manganese, superoxide, dismutase, gamma

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