ABSTRACT

Title of Dissertation: BIOLOGICAL CHARACTERIZATION OF RECD MUTANTS IN *DEINOCOCCUS RADIODURANS*

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The RecD protein in *E. coli* and other gram negative bacteria functions as a subunit in concert with RecB and RecC to form the heterotrimeric RecBCD complex. It is involved in degrading foreign linear DNA, providing a block against viral invaders, and has been shown to inhibit recombination. This complex also catalyzes the first step in the highly regulated enzymatic process of DSBR via homologous recombination (HR). DSBR via HR is thought to be the major route for processing double strand breaks in *E. coli*. The first step is known as initiation and generates the substrate for the next enzyme in the pathway, RecA. In contrast to *E. coli*, most gram positive bacteria, use a set of genes known as the AddAB genes to accomplish this same task, to produce the substrate for RecA. The gram positive bacterium *Deinococcus radiodurans* is remarkable in its ability to withstand DNA damaging agents such as MMC, gamma irradiation, hydrogen peroxide and UV irradiation. While *D. radiodurans* has a RecA homolog that has been shown to be critical for repair of double strand breaks, sequence analysis has not revealed homologs of AddAB or RecBC. A sequence with high similarity to *recD* was found. *D.*

radiodurans RecD has an extended N-terminus grouping it with a sub-family of recD-like genes. The gene was expressed in *E. coli* and purified. Its biochemical activities were published by Wang and Julin, 2004, showing it had 5'-3' helicase activity like RecD proteins from other organisms. In this study a *recD* mutation in *D. radiodurans* was generated. The mutant was sensitive to gamma and UV irradiation, and hydrogen peroxide. The mutant was insensitive to MMC and MMS. Additionally the mutant showed an increased capacity to be transformed by exogenous DNA. These results imply a role in DNA repair through some form of recombinational repair. This finding is the first published result of a member of the recD-like sub-family of proteins that suggests role in DNA repair.

BIOLOGICAL CHARACTERIZATION OF RECD MUTANTS
IN *DEINOCOCCUS RADIODURANS*

By

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LIST OF ABBREVIATIONS

amp	Ampicillin resistance gene
ATP	Adenosine triphosphate
BER	Base Excision Repair
BSA	Bovine serum albumin
cam^{r}	Chloramphenicol resistance gene
DIG	digoxygenin
dNTP	deoxyribonucleic acid triphosphate
dUTP	deoxyuracil triphosphate
DSBR	Double stranded DNA break Repair
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
ESDSA	Extended synthesis dependent strand annealing
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HisRecD	His-tagged RecD
H_2O_2	Hydrogen Peroxide
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Ionizing radiation
kan^{r}	Kanamycin resistance gene
kb	Kilo base pairs
LB	Luria-Bertani
MMC	Mitomycin C
MMS	Methyl methane sulfonate

MMR   Methylation dependent Mismatch Repair

NER   Nucleotide Excision Repair

NHEJ   Non-Homologous End Joining

PAGE   Polyacrylamide gel electrophoresis

PCR   Polymerase chain reaction

PEG   Polyethylene glycol

PFGE   Pulsed field gel electrophoresis

PIPES   1,4 piperazine bis-(2-ethanosulfonic acid)

PMSF   phenyl methyl sulfonyl fluoride

SDS   Sodium dodecyl sulfate

SSA   Single stranded annealing

str^r Streptomycin resistance gene

TE   Tris EDTA buffer

TGY   Tryptone-glucose-yeast

UV   Ultraviolet

Chapter 1 INTRODUCTION

The bacterium *Deinococcus radiodurans* is remarkable in its ability to tolerate a variety of cellular insults, particularly those which cause DNA damage. Most notable is its capacity to recover from the deleterious effects of ionizing radiation. Ten percent of *D. radiodurans* cells survive an acute dose of 16 kGy compared to *E. coli*, for which the 10% survival dose is only 0.7 kGy. It is also extremely resistant to desiccation, presumably the evolutionary rationale for gamma radiation resistance, given the low amount of gamma radiation that penetrates the ozone layer (1, 2, 3 ,4). Ionizing radiation causes a variety of damage to cellular macromolecules, including double strand breaks in DNA. According to pulse field gel electrophoresis (PFGE), a dose of 10 kGy of ionizing radiation results in at least 100 double strand breaks per chromosome in *D. radiodurans*. The organism can survive this dose without mutation, fully reassembling its genome within 29 hours (2, 5, 6), while many organisms cannot withstand more than two or three double strand breaks occurring from radiation (7).

D. radiodurans uses RecA catalyzed DSBR to reconstruct chromosomes shattered by ionizing radiation, as evidenced by the severe sensitivity to this form of damage in *recA* mutants (3, 6). In gram negative bacteria, the heterotrimeric RecBCD enzyme plays a crucial role in preparing the DNA substrate for RecA (7, 8, 9). In many gram positive bacteria, a heterodimeric enzyme AddAB performs essentially the same function (10, 11).

In 1999, White et al. sequenced the genome of *D. radiodurans* (12). It revealed many DNA repair enzymes common to other bacteria, suggesting that *D. radiodurans* uses many of the same DNA repair pathways previously characterized in other organisms

(13, 14). Interestingly however the genome sequencing failed to detect a homolog of AddAB or RecBC. There was however a protein of significant homology to the RecD subunit of the RecBCD complex. This enzyme *D. radiodurans* RecD was cloned and purified. Biochemical analysis revealed that it has enzymatic properties comparable to the RecD subunit of RecBCD (15). The goal of this study is to determine the biological role of the RecD protein of *D. radiodurans*.

1.1 RecBCD in *E. coli*

The RecBCD enzyme is a 330 kDa heterotrimer, originally denoted exonuclease V. It is composed of three polypeptides, called RecB (134 kDa), RecC (129 kDa) and RecD (67 kDa), respectively. It has both helicase and nuclease activities. Helicases are molecular motor proteins. Helicases have three fundamental biochemical properties: binding of nucleic acids, NTP binding, and NTP hydrolysis dependent unwinding of nucleic acids (16-18). Nucleases are enzymes that cut DNA by cleaving the phosphodiester bonds between the bases. The RecBCD enzyme is capable of binding free double strand DNA ends, and unwinding the duplex. It has 3'-5' helicase activity from the RecB subunit (19), and 5'-3' helicase activity from the RecD subunit (20). The RecB subunit is responsible for the nuclease activity of the complex (21). Lastly, the enzyme is capable of loading the DNA recombinase RecA (7, 8, 9).

1.2   Functions of RecBCD in *E. coli*

RecBCD plays a critical role in defending the organism against viruses, such as T4 phage. In this capacity RecBCD rapidly can degrade several thousand base pairs of linear double stranded DNA in a matter of seconds (972 ± 172 bp / sec), in a processive manner (at least 30,000 bp per binding event), rapidly destroying the viral invader's DNA (8). To prevent the enzyme from attacking double strand breaks in its chromosomal DNA, a specific sequence called Chi (5'-GCTGGTGG-3') is located periodically throughout the genome (22). When the enzyme encounters the sequence, the nuclease activity of RecBCD is altered allowing for repair of the broken chromosome (23).

1.2.2   Double Strand Break Repair in *E. coli*

Double strand breaks can arise directly from a variety of DNA damaging agents, and indirectly as a result of repairing or replicating damaged DNA (24, 25). They are thought to be one of the most deleterious forms of DNA damage as they expose the genetic material to the cell's exonucleases which can rapidly degrade vital genetic information. Repair of double strand breaks occurs through homologous recombination. In this process, a double strand break is repaired by DNA synthesis using another "homologous" piece of DNA as a template to ensure correct repair of the break. The mechanism requires that there are redundant copies of the genetic material. The end result of this process is two repaired versions of the molecule with portions of each recombined into the other (8, 25).

In *E.* coli the RecBCD complex is responsible for catalyzing the first step in homologous recombination, initiation. In this step the blunt ends of the double strand

break are converted to 3' overhangs which are coated by RecA (26). This is accomplished through the collective and coordinated activities of the three subunits of RecBCD. The enzyme binds to the end of double strand break (Fig. 1.1, step 1), and the two helicases appear to work together to unwind DNA in the same direction, each operating on different strands (Fig. 1.1 step 2). The cooperativity of the two motors may explain its extremely high rate of unwinding and processivity. While unwinding DNA the enzyme also degrades the 3' to 5' strand using the nuclease activity of RecB (Fig.1.1, step 2). When the enzyme encounters the Chi sequence, step 3 in figure 1.1, it pauses briefly making its final cut on the 3' to 5' strand just prior to the Chi site step 3 in figure 1.1 (25, 27).

This encounter drastically changes the activity of RecBCD. When it resumes translocating, it does so at approximately half its initial rate (28). The polarity of the nuclease activity changes from the 3' to 5' strand to 5' to 3' strand. This results in a DNA end with a single strand 3' overhang, terminating in the Chi sequence. Also, after encountering the Chi site, RecBCD loads the recombinase enzyme RecA onto the 3' single strand overhang generated by the combined helicase and nuclease activity of RecBCD. This is shown in step 3 in figure 1.1 (5, 20, 27, 29). It has been postulated that the changes in activity of the enzyme are due to either a loss of RecD from the holoenzyme, or a conformational change in the subunit, inactivating it (29). This is supported by experiments demonstrating that RecD inhibits RecA loading and recombination in a particular *recB* mutant background (30). Also, the slowing of translocation by the enzyme after Chi could be explained by the loss of function or presence of one of the two molecular motors.
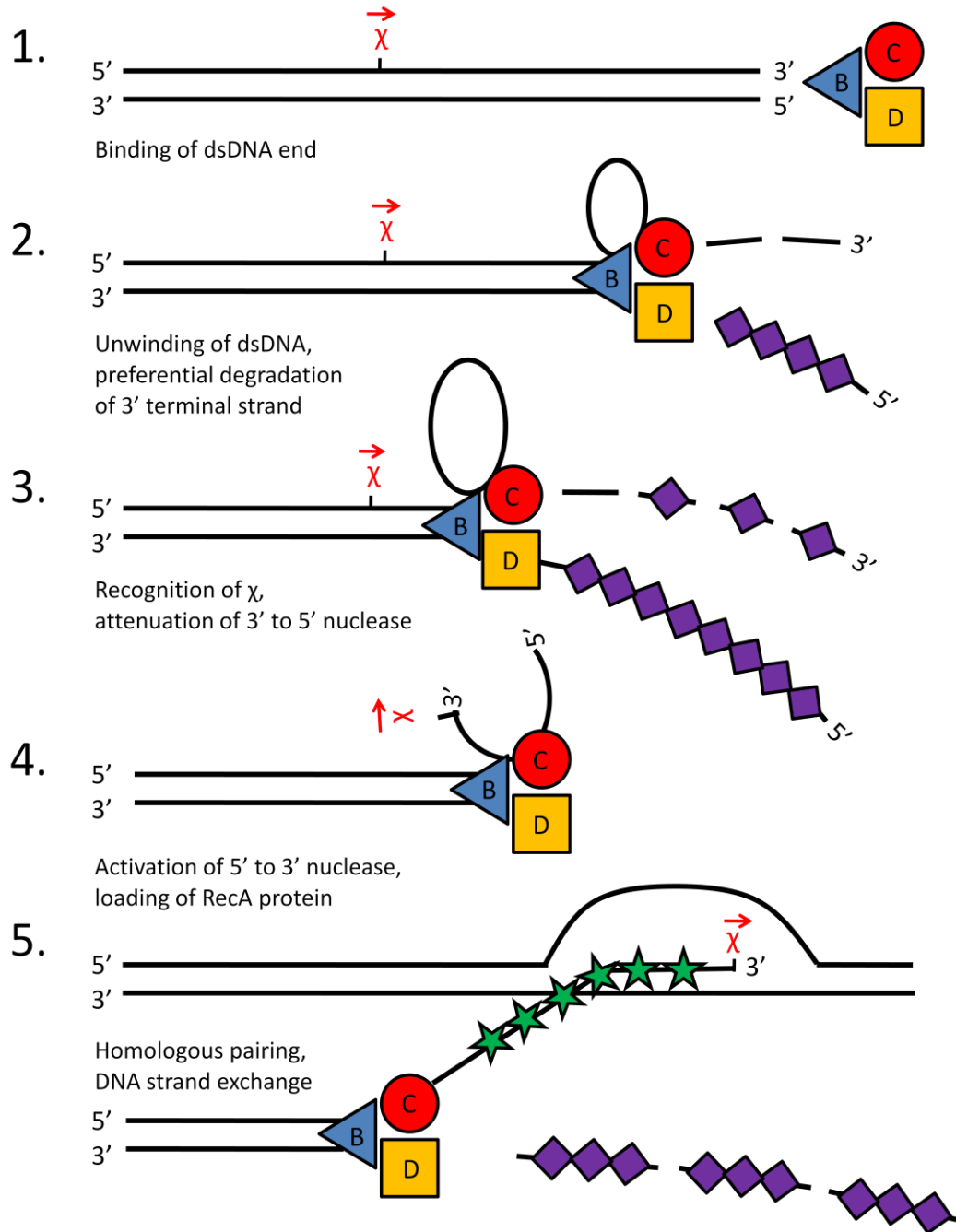
Figure 1.1 Enzymatic activities of RecBCD. Steps 1 through 5 of this diagram detail the involvement of RecBCD (labeled B, C, and D), RecA (green stars), single stranded binding protein (purple diamonds), and the recombination hotspot chi (χ) in the early stages of homologous recombination. The production of RecA-coated, Chi-containing single stranded DNA results from the coordinated nuclease and helicase action of RecBCD. Figure adapted from Kowalczykowski, 2000 (25).

The subsequent steps in the process are RecBCD independent. The second step in the pathway is mediated by RecA. This enzyme is capable of locating another piece of DNA homologous to the one that is currently bound. It then unwinds the homologous stretch of DNA allowing the bound strand to invade the duplex strand (Fig. 1.2A). The RecA bound strand binds to its complement, while displacing the homologous strand of the duplex forming a "D-loop." Next, DNA polymerase I (Pol I) polymerizes new DNA, using the end of the Chi sequence as a primer. Ultimately the polymerase resynthesizes the DNA that was degraded. The regions of homology hold the broken strands in place so DNA ligase can actually repair the DNA cleavage. Branch migration is mediated by another helicase, the RuvAB heterodimer, and performs the function of checking for erroneous recombination. The resolvase (RuvC) catalyzes the final step, which is resolution of the Holiday Junctions, separating the strands from each other, shown in figure 1.2B (8, 25).
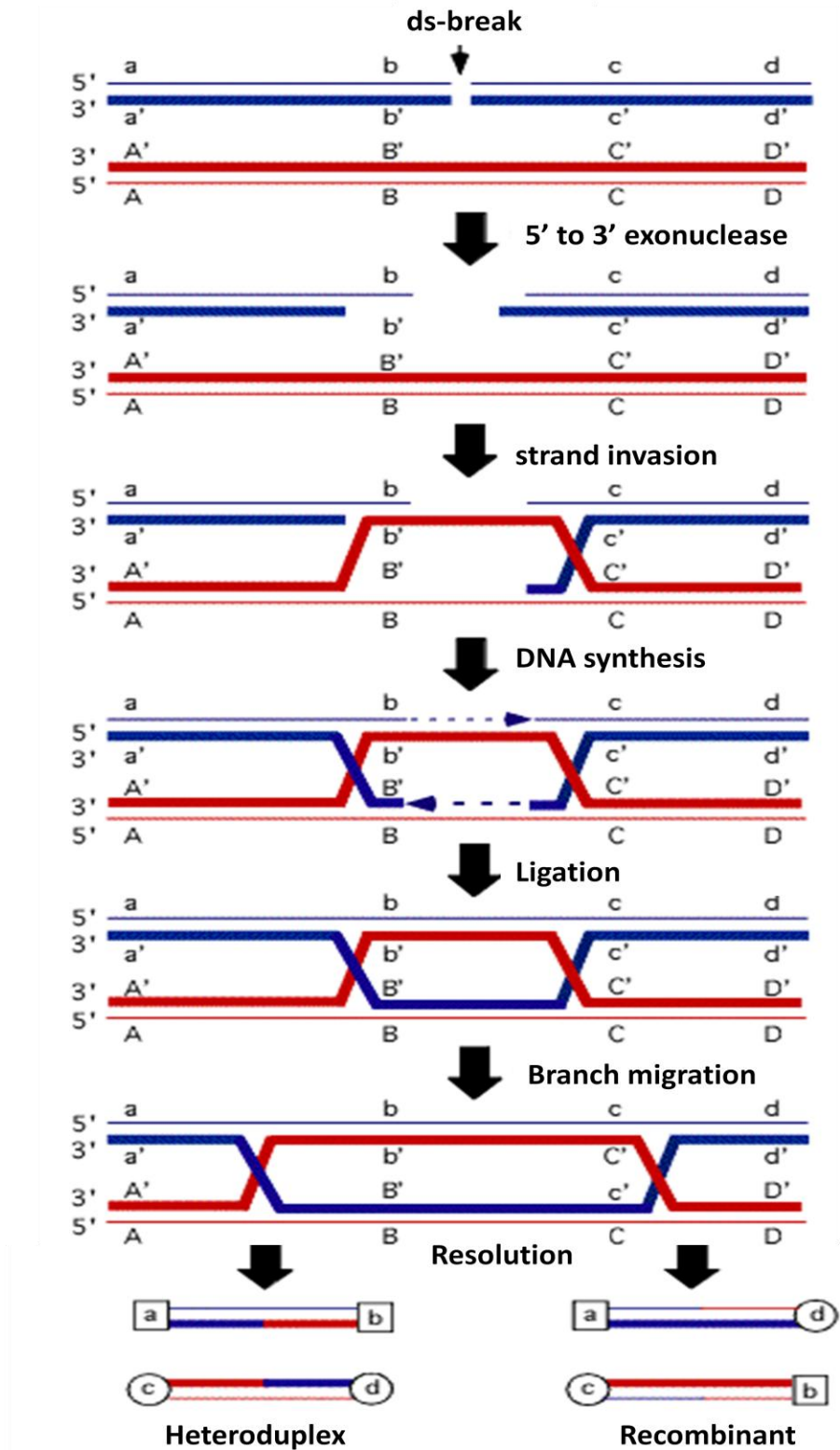
Figure 1.2 A general model for Double Strand Break Repair by homologous recombination adapted from Kowalczykowski, 2000 (25).

1.2.3 Phenotypes of RecBCD Mutants in *E. coli*

Null mutations mapped to *recB* and *recC* are sensitive to DNA damaging agents, reflecting the importance of DSBR in *E. coli* (31-33). Additionally, cultures of these strains result in many nonviable cells (34). These strains are also sensitive to infection by phage, and in certain mutant backgrounds are transformable with linear double strand DNA (35). In contrast, null mutations in *recD* leave strains highly viable and proficient in recombination and DNA repair, as they retain their proficiency in homologous recombination (25). Unexpectedly, *recD* mutants lack exonuclease activity, despite the nuclease activity of RecBCD being mapped to the RecB subunit. This result suggests that RecD modulates RecB nuclease activity (36).

1.3 Extremophile *Deinococcus radiodurans*

Deinococcus radiodurans is a small, pink, non-motile, gram positive bacterium originally isolated as a contaminant of gamma irradiated meat (37). Unlike most gram positive bacteria, it has a complex membrane reminiscent of gram negative bacteria. It is easily amenable to genetic studies as it is innately transformable, taking in DNA and incorporating it into its genome naturally. All known *Deinococcus* species are extremophiles capable of surviving a variety of harsh conditions. *D. radiodurans* is particularly resistant, withstanding UV radiation (~20 fold), ionizing radiation (~200 fold), and hydrogen peroxide (~100 fold) better than *E. coli* (38, 39). The organism is capable of resurrecting its genome from doses of gamma radiation determined to induce over 100 double strand breaks per chromosome without mutation or loss of survival (41-40).

RecA mediated DNA repair is critical to repairing double strand breaks in DNA in the model organisms *E. coli* and *B. subtilis* (7-11). RecA repairs double strand breaks in DNA by using an unbroken piece of DNA homologous to broken piece. It is therefore necessary to have redundant copies of genetic material to utilize this repair process. *D. radiodurans* maintains multiple copies of its DNA which facilitates repair of double strand breaks by homologous recombination. The bacterium maintains 8-10 copies of its chromosomes in exponentially growing cultures, and 4 in stationary phase cultures (128-130). The *D. radiodurans* genome consists of two chromosomes, one has 2,649 kbp and the other 412 kbp. It also has one megaplasmid of 177 kbp, and one plasmid of 46 kbp. The sequences of these genetic elements contain 3,195 predicted genes (12, 43).

Depicted in figure 1.3, Daly and Minton provided evidence for the importance of RecA mediated recombinational repair using pulsed field gel of *D. radiodurans* DNA at various time points following an acute dose of gamma radiation (17.5 kGy) at a dose rate of 10 kGy/hr (2). Before irradiation, the genomic DNA appears as a smear between 290 and 23 kbp (Fig. 1.3). Immediately following the exposure the genome is reduced to fragments of less than 23 kbp. Both wild type and recA mutant cells begin repairing DNA by a RecA independent mechanism. By 29 hours of recovery in TGY medium, the pre-radiation pattern returns in wild type alone (2). Clearly the organism has an amazing ability for withstanding ionizing radiation, and the capacity for repairing the DNA damage incurred from this assault (6, 7, 8). The reason for this is currently a topic of intense debate in the field of radiation biology.

A. Wild type

B. *recA* mutant
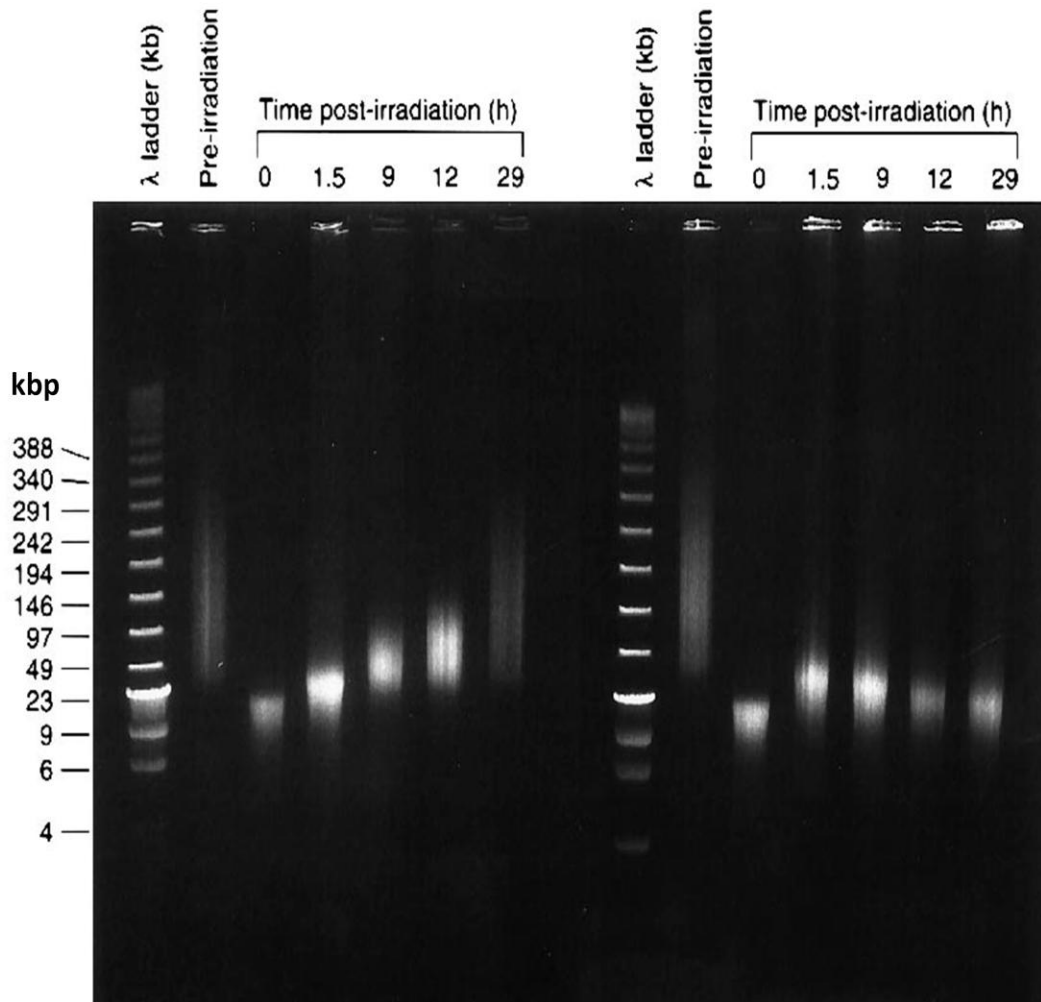
Figure 1.3 Pulsed Field Gel Electrophoresis (PFGE) analysis of chromosomal DNA purified from *D. radiodurans* R1 and the *rec30* mutant strain, recovering from a dose of 17.5 kGy of gamma radiation. (A) Wild type R1. (B) A *recA* mutant strain (designated *rec30*) isolated by MNNG mutagenesis. Cells were grown to early stationary phase and treated with 17.5 kGy of gamma radiation on ice, and returned to standard culture conditions for *D. radiodurans* for recovery. At various time points the cells were harvested and chromosomal DNA was purified, and run on a pulsed field gel (6).

1.3.1 Cellular Damage Resulting From Gamma Irradiation

Gamma radiation causes multiple forms of cellular damage importantly damage to

DNA. One of the major difficulties in studying these effects is gamma radiation results

in so many modifications to DNA (131). Irradiating thymine in dilute oxygenated

solutions produced 24 different identifiable variations of the base (132). Further

complicating the understanding of gamma irradiation induced DNA damage, are the

variety of conditions used to assess the products formed. Irradiating DNA under oxygen

rich or anoxic solutions results in different types of damage. The pH at which the

experiments were performed also influences the results. Lastly, irradiating DNA in an

anhydrous state leads to different proportions of certain types of damage compared to

experiments performed in solution. Therefore it has been estimated that gamma radiation

results in hundreds of forms of DNA damage (131).

Gamma rays transfer energy to electrons of irradiated samples. An electron can

be excited to a higher energy state if the energy transferred is less than its ionization

energy. If the energy transferred to the electron exceeds its ionization energy, the

electron is ejected from the atom. This energy transfer can occur directly in the DNA

molecule itself resulting in direct action on the molecule. Alternatively, it can react with

the solution in which the DNA is dissolved creating reactive species that can

subsequently damage DNA, a process known as indirect action. In aqueous solutions

gamma rays split water creating the hydroxyl radical ($HO^{\bullet}$), the hydrated electron (e^-),

the H atom. Two hydroxyl radicals can react forming H_2O_2, and two hydrogen atoms

combine to form H_2. In the presence of oxygen, superoxide radical ($O2^{\bullet -}$) is formed

from combining dioxygen with the hydrated electron. Superoxide radical and hydrogen

peroxide are produced at relatively low levels. They are relatively unreactive with DNA and are not considered to play a significant role in damaging DNA in oxygenated solutions. Hydroxyl radical however is capable of causing significant amounts of various types of DNA damage (60, 131).

Both the direct and indirect action of gamma radiation damage DNA resulting in altered bases, as well as cleavage of the sugar phosphate backbone. It is difficult to differentiate the products and extent of DNA damage resulting from direct and indirect action. Analysis of gamma irradiated samples of DNA and DNA bases in their solid state allow for crude inferences into the types of damage due to direct action. DNA strand breaks can be caused directly from gamma radiation by excitement of an inner shell electron in DNA. These breaks were detected by irradiating supercoiled covalently closed ΦX 174 DNA, followed by sedimentation analysis (133). Direct damage to bases has been detected by irradiating frozen purified thymine. Thymine dimers make up the majority of the gamma irradiation induced products (134).

Indirect damage of DNA by hydroxyl radicals is thought to be the major route of DNA damage resulting from gamma irradiation in bacteria, as they are composed of 50-70% water which would absorb most of the energy (135). Hydroxyl radical is capable of abstracting a hydrogen atom from any of the five carbons in deoxyribose. Extraction of 4'-C and 5'-C hydrogen atoms leads to alkali-labile bonds that can be broken, ultimately resulting breakage of both the 5' and 3' sugar-phosphate bonds from the attacked sugar (131). Extraction of 1'-C and 2'-C hydrogen atoms followed by addition of oxygen leads to breakage of the 3' sugar-phosphate bond from the attacked sugar (131). The 5' and 3'

ends of double strand breaks commonly found following hydroxyl radical mediated strand cleavage are depicted in figure 1.4 (136).
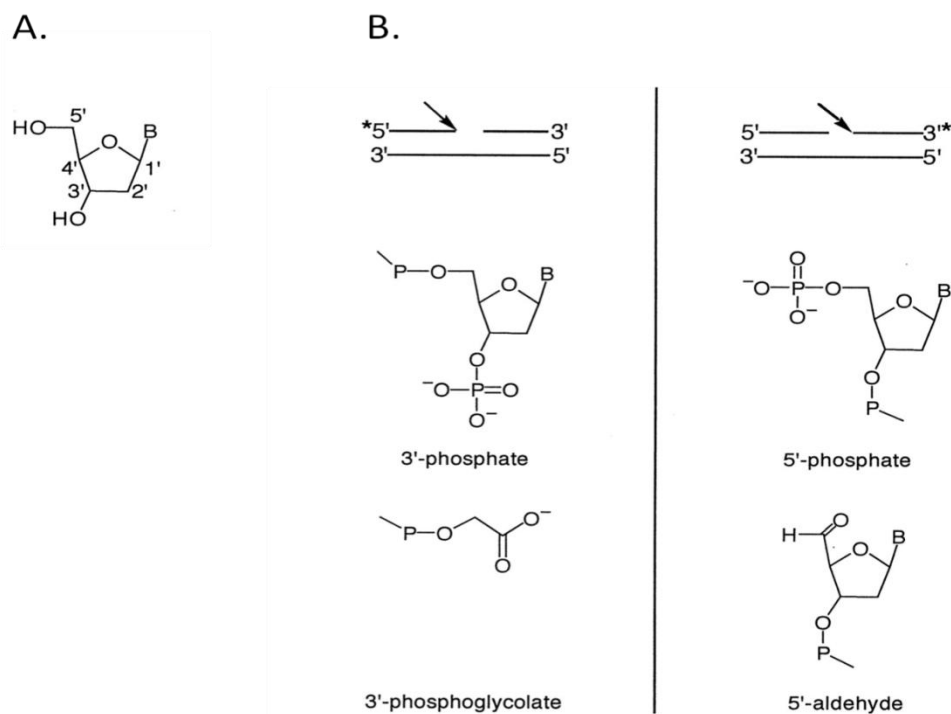
Figure 1.4 Free ends of double strand breaks resulting from hydroxyl radical mediated cleavage. (A) Numbering scheme for the carbon atoms of deoxyribose. (B) Structures of the products of hydroxyl radical mediated DNA strand cleavage. (Left) Structures at the 3' end of the DNA strand at the site of cleavage. (Right) Structures at the 5' end of the DNA strand at the site of cleavage. Locations of cleavage ends are marked by arrows above structures. Figure adapted from Balasubramanian et al, 1998 (136).

Hydroxyl radicals are capable of directly modifying DNA bases through oxidation. All of the bases are capable of being modified, however the formation of 8-hydroxyguanine (fig. 1.5 structure 8) is the most prevalent. Guanine has the lowest reduction potential of the four bases and is capable of reversing the radical formed at the initial site of oxidation, ultimately forming 8-hydroxyguanine (60). Oxidation of purine bases results initially in hydroxylation at the 8-position. Further rearrangement can lead to the formation of ring-opened derivatives. Oxidation of the pyrimidine bases results in a

more complex product mixture involving primarily initial addition to the 5'-6' double

bond (137). Both purine and pyrimidine products have been used as biomarkers to

monitor oxidative stress resulting from oxidative damage (138, 139).
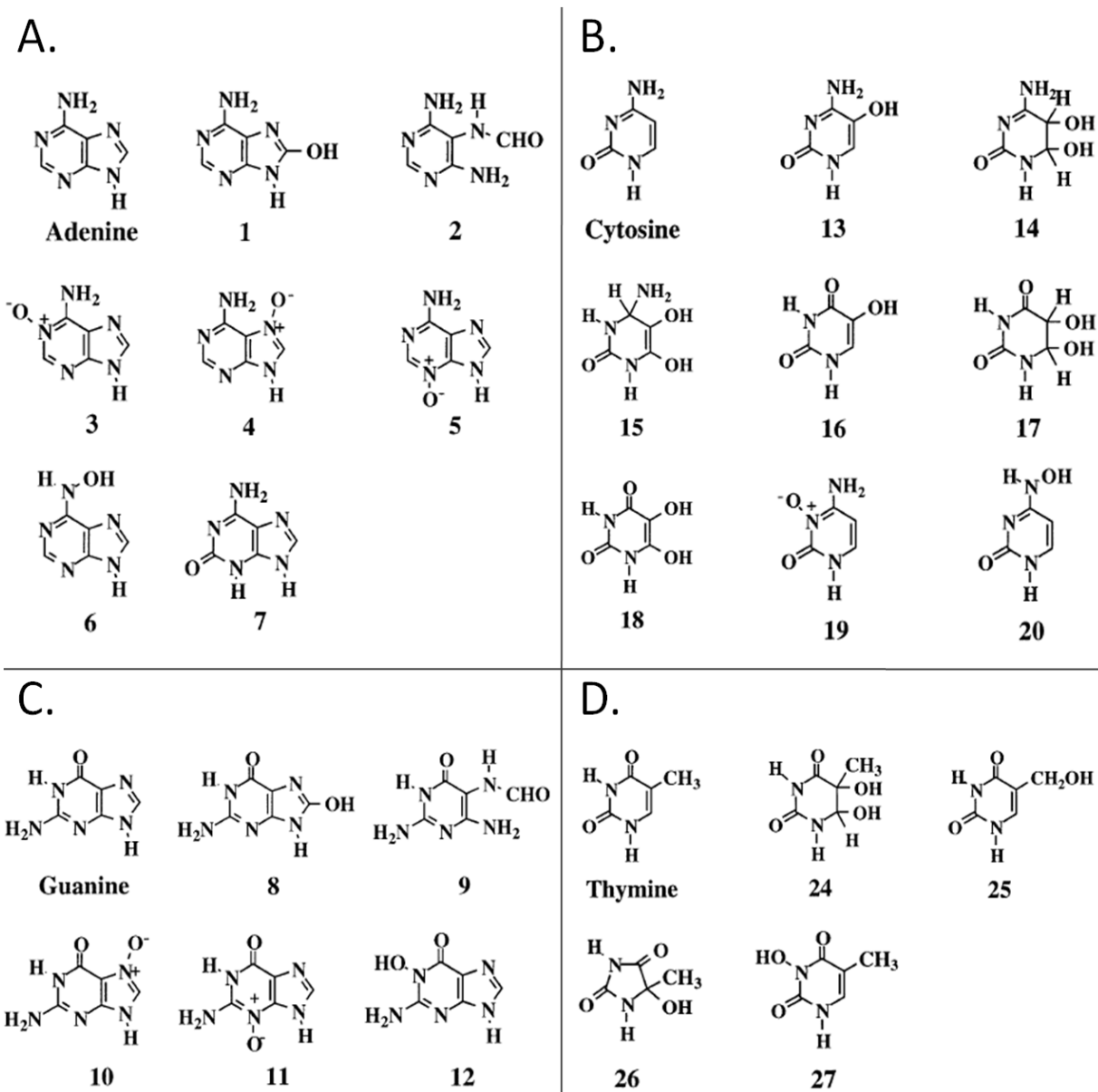
Figure 1.5 DNA bases and their hydroxyl radical mediated modifications. (A) Adenine, (structures1-7) modifications of adenine. (B) Cytosine, (structures13-20) modifications of cytosine. (C) Guanine, (structures8-12) modifications of guanine. (D) Thymine, (structures24-27) modifications of thymine. Figure adapted from Sidanman et al, 1998 (137).

While DNA damage is thought to be the lethal effect of gamma irradiation, it also damages other cellular macromolecules both directly by damaging any molecule in the cell as it does to DNA, and indirectly through the products of water radiolysis. Superoxide and hydrogen peroxide are capable of damaging iron-sulfur containing proteins inactivating them, releasing their bound iron and sulfur. Hydrogen peroxide can oxidize cystinyl residues creating disulfide crosslinks in proteins. Hydroxyl radicals can catalyze the carbonylation of proteins and initiate the peroxidation of lipids (60).

1.3.2 DNA Repair in *D. radiodurans*

D. radiodurans exhibits a remarkable ability to withstand DNA damaging agents. Many of the well characterized DNA repair pathways are present in its genome. It has members of the Methylation-dependent Mismatch Repair (MMR) pathway, including MutS and MutL. While it does not have typical Dcm and Dam methylases, it does have other predicted methylases that may perform the functions of Dcm and Dam in MMR. Components of the Nucleotide Excision Repair (NER) pathway of *D. radiodurans* include UvrABC and D. Transcriptional coupled repair is also present as evidenced by the presence of mfd helicase (transcriptional repair coupling factor). Base Excision Repair (BER) enzymes such as MutM, Y, and T are also present, along with a variety of other endonucleases and glycosylase. Recombinational repair enzymes are also well represented, including many of the Rec genes, including RecA, RecF, RecO, RecR, RecQ, and RecX (68-71). Additionally *E. coli* PolI and UvrA can completely complement *polI* and *uvrA* mutations in *D. radiodurans* (43).

Notable exceptions are a lack of photolyase and endonuclease IV, specific to the repair of UV irradiation induced damage. Additionally, there does not seem to be an SOS pathway. This pathway is activated in response to cellular stress, and activates the transcription of a number of genes, many involved in DNA repair. LexA is a key mediator of the SOS response (44). While *D. radiodurans* has two genes designated as LexA homologs, the organism appears to lack a typical SOS response. However, PprI has been identified and shown to upregulate RecA and PprA, both DNA repair enzymes with significant contributions to radioresistance in *D. radiodurans* (46, 47, 67). The lack of unique DNA repair enzymes, and the fact that some *E. coli* enzymes can complement for mutations in *D. radiodurans* homologous enzymes, has led to many interesting hypotheses attempting to explain the organism's phenomenal DNA repair capacity.

A highly controversial theory is that Non Homologous End Joining (NHEJ) facilitated by the structure assumed by *D. radiodurans* nucleoids aids the reassembly of chromosomes following gamma irradiation. Nucleoids in D. *radiodurans* and other radiation resistant bacteria were suggested to assume a tightly compacted (toroidal) structure based on their nucleoid's general shape in transmission electron microscopy (48-50). This type of structure has been reported for lambda phage by cryoelectron microscopy (51). This structure was postulated to assist in NHEJ and be a key to extreme radioresistance (49). This statement has been refuted as the evidence to support toroidal nucleoid structure provided by those suggesting this model, is not at a resolution to make this claim. They employed a different technique, cryoelectron microscopy on vitreous sections of nucleoids of *D. radiodurans* (52-54). This technique gives greater resolution, and provided evidence against the toroid structure of *D. radiodurans* nucleoids.

Single Stranded Annealing (SSA) was postulated as a key process for DNA repair that preceded RecA dependent repair (2). This pathway (shown in Fig. 1.6) connects chromosomal fragments with overlapping regions of homology. *D. radiodurans* has multiple copies of its chromosomes, allowing for such homology in the event of chromosomal shattering. The ends of DNA fragments are made single stranded by exonucleases. There is evidence that *D. radiodurans* degrades and removes DNA from its cells following gamma irradiation support for this nuclease activity in the SSA model (72). The complementary single-stranded regions from two different fragments are able to hybridize with each other simply by annealing their complementary sequences. After annealing, any non-homologous overhangs could be resected by nucleases, and any single-stranded gaps can be filled in by a DNA polymerase, and ligation by DNA ligase. Incorporation of multiple fragments in this manner will increase the physical length of chromosomal DNA fragments. Ultimately these fragments can be resolved into intact chromosomes by RecA mediated recombination (2, 55, 56).

The key evidence for this was provided by two experimental observations. First, shortly after an acute dose of gamma irradiation was applied, RecA mutants displayed a slight capacity to reform its chromosomal DNA (Fig. 1.3). Secondly, insertions were made into *recA* mutant *D. radiodurans* with or without repetitive DNA from *E. coli* derived plasmids. The constructs were generated in a manner allowing SSA to make circular plasmids from radiation induced fragmentation of the chromosomes harboring them as insertions. Only the insertions with repetitive sequence were capable of forming circular plasmids (2).

Zahradka et al. proposed a model called Extended Synthesis-Dependent Strand Annealing (ESDSA). They postulate that the same beginning step that occurs in SSA occurs in ESDSA. That is that 5' ends of double strand breaks are chewed back generating 3' overhangs, step 2 in figure 1.6. These overhangs are capable of priming DNA synthesis using homologous regions of overlapping sequence from other recessed fragments as a template, step 3 in figure 1.4. DNA polymerase I can synthesis DNA, proceeding to the end of the fragment used as a template. This results in fragments of DNA with even longer 3' overhangs that can find and anneal to complementary sequences in other similarly generated fragments. The annealing occurs as in the SSA model, step 4 in figure 1.6. The difference is in ESDSA, newly synthesized DNA establishes more regions of homology, aiding in accurate annealing of fragmented sections of DNA. The remaining steps in ESDSA are the same as in the SSA model (57).

The initial observation suggesting this model is the extreme sensitivity of DNA polymerase I mutants. The primary physical evidence supporting this more complex model is the extensive amount, and pattern of newly synthesized DNA found in *D. radiodurans* chromosomes in cells recovering from ionizing radiation. The newly synthesized DNA was found on only one strand of some stretches of DNA and coinciding on both strands in other stretches of DNA. The extent and pattern of DNA synthesis immediately following irradiation was monitored by the incorporation of a heavy analogue of thymidine, 5-bromo-deoxyuridine (BrdU) on cesium chloride equilibrium density gradients. This suggests that extended DNA synthesis occurs prior to annealing of fragmented chromosomes. It is notable that not all the repair enzymes required to mediate this process have been determined.
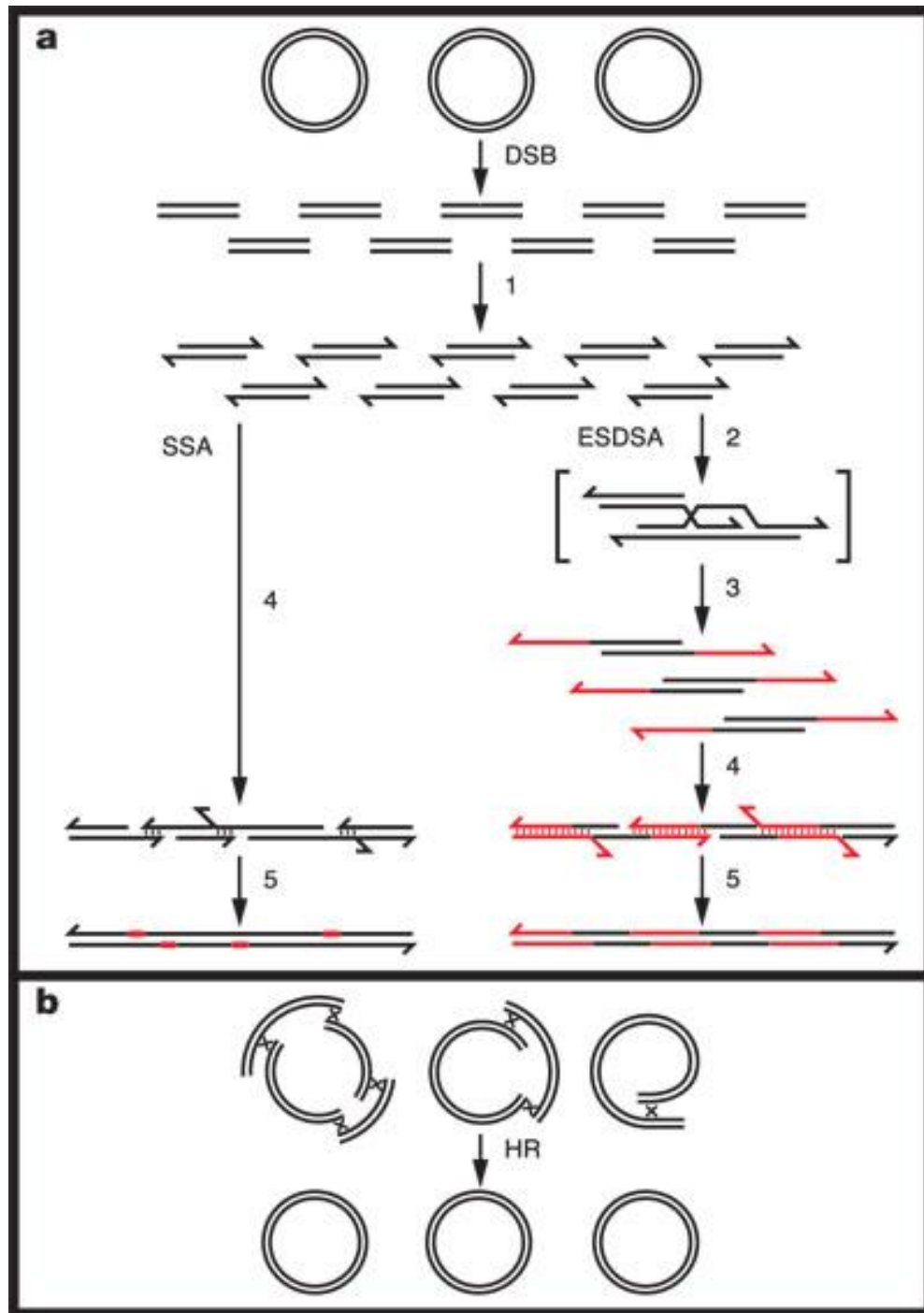
Figure 1.6 Schematic model of ESDSA compared with SSA. Step 1, The 5' ends of double strand breaks are chewed back generating 3' overhangs. These overhangs are capable of priming DNA synthesis using homologous regions of overlapping sequence from other recessed fragments as a template (steps 2 and 3).   The 3' overhangs find and anneal to complementary sequences in other fragments (step 4). Finally RecA mediated homologous recombination reassembles the annealed fragments. Zahradka et al. (57)

1.3.3 Protection of Proteins from Oxidative Damage

Daly et al. have postulated that while *D. radiodurans* has an exceptional capacity to repair DNA, but what makes it so resistant to ionizing radiation (IR) is its ability to reduce IR generated oxidative stress. It was noted that many bacteria with substantial radioresistance such as *Deinococcus* sp. had high ratios of manganese to iron. Conversely many bacteria particularly sensitive to ionizing radiation have low manganese to iron ratios (58). In addition to directly damaging cellular components, gamma radiation induced-lysis of water forms the reactive oxygen species hydroxyl radical $OH^{\bullet}$, superoxide radical $O_2^{\bullet -}$, and hydrogen peroxide (59, 131, 136). Hydroxyl radicals are known to damage DNA and other cellular macromolecules such as lipids, and proteins (60, 61). Superoxide radicals are not reactive to DNA damage, but damage iron-sulfur containing proteins, causing them to release iron (60, 61). Fe(II) reacts with hydrogen peroxide creating Fe(III), hydroxyl anion an hydroxyl radicals which damage cellular components (60, 61). Therefore limiting free iron prevents additional formation of $OH^{\bullet}$ resulting from reactive oxygen species generated from gamma radiation.

In Daly's theory, manganese cycles between Mn(II) and Mn(III), reducing $O_2^{\bullet -}$ levels inside the cell. Additionally, Mn(III) reacts with hydrogen peroxide converting it to H_2 and O_2. The reduction of superoxide radicals and hydrogen peroxide by manganese ultimately results in protein protection by limiting hydroxyl radical formation. DNA repair enzymes being proteins are therefore protected and capable of performing their functions. Daly proposes that gamma radiation induced protein damage causes a number of critical cellular processes such as protein synthesis, and DNA repair to fail, killing the

cell. In radiation resistant organisms, DNA damage is a repairable consequence of gamma radiation as the organism still has functioning DNA repair proteins (61).

The level of carbonyl groups detected on proteins can be used as a marker of oxidative protein damage. It has been shown to be irreversible and not repairable, and detectable by monoclonal antibodies (61, 62). Daly performed Western blots on gamma irradiated cells of radioresistant (*Deinococcus sp. D. radiodurans,* and *D. Geothermophilus*) and radiosensitive species (*E. coli*, and *Shewanella oneidensis*). Protein damage as detected as protein carbonylation occurred at much lower doses in radiation sensitive species than radiation resistant species (61).

1.4 RecD Protein in *D. radiodurans*

D. radiodurans RecD is a helicase with significant homology to other RecD family members (15). It is classified in the Clusters of Orthologous Groups (COG) data base as a member of the RecD family (COG0507) of Superfamily I helicases (12, 63). Typical of this Superfamily of helicases, it has seven conserved helicase motifs 1-6 and 1a which are involved in DNA binding and nucleotide hydrolysis (13). *D. radiodurans* RecD contains an extended N-terminus (370 amino acids), distinguishing itself from the typical RecD proteins which are members of RecBCD complexes. This N-terminus preceding the seven helicase motifs is not well conserved among other RecD members of RecBCD complexes and is unlike sequences found in other helicases. Therefore it is listed in the TIGR Protein Families data base (http://www.tigr.org/TIGRFAMs/) as a subgroup of this family along with other RecD members containing extended N-termini that are not part of RecBCD complexes (Fig. 1.7).

A. Components of RecBCD complexes
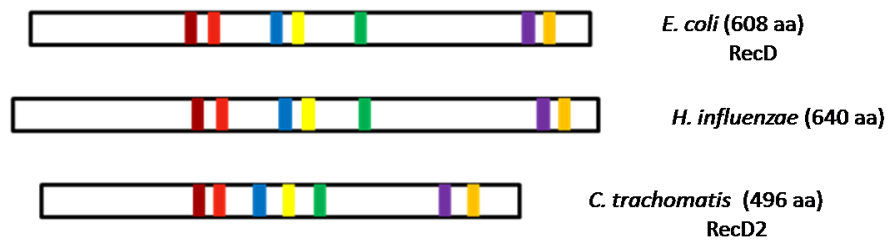
A. Members of RecD Related Proteins
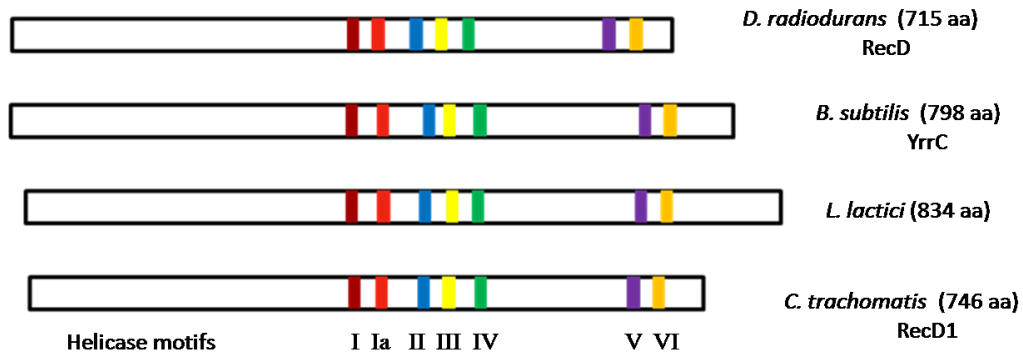
Figure 1.7 Schematic representation of selected RecD Superfamily I helicases, depicting the seven conserved sequences, aligned using motif I. (A) RecD family members from RecBCD enzymes. (B) RecD related family of helicases not associated with RecBCD enzymes, with which *D. radiodurans recD* is grouped. Families are according to TIGR Protein Families database. RecD family is accession number (TIGR01447), RecD related is(TIGR01448). Adapted from Wang, Julin 2004 (15).

RecD homolog from *D.radiodurans* is expressed at the transcriptional level as determined by microarray.   Its transcriptional expression is found to not be up regulated following gamma irradiation (64). This is not necessarily a negative finding, as the RecBCD subunits of *E. coli* are not upregulated after gamma irradiation (65). A proteome experiment using mass spectrometry has revealed the expression of the protein under certain conditions. Gamma irradiation failed to up regulate the level of RecD protein (66). Additionally, the biochemical characterization of this protein was recently

published. The gene was cloned, and expressed in *E. coli* with a C-terminal His-tag for purification over a nickel column. A final purification involving a single stranded DNA column was employed. The purified protein was shown to unwind 20mer DNA duplexes that have either a forked end or 5' overhang, while not unwinding 3' or blunt ended molecules. This is in keeping with *E. coli* RecD subunit which also displays 5' to 3' polarity (4). On the other hand, *Deinococcus* RecD possesses low processivity *in vitro*, contrary to the incredible processivity of the RecBCD complex. *D.radiodurans* RecD unwinds longer substrates (52 and 76 bp) much less efficiently than *E. coli* RecBCD (15).

1.5 Importance of Studying the Biological Function of *D. radiodurans* RecD

The means of resistance to high doses of gamma irradiation in *D.radiodurans* are still poorly understood. Many theories have been proposed however none can completely explain bacterium's extreme radioresistance. It is likely that there are many factors that are crucial to radiation resistance. Notably, efficient DNA repair and resistance to oxidative damage are leading candidates for explaining tolerance of gamma rays. Whether DNA or protein damage is the limiting factor for surviving high doses of irradiation does not decrease the importance of DNA repair in surviving such an assault.

Evolving a means of dealing with a cellular assault that damages many cellular components requires that the organism is adept at minimizing the damage, and repairing or replacing these damaged components. The damage to DNA due to gamma irradiation is extensive. The evolution of resistance to radiation in *D. radiodurans* must have similarly involved the evolution of an extremely efficient DNA repair system. Advances in the understanding of DNA repair in *D. radiodurans* have been made. However some

elements of the process are incompletely characterized or unidentified. *D. radiodurans* RecD helicase has homology to other DNA repair enzymes. It also has biochemical activities that make it suitable for DNA repair. It is therefore important to determine the biological role of RecD in *D.radiodurans*.

1.6 Specific Aims

Extremely efficient DNA repair has been suggested to be a major determinant of radiation resistance in *D. radiodurans*. To test this hypothesis many *D. radiodurans* genes with homology to known DNA repair genes in other more characterized organisms have been studied. One approach is to attempt to increase the radiation resistance of more sensitive organisms by genetically engineering them to express *D. radiodurans* DNA repair genes. Another strategy is to make null mutations of potential DNA repair genes in *D. radiodurans*, and test for sensitivity to DNA damaging agents.

One such potential DNA repair gene in *D. radiodurans* is a gene with homology to the RecD family of helicases. In many bacteria RecD helicases work in conjunction with RecB, RecC and RecA to initiate repair of double strand breaks in DNA via homologous recombination. *D. radiodurans* is missing homologs to *recB* and *recC* but has a *recA* gene. It is therefore unclear how the organism performs the initial steps of double strand break repair. One hypothesis is that the RecD-like protein is somehow involved in the initiation of double strand break repair.

The specific aim of this research is to further understand the biological role of RecD in *D. radiodurans*. To this effect a *recD* null mutation in the organism was generated. In order to assess a role in DNA repair the mutant was tested against a panel

of DNA damaging agents. The mutant was also tested for growth defects and changes in transformation efficiency, as certain helicase mutants seem to be involved in both these processes in bacteria (100, 101, 141). It was also tested for altered cellular and chromosomal structures as found in certain mutants of DNA repair genes in *E. coli* (142).

Recently Zhou et al reported that *D. radiodurans* RecD plays a role in an oxidation resistance pathway (103). They generated a *recD* null mutant by insertional mutagenesis, and found that the mutant is sensitive to hydrogen peroxide, a chemical known to induce oxidative stress. Additionally the mutant displayed a reduction in catalase activity, a way by which cells eliminate hydrogen peroxide. We tested our *recD* mutant for altered catalase activity as found in the *D. radiodurans recD* mutant generated by Zhou et al (103). Lastly the purified protein was immunoprecipitated to determine if functional binding partners could be co-immunoprecipitated that might suggest a physiological role for *D. radiodurans* RecD.

The *recD* mutant was determined to be sensitive to DNA damaging agents including gamma and UV irradiation and hydrogen peroxide, but not to Mitomycin C or Methyl methanesulfonate. It was also found to be hyper-transformable with exogenous DNA. The mutant did not reproducibly demonstrate the extent of catalase activity loss found by Zhou et al (103), but did show marginal loss of catalase activity. Immunoprecipitations failed to identify functional binding partners of RecD. These results suggest a role for RecD in DNA repair in *D. radiodurans*.

Chapter 2 Generation and Phenotyping of *recD* Mutants in *Deinococcus radiodurans*

2.1 Introduction

To determine if a gene is involved in the DNA repair system of an organism, a null mutation of the potential DNA repair gene is often made. The mutant is then tested for sensitivity to a variety of DNA damaging agents. The mutant will be sensitive to DNA damaging agents specific to the DNA repair pathway it is involved, providing the organism does not have a means of compensating for the loss of the gene.

To study the biological function of the RecD protein of *D. radiodurans* a mutation in the *recD* gene was made. As in many organisms, this was accomplished by insertional mutagenesis. In this method a gene is disrupted or excised by the insertion of an antibiotic resistance gene driven by a promoter that is functional in the organism studied. This sequence is then incorporated into the target sequence by homologous recombination.   Insertional mutagenesis has been used to study DNA repair enzymes such as RecA, Q, N, and X in *D. radiodurans* (1, 68-71).

A null mutant was generated (denoted *recD*::*kan*) by modification of a published protocol used to make a *recN* mutant in *D. radiodurans* (1). A *D. radiodurans recD* specific disruption cassette was made by first cloning the gene into an *E .coli* plasmid. Next, the cloned gene's reading frame was disrupted by inserting a kanamycin resistance gene and promoter (*kan*^{r}) into the *recD* sequence. Mutants were selected on TGY plates supplemented with kanamycin. The disrupted gene was PCR amplified and transformed into competent D. radiodurans. In some cells the wild type *recD* gene is replaced with the disrupted *recD* gene by homologous recombination. These cells are selected for using TGY plates containing kanamycin.

The *recD*::*kan* mutant retains portions of the *recD* coding sequence. As a result, it is possible that a portion of the RecD protein could be translated into protein, which could produce unintended effects in the mutant. Also, this portion of the RecD protein could interfere with complementation of the mutation by blocking the complementing RecD protein from performing its function. Furthermore, having a *recD* mutant in another antibiotic resistant background allows *recD* double mutants to be generated with other mutants that are resistant to the same antibiotic used to make the initial *recD* mutation. Therefore, a complete deletion mutant of the *recD* gene called the *ΔrecD*::*cam* mutant was made using chloramphenicol for antibiotic selection.

D. radiodurans has genes ascribed to many characterized DNA repair pathways including DSBR, MMR, NER and BER. Helicases are involved in the first three of these repair pathways, and RecD could be involved in one or more of them. Therefore the *recD* mutant was tested against a panel of DNA damaging agents known to create DNA damage repaired by these pathways. It was also tested for defects in growth, cell structure and segregation of chromosomes as found in mutants that effect RecBCD function in *E. coli* (32). Gamma radiation is known to cause double strand breaks as well as a variety of chemical modifications to DNA. Hydrogen peroxide is also thought to cause single strand breaks and oxidative modifications to DNA through the generation of reactive oxygen species. UV light produces cyclobutyl pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts(13). Methyl methanesulfonate, and Mitomycin-C are known to methlylate and alkylate DNA respectively. Lastly, the possibility of RecD impacting the innate competency of *D. radiodurans* was tested by transformation with chromosomal and plasmid DNA.

2.2 Materials and Methods

2.2.1 Bacterial Strains and Plasmids

The *Deinococcus radiodurans* wild type strains used were R1(ATCC 13939) (74), acquired from the American Type Culture Collection (ATCC), Manassas, Va., and (ATCC BAA-816) (75), a gift from Michael Daly, Uniformed Services of the Health Sciences, Bethesda, Md. *Deinococcus radiodurans* strain LS18 is an uncharacterized streptomycin resistant strain of (ATCC 13939) and was a gift from John Battista (Louisiana State University, Baton Rouge, Louisiana). *Deinococcus radiodurans* strains were grown at 30°C in TGY media (5% tryptone, 1% glucose, 3% yeast extract), shaking at 225 rpm, or on TGY agar (1.5%) plates. Antibiotics used for *D. radiodurans* were kanamycin (8 µg/ml), streptomycin (5 or 50 µg/ml), and chloramphenicol (3 µg/ml). Plates used for maintaining strains contained appropriate antibiotics, plates for sensitivity assays did not contain antibiotics unless otherwise stated. Cells plated on TGY agar plates were incubated at 30°C for three days prior to scoring. Overnight cultures of mutants were grown in TGY with appropriate antibiotics. Cultures for phenotypic analysis were started from 1:10 dilutions of the overnight culture into fresh TGY.

D. radiodurans shuttle plasmids p11530 (76), pRAD1(77), and pI3 (78) were a gift from Mary Lidstrom, University of Washington, Seattle Washington. All three plasmids provide chloramphenicol resistance in *D. radiodurans*, and ampicillin resistance in *E. coli*. For routine cloning, *E. coli* DH5α was used. It was grown in at 37°C in Luria-Bertani (LB) broth or on (LB) plates (1.5% agar). Antibiotics used for *E. coli* were kanamycin (30 µg/ml), ampicillin (100 µg/ml) and chloramphenicol (15 µg/ml). Bacterial strains and plasmids used in this chapter are listed in Table I.

Table I. Bacterial strains and plasmids

Strain	Description	Reference
D. radiodurans R1	ATCC 13939	White et al. (1999)
D. radiodurans BAA-816	ATCC BAA-816	White et al. (1999)
D. radiodurans LS18	ATCC 13939, with uncharacterized streptomycin resistance	Earl, Battista (2002)
recD::*kan* mutant 1	ATCC 13939 with recD kan^r insertional mutation made from a PCR product using plasmid pRecDKanDis and primers RecD1 and RecD2	This study
recD::*kan* mutant 2	ATCC BAA-816 with recD kan^r insertional mutation made from genomic DNA isolated from strain *recD::kan* mutant 1	This Study
recD::*kan* mutant 3	ATCC BAA-816 with recD kan^r insertional mutation made from PCR product using DNA isolated from strain *recD::kan* mutant 2, and primers RecD1 and RecD2	This Study
Δ*cam*::*recD* mutant	*recD::kan* mutant 3 with recD cam^r null mutation made from a PCR product using plasmid pRecDCamDis and primers CamDis1 and CamDis4	This Study
E. Coli DH5α	Strain used for cloning	Invitrogen Corp.

Plasmid	Description	Reference
pDr-RecD.ptz	ptz.19r containing *D. radiodurans* recD	Wang, Julin (2004)
pCR-Blunt	Cloning Vector	Invitrogen Corp.
pRecDKanDis	pDr-RecD.ptz with kan^r inserted into *D. radiodurans* recD	This study
pCR-2.1	Cloning Vector	Invitrogen Corp.
p11530	Shuttle vector for *D. radiodurans* and *E. coli*	Lecointe et al. (2004)
pCR-*cam*	pCR-2.1 with *cam*^{r} cloned from p11530	This study
pRecDCamDis	pCR- pCR-*cam*^{r} , with *cam*^{r} flanked by 500bp sequence up and down stream of *recD*	This study
pRAD1	Shuttle vector for *D. radiodurans* and *E.coli*	Meima et al. (2000)
pRAD1-*recD*	pRAD1 with *D. radiodurans* recD and 948 bp upstream of the *recD* sequence	This Study
pI3	Shuttle vector for *D. radiodurans* and *E.coli*	Lindler et al. (1998)

2.2.2   Molecular Biological Techniques

Chromosomal DNA from *D. radiodurans* was purified according to Earl and Battista, 2002 (79). A five ml culture of our wild type strain was grown overnight at 37°C. The culture was spun down into 2.0 ml centrifuge tubes by repeatedly centrifuging at $10,000 \times G$ for 30 seconds, and removing the supernatant. The cell pellet was resuspended with 1.0 ml 95% ethanol, and incubated at room temperature for 10 min to remove the outer membrane. Cell ghosts were gently pelleted again at $3,000 \times G$ for 15 min and the ethanol was pipeted away. The husks were then resuspended in 1.0 ml TE buffer (10 mM Tris, 0.1mM EDTA pH-8.0), with 250 µg/ml lysozyme (Sigma-Aldrich), and incubated at 37°C for 30 min. The cell lysate was then supplemented with 80 µl of 10% SDS and 8.0 µl of a 20 mg/ml stock solution of proteinase K (Fermentas) and incubated at 57°C for 4 hours. The lysate was extracted three times with phenol:chloroform: isoamyl alcohol 25:24:1 (Sigma-Aldrich). To precipitate the DNA, three volumes of 100% ethanol and one tenth volume of 3.0 M Sodium Acetate pH 7.0 were added and incubated at -20°C overnight. The next day the tube was spun for 30 min at $14,000 \times G$ at 0°C in a refrigerated microcentrifuge tube. The pellet was then washed three times with 1.0 ml of ice cold 70% ethanol to wash away the remaining salt. After the final wash was removed, the pellet was allowed to air dry, and then resuspended in 300 µl TE (10mM Tris, 1mM EDTA).

Competent *D. radiodurans* cells were made from an adaptation of a procedure found by Masters *et al*. (80). An overnight culture was used to seed a 3.0 ml TGY culture at a 1:10 dilution. This daughter culture was grown for about 3 hrs until an OD_{600} ~ 0.5 was achieved. Cells were then spun down and resuspended in 100 µl of 65% TGY,

25% 100mM CaCl$_2$, 10% glycerol, and stored at -80°C. On the day of transformation, the cells were thawed on ice. Transforming DNA was added to the tube of cells, mixed by flicking and incubated on ice for 10 min. Subsequently the cells were incubated shaking for 30 min at 30°C. One ml of fresh TGY was then added to the tube, mixed, and transferred to a new culture tube. The cells were allowed to recover 18 hours and plated the following morning on TGY plates containing 1.5% bacto-agar supplemented with appropriate antibiotics. Three days later, plates were checked for transformants.

PCR reactions were carried out in 50 µl reactions using 0.5 µM of each primer, and each 200 µM of each dNTP. Template DNA concentrations were 100 ng for genomic DNA and 0.1 ng for plasmid DNA. Pfu polymerase (Stratagene) was used with supplied buffer according to manufacturer's protocol unless otherwise specified. Thermocycling was performed using an Ericomp Powerblock 2 thermocycler. Cycling conditions were as follows.   Cycle 1 had one step, 96°C for 4 min initial denaturation. Cycle 2 was repeated 30 times; step 1, denaturation (96°C for 1 min); step 2, annealing (60 °C 1min); step 3, extension (72 °C for 6 min). Cycle 3 had 1 step (72 °C for 6 min). Primers used in this study are listed in table II.

To analyze and purify DNA, fragments were run on 0.9% agarose (Invitrogen) gels supplemented with 1 µg/ml ethidium bromide (Sigma). The gels were visualized using a UV transilluminator (UVP inc.) and photographed using a Polaroid camera, with Polaroid instant film. Bands were excised with a fresh razor blade and purified using the Qiagen Qiaquick Gel Extraction kit. DNA concentration was assessed using a UV Spectrophotometer (Varian). Digestions were performed using New England Biolabs restriction enzymes according to package instructions. The vector and insert were ligated

together in a 1:1 ratio using T4 DNA ligase (Fermentas) overnight at 16°C. Ligation

mixtures were transformed into electrocompetent *E. coli* strain DH5α using a 0.1 mM gap

length electroporation cuvette (BioRad), and electroporated at 1700 volts in an eppendorf

electroporator (model 2510).

Table II. Primers used in this study

Prom1
5'-ACATAATCCACCTTCCGCG-3'

*Sac*II
↓
KanRev
5'-GCGGGACAAAAGTCCGCACAGATGCGTAAGGAG-3'

*Psh*AI
↓
KanFor
5'-GCAACCGCGGACAGCAAGCGAACCGG-3'

*Eco*RI   *Nde*I
↓   ↓
RecD1
5'-CGCGGAATTCATATGTCTGCTGCCCCTGCC-3'
— Start

*Bam*HI
↓
RecD2
5'-AGTGGGATCCCTGACAGAACTCTTAAGGCGTCTTAATG-3'
Stop

*Sac*I
↓
RecDUpStr
5'-AAGAGCTCTGAAGCTGCCCCTGCAACTGC-3'

CamFor
5'-AGATCAGTCTACTCTCCCTCATGGTCCTCG-3'

CamRev
5'-AGATCAGACCGAAGTCCCGACGACCGGGTCG-3'

*Hind*III
↓
CamDis1
5'-TAGCTAAAGCTTCCGCGAATGTCAGCAGCG-3'

*Bam*HI
↓
CamDis2
5'-ACTAGTGGATCCCTGGACCGAGTTAAGTCACG-3'

*Xho*I
↓
CamDis3
5'-TAGCTACTCGAGCCCCAGGAACTTCTGTCG-3'

*Nsi*I
↓
CamDis4
5'-ATCAGTATGCATGCCCGCCTGTTCCAGC-3'

2.2.3   Construction of the *Deinococcus radiodurans recD*::*kan* mutant by Insertional

Mutagenesis

A null mutant was generated by insertional mutagenesis using a kanamycin

resistance gene for selection. The disruption cassette was made by first cloning the *D.*

radiodurans recD gene. This was accomplished previously by Jianlei Wang. Primers

RecD1 and RecD2 were used to clone the *recD* gene into pTZ19r generating a 5 kb

plasmid designated pDr-RecD.ptz (81). In the *recD* coding sequence there is a *PshA*I site

that is 589 bp from the start of translation, and a *Sac*II site that is 637 bp from the start of

translation. These two restriction sites were employed to clone a kanamycin resistance

gene with promoter (*kan*^r) into the *recD* sequence in the opposite orientation. To obtain

the *kan*^r DNA fragment, PCR was performed using primer set KanFor and KanRev on

plasmid pCR-Blunt (Invitrogen Corp.). Plasmid pCR-Blunt contains a *kan*^r that had been

proven in our lab to function in *D. radiodurans*. Primer KanFor contains a 5'-terminal

*Sac*II site and anneals just upstream of *kan*^r in pCR-Blunt. Primer KanRev contains a 5'-

terminal *PshA*I site and anneals shortly after the stop codon for *kan*^r in pCR-Blunt. The

resulting plasmid designated pRecDKanDis, has a *recD* coding sequence that reads

correctly for 588 bp, but then encounters a TAA stop codon 18 bp into *kan*^r sequence.

Verification of the plasmid was performed by double digestion using *Sac*II and *PshA*I.

PCR using primers RecD1 and KanFor were used to determine the orientation of the

insertion. PCR using primers RecD1 and RecD2 generated a linear fragment used to

transform *D. radiodurans*. Hundreds of transformants were obtained when 100 µl of the

transformation mixture was plated on TGY kanamycin plates. A double cross over event

produced the *recD*::*kan* mutant (figure 2.1).
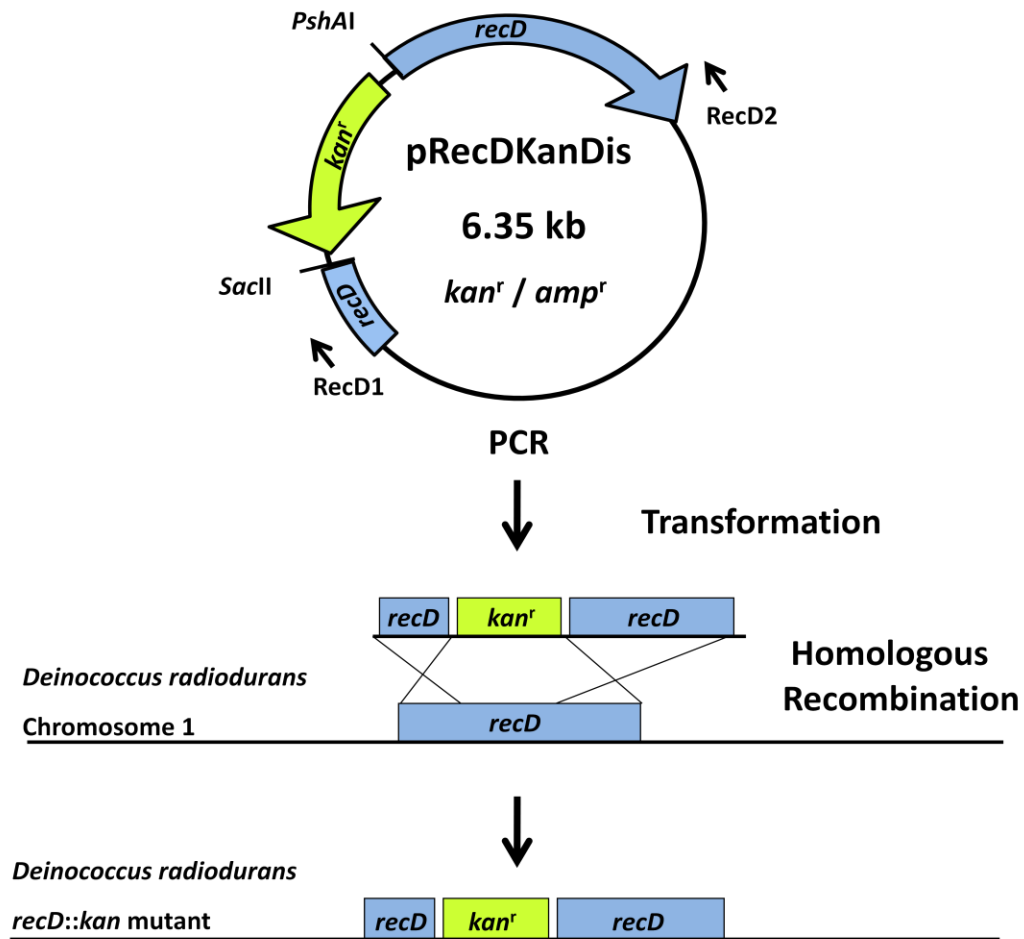
Figure 2.1 Schematic representation of the Generation of *D. radiodurans recD*::*kan* mutant. Primers KanFor and KanRev were used to amplify *kan*[r] from plasmid pCRBlunt. Restriction sites *PshA*I and *Sac*II were included at the ends of the PCR product and were used to clone the gene into plasmid pDr-recD.ptz, assembling plasmid pRecDKanDis. Primers RecD1 and RecD2 were used to amplify the disruption cassette which was transformed into competent *D. radiodurans*. A double cross over event results in a disrupted copy of the *recD* gene.

Several versions of the *recD*::*kan* mutation were made in an effort to confirm that the phenotypes detected were a result of the *recD* disruption and not the result of an additional random mutation. The second version of the mutation was made by transforming the wild type strain BAA-816 (75), with genomic DNA from *recD*::*kan* mutant 1, resulting in a few dozen transformants per 100 µl of transformation mixture. This second strain was designated *recD*::*kan* mutant 2. The genomic DNA from the second mutant was extracted and used as template DNA for PCR using primers recD1 and recD2. The 3,000 bp fragment was gel purified and used to transform the wild type strain BAA-816 again, resulting in hundreds of transformants from 100 µl of transformation mixture. It was designated *recD*::*kan* mutant 3. The first two kanamycin mutants were genotyped by PCR. The *recD*::*kan* mutant 3 was the only kanamycin resistant mutant genotyped by Southern blot. Southern blot is a better method of genotyping than PCR. PCR amplifies and identifies certain sequences of DNA, and is influenced by factors such as copy number of a target sequence. This means that low copy numbers of wild type copies of the gene could go undetected.   Southern blot allows the entire genome of a strain to be examined increasing the likelihood of detecting the wild type gene. According to the DIG probe detection kit one copy of the wild type gene per cell could be detected in the samples probed in this study.

2.2.4   Construction of the *D. radiodurans* Δ*recD*::*cam* mutant by Insertional Mutagenesis

A deletion mutant of the *recD* gene in *D. radiodurans* was made by insertional mutagenesis using a chloramphenicol resistance gene, to determine if complete removal

of the gene altered the phenotypes found in the first mutant. This was done by generating

a plasmid containing a resistance cassette flanked on either side by DNA upstream and

downstream of the *recD* sequence. The disruption sequence was PCR amplified and

transformed into *D. radiodurans* strain *recD*::*kan* mutant 3. A chloramphenicol

resistance gene and promoter (*cam*[r]) from *D. radiodurans* shuttle vector p11530 (76) was

PCR amplified using primers CamFor and CamRev, using *Taq* DNA polymerase

(Fermentas) so the PCR product would be compatible (having 3'-A overhangs) with the

TA cloning vector (Invitrogen Corp.). The kit was used as per package instructions and

the reaction conditions and thermocycling conditions were the same as described

previously. The PCR product was gel purified and ligated into the TA cloning vector

pCR2.1 (Invitrogen Corp.) using kanamycin and chloramphenicol for selection. The

orientation of the insert was determined by digestion with *Xho*I, which cuts in the

plasmid close to the site of insertion, and *Acc*I, which cuts near the 5' end of the

resistance gene. The resulting plasmid was designated pCR2.1-*cam*.

A 558 bp section of *D. radiodurans* chromosome 1 DNA upstream of the *recD*

gene was PCR amplified. Primers CamDis1 (containing a *Hind*III site), and CamDis2

(containing a *BamH*I) site were used. A 662 bp section of *D. radiodurans* chromosome 1

DNA upstream of the *recD* gene was PCR amplified using primers CamDis2 (containing

an *Xho*I) site and CamDis4 (containing an *Nsi*I site). Plasmid pCR2.1-*cam* contains a

*Hind*III and *BamH*I site just before the *cam*[r] sequence, and *Xho*I and *Nsi*I sites just after

it. The PCR products were double digested with the appropriate enzymes and cloned into

plasmid pCR2.1-*cam*. The resulting plasmid was designated pRecDCamDis.

PCR on plasmid pRecDCamDis using primers CamDis1 and CamDis4 produced

the linear fragment used to generate *D. radiodurans* strain Δ*recD*::*cam* mutant. The

fragment was transformed into the *recD*::*kan* mutant 3. A double cross over event

removed the *kan*[r] and remaining *recD* sequence. The transformation generated hundreds

of transformants from 100 μl of transformation mixture. One transformant was

genotyped by Southern Blot and used for phenotypic analysis.
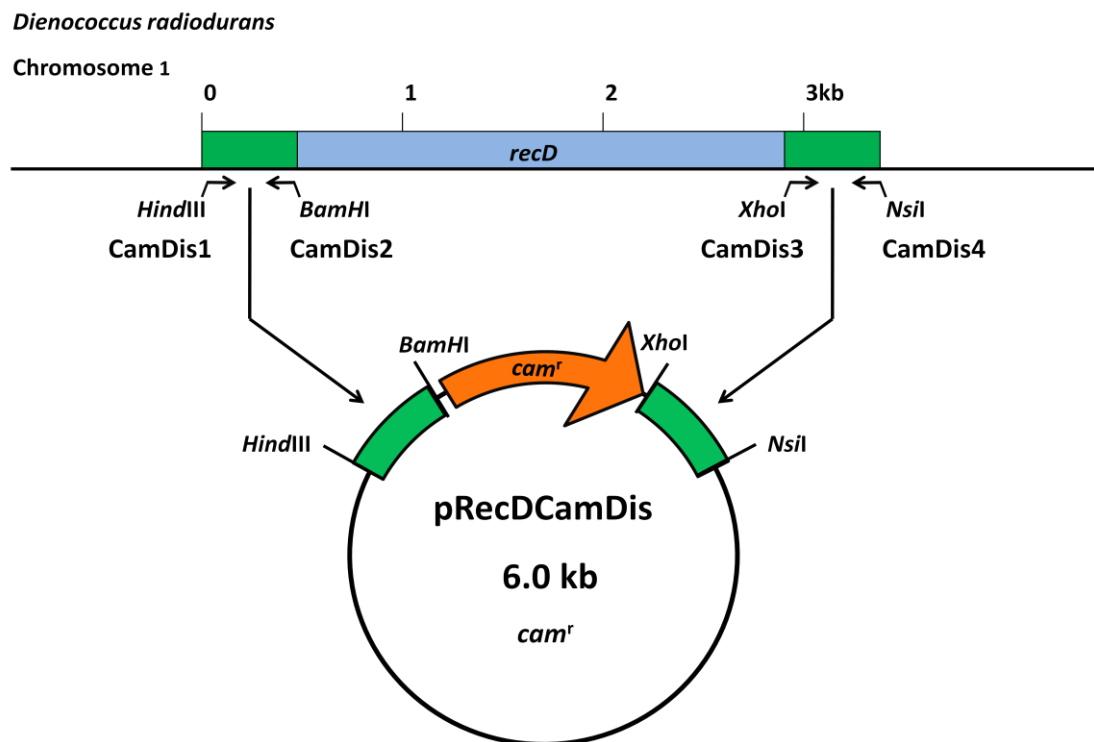
Figure 2.2 Schematic representation of the construction of pRecDCamDis. PCR
using primer pair CamDis1 and CamDis2, and primer pair CamDis3 and CamDis4 were
used to amplify approximately 600 bp upstream and downstream of the *D. radiodurans*
recD gene. These fragments were successively ligated into plasmid pCR2.1-*cam*.

2.2.5 Genotyping *D. radiodurans* Mutants by PCR and Southern Blotting

D. radiodurans recD* mutants were genotyped to assess the mutations. The

recD::*kan* mutants were genotyped by PCR using primer pair Prom 1 and RecD2, and

primer pair Prom1 and KanFor. Reactions were visualized on 0.9 % agarose gels.

Primer Prom1 is outside of the sequence used to construct the mutant and should only

amplify the disruption sequence if the mutation occurred in the correct location and

orientation. PCR amplifies a target sequence of DNA. It therefore it does not provide a

complete picture of all DNA sequences present in a sample. Due to the fact that

individual *D.radiodurans* cells have multiple copies of its genome, Southern blots were

performed as they provide a means of directly examining all the DNA of a purified

genomic sample. According to the instructions provided in kit used for the Southern blot,

one copy of the wild type gene per cell could be detected under the condition used.

Southern blots were performed using a method developed by Roche. The PCR DIG

probe synthesis kit (cat. # 11 636 090 910), was used to make the probe, and the DIG

luminescent detection kit (cat. # 11 363 514 910) was used to develop the blot. A probe

complementary to a sequence of interest is generated by PCR using a dNTP mix which

includes a derivative of dUTP, called DIG-11-dUTP which includes a digoxygenin

steroid ring. Genomic DNA from strains to be tested was digested with appropriate

restriction endonucleases and electrophoresed on an agarose gel. The DNA was

transferred to a reinforced nitrocellulose membrane by the capillary method. The

membrane is blotted with a solution containing nonspecific blocking DNA and the DIG

labeled probe. The probe hybridizes with the target DNA on the membrane and is

visualized immunologically, using an anti-digoxygenin antibody conjugated with alkaline

phosphatase. This antibody binds to the DIG-11-dUTP found in the probe. The

membrane was then incubated with a solution containing the chemiluminescent substrate

CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricycle[3.3.1.1^{3,7}]-decan}-4-yl)phenyl phosphate).

. The alkaline phosphatase conjugated to the antibody cleaves the CSPD, emitting a light wave with a maximum wavelength of 477 nm, which was imaged using a STORM phosphorimager (GE Healthcare Life Sciences).

Three probes were synthesized, one complementary to the full length *recD* gene using primers RecD1 and RecD2, one to *kan*[r] using primers KanFor and KanRev, and one to *cam*[r] using primers CamFor and CamRev. To produce a probe by this method, two PCR reactions are carried out. One reaction included DIG-11-UTP and was used to synthesize the actual probe, while the other was used as a control to ensure the PCR reaction was successful. The PCR reactions for probe synthesis were 50 µl total volume including, 1 pg template DNA, 0.5 µM of each primer. Components from the kit in the reaction were 1 x PCR buffer with MgCl$_2$, 2.6 units of expand high fidelity enzyme mix, and 100 µM each of dATP, dCTP, dGTP, and 65 µM dTTP and 35 µM DIG-11-dUTP. The thermocycling conditions were the same as previously described. The control reaction was run under the same conditions except 200 µM of dNTPs, and no DIG-11-dUTP was used. As expected the probe bands migrated slightly slower than their unlabelled counterparts due to the additional steroid moiety, but are close in size. In initial experiments, the probe bands were gel purified, but this was later determined to be unnecessary. Probes were stored frozen at -80°C until needed.

Genomic DNA was isolated from *D. radiodurans* wild type strain BAA-816, and *recD*::*kan* mutant 3, and Δ*recD::cam* mutant cells. Five ng of purified DNA was digested individually with three different restriction endonucleases and one pair of

restriction endonucleases. The enzymes used singly were *Eag*I, *EcoR*I, *Apa*I, and the double digestion was *Nco*I and *Pst*I. The digestions were loaded and separated on a 0.9% agarose gel without ethidium bromide. The gel was run at 60 volts for 1.5 hrs and visualized as above (Fig. 2.8). The gel was photographed next to a ruler as the molecular weight markers used were not designed to be visualized by the DIG detection kit. Therefore the molecular weight of the bands on the membrane was estimated based on the distance they traveled down the gel compared to that of the markers indirectly. This was accomplished by measuring the distance the bands travelled on the blot from the top of the membrane (aligned with the wells on the gel during transfer) and estimating the bands in the molecular weight marker from the picture of the gel.

The digested DNA was transferred to a reinforced nitrocellulose membrane, Optitran BA-S 85, pore size 0.45 um (Whatman, Schleicher & Schuell #10439251), according to the instructions. The gel was incubated for forty min in 0.25 M HCl to depurinate the DNA. The gel was then rinsed 2 times in distilled water followed by two 20 min incubations in denaturation buffer (1.5 M NaCl, 0.5 M NaOH). Finally the gel was soaked in neutralization buffer (3 M NaCl, 0.5 M Tris pH = 6.8) for 30 min. The transfer was performed in $10 \times$ SSC, composed of 1.5M Tris, 4.4% Sodium Citrate, pH 7.0. To construct the transfer apparatus, a pyrex baking dish was filled ¾ full with $10 \times$ SSC. A plastic support wider than the gel was laid across the top of the dish, covering the middle third of open space. A sheet of Whatman paper soaked in $10 \times$ SSC, cut one inch shorter than the width of the dish and one inch shorter than the length of the dish was laid across the plastic support so that both ends of the paper hang down into $10 \times$ SSC in the dish on either side of the support. The gel was prepared with 0.5 cm of

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additional space surrounding the pertinent portion of the gel, removing the wells. The gel

was then placed upside down on the center of the Whatman paper on the transfer rig.

Plastic wrap is carefully laid over the four edges of the gel covering the 0.5 cm of

extraneous gel and forming a barrier covering the baking dish leaving only the portion of

the gel to be transferred exposed. A sheet of Optitran membrane cut to the size of the gel

with the upper right corner nicked and soaked in $10 \times SSC$, was laid over the gel. Care

was to insure that bubbles were not trapped between the gel and the membrane. Two

pieces of filter paper cut to the size of the gel and soaked in $10 \times SSC$ were place on top

of the membrane again removing any trapped bubbles. A six inch stack of paper towels

cut to the size of the gel was placed on the two sheets of filter paper. A plastic support

slightly larger than the gel was placed on the paper towels. Lab tape was attached from

all four corners of the dish to all four corners of the top plastic support. Last of all a

small aluminum heating block was taped to on top of the plastic support to keep pressure

on the stack. The rig is allowed to sit overnight and acts as a wick pulling the SSC and

DNA from the dish up into the paper towels pulling, which clings to the membrane.

The next morning the gel was stained with ethidium bromide as previously

described to determine if the transfer was complete. Then the membrane was baked

DNA side up (the nick is now in the upper left hand corner) in an oven for 2 hrs at $80^{\circ}C$

on a piece of Whatman paper. The membrane was then blocked for an hour at $42°C$ in 20

ml pre-hybridization buffer in a sealed sandwich bag. The pre-hybridization buffer was

composed of 50% deionized formamide, 0.5% SDS, 0.8 M NaCl, 20mM PIPES, pH 6.5,

supplemented with 100 µg/ml sonicated salmon sperm that had been boiled for 10 min

(and rapidly cooled on ice). Hybridization was carried out in 20 ml of pre-hybridization

buffer supplemented with 40 µl of probe, overnight at 42°C. The pre-hybridization

buffer with probe was saved in a -20°C freezer for reuse. The blots were transferred to

glass staining dishes containing 100 ml of room temperature low stringency buffer (2 ×

SSC, 0.1 % SDS) shaking for 10 min. The blots were then washed in two changes of

high stringency buffer (0.1 % SSC, 0.1% SDS) shaking for 15 min at 55°C. At this point

the blots were ready for immuno-detection. Blots were rinsed briefly with 50 ml wash

buffer (0.15M NaCl, 0.3% Tween 20, 0.1M Maleic acid pH 7.5). Blots were then

transferred to 25 ml blocking buffer which was the wash buffer with 1 × blocking

solution provided in the kit. After blots were agitated at room temperature in blocking

buffer for 30 min, 2.5µl of the anti-DIG antibody was added, and the blots agitated for

another hour. The blots were then washed three times in washing buffer, and one time in

developing buffer (0.1M NaCl, and 0.1M Tris pH 9.0). To develop, blots were overlaid

with 2.0 ml of developing buffer plus 20 µl CSPD, and left in a light proof box for 5 min.

The blots were then imaged using a STORM phosphorimager.

2.2.6 Growth Assay

Overnight cultures of wild type and *recD*::*kan* mutant 1 were diluted 1:200 into a

fresh culture flask without antibiotics. Cells were grown under standard conditions for a

total of 26 hours. Cell density was monitored spectrophotometrically at OD_{650} using a

Perkin-Elmer Lambda Bio spectrophotometer and a 1-cm-path-length quartz cuvette

every 2 hours. Aliquots of the culture were plated after dilution if necessary to ensure

optical density reflected viable cell numbers. Verification of the strains was performed

by PCR as described previously from genomic DNA prepared from the culture after the last optical density was obtained.

2.2.7 Mitomycin C Sensitivity Assays

Wild type BAA-816 and *recD*::*kan* mutant 3 were grown to mid log phase, OD_{600} of 0.5. A sample was serially diluted in triplicate, and 100 µl of cells were plated on TGY plates as a control for initial viable cells. The remaining culture was exposed to 10 µg/ml of MMC, shaking at 30°C. At various time points, one ml of the culture was pulled, spun down for one min (16,000 × G) and resuspended in one ml of fresh TGY, and serially diluted and plated as in the control. Dilutions were as follows; 1:5, 1:25, 1:250, 1:2,500, 1:25,000 and 1:50,000; the last of which was the control dilution.

2.2.8 Methy Methanesulfonate Sensitivity Assay

Wild type BAA-816 and *recD*::*kan* mutant 3 were grown to mid log phase, OD_{600} of 0.5. A sample was serially diluted in triplicate, and 100 µl of cells were plated on TGY plates as a control for initial viable cells. The remaining culture was exposed to 30 mM MMS with shaking at 30°C. At various time points, 350 µl was pulled, spun down for one min (16,000 × G), resuspended in the same volume of TGY, serially diluted and plated as in the control. Dilutions were the same as in the MMC sensitivity assay.

2.2.9 Gamma Irradiation Sensitivity Assays

Wild type BAA-816, *recD*::*kan* mutant 3, and Δ*cam*::*recD* mutant were grown to mid log phase, OD_{600} of 0.5. A sample was pulled and serially diluted in triplicate, and 100 µl of cells were plated on TGY plates as a control for initial viable cells. The cells were then exposed to gamma irradiation at a dose rate of 60 Gy/min, on ice in 1ml centrifuge tubes from a ^{60}Co source (Neutron Products, model 200324). Post irradiation, the cells were diluted in triplicate and 100 µl of cells were plated on TGY plates. Dilutions were as follows; 1:10, 1:25, 1:250, 1:2,500, 1:25,000 and 1:50,000; the last of which was the control dilution.

2.2.10 Hydrogen Peroxide Sensitivity Assays

Hydrogen peroxide sensitivities were performed using two methods. In the first, *D. radiodurans* strains wild type BAA-816 and *recD*::*kan* mutant 3 were grown to mid log phase, OD_{600} of 0.5. A sample was serially diluted in triplicate, and 100 µl was plated on TGY plates to find the initial cell number. The culture was split into separate tubes, three for each concentration of hydrogen peroxide used. Hydrogen peroxide was added, and the cells were incubated at 30°C without shaking. After one hour, the cells were diluted as in the control and plated. Dilutions were as in the gamma irradiation sensitivity.   Alternatively, *D. radiodurans* strains wild type BAA-816 and *recD*::*kan* mutant 3 were grown to mid log phase, OD_{600} of 0.5, and then pelleted ($5,000 \times G$ for 10 min). The cell pellet was then resuspended in an equivalent volume of ice cold PBS (20 mM $Na(PO_4)$, 150 mM NaCl, pH 7.4). A sample was pulled and serially diluted in triplicate, and 100 µl was on TGY plates to ascertain initial cell number. The remaining

cells were aliquoted into separate tubes for different hydrogen peroxide doses. Hydrogen

peroxide was added and the cells were incubated on ice for one hour. The tubes were

then treated with 1,000 units of catalase/ml (Sigma) on ice for 30 min to destroy the

remaining hydrogen peroxide, then serially diluted and plated in triplicate as in the first

method.

2.2.11 UV Irradiation Sensitivity

D. radiodurans strains, type BAA-816, *recD*::*kan* mutant 3, and Δ*cam*::*recD*

mutant were grown to mid log phase, OD_{600} of 0.5. The cells were then diluted in

triplicate and 50 µl was plated on TGY plates and allowed to dry for one min. Dilutions

were the same as in MMC sensitivity assay. The plates were then exposed to UV light

from a 15 watt germicidal lamp (FG15 T8, Fisher) at a rate of 90 J/m^2/min, measured

with a UVX radiometer (UVP, Upland, Ca.).

2.2.12 UV Irradiation Sensitivity Complementation Assay

The *D. radiodurans recD* gene, along with 948bp of DNA upstream of the RecD

start codon, was PCR amplified from genomic wild-type DNA using primer pair

RecDUpStrSacI and RecD2, which contain *Sac*I and *BamH*I restriction sites respectively.

The fragment was gel purified and ligated into *Sac*I/*BamH*I digested pRAD1 to make

pRAD1-*recD*. Shuttle vector pRAD1 replicates autonomously in *D. radiodurans* and

confers chloramphenicol resistance. The *recD* gene and upstream DNA inserted into this

plasmid were sequenced. No differences from the sequence in GenBank (accession

number AE000513) were found. The pRAD1-*recD* plasmid was transformed into *D.*

radiodurans cells *recD*::*kan* mutant 3 strain as described above, and transformants were selected for their ability to grow on chloramphenicol and kanamycin.

Two complementation strains were initially generated by independently transforming the mutant strain with plasmid pRAD1-*recD*. The plasmids used for these strains were generated from separate *E. coli* DH5α colonies from the plate originally used to select for the ligation that constructed pRAD1-*recD*. Colonies 1 and 3 were selected for plasmid purification and designated pRAD1-*recD*-1 and pRAD1-*recD*-3. The two pRAD1-*recD* transformed *recD*::*kan* mutant 3 strains were designated *recD*::*kan* mutant 3-(pRAD1-*recD*-1) and *recD*::*kan* mutant 3-(pRAD1-*recD*-3). Master plates from the selected clones of the initial transformation of *recD*::*kan* mutant 3 were made. These strains were designated *recD*::*kan* mutant 3 (pRAD1-*recD*-1)-MP, and *recD*::*kan* 3 (pRAD1-*recD*-3)-MP. Plasmids pRAD1-*recD* 1 and 3 were retransformed into *recD*::*kan* mutant 3 and were designated *recD*::*kan* mutant 3 (pRAD1-recD-1)-FT, and *recD*::*kan* mutant 3 (pRAD1-*recD*-3)-FT. Sensitivity of the *recD*::*kan* mutant 3 (pRAD1-*recD*) strains to UV irradiation was tested as described above.

2.2.13 Gross Cellular and Nucleoid Morphology Visualized by Microscopy

The *recD* mutant was tested for defects in cellular and chromosomal morphology, as certain DNA repair gene mutants display defects in these aspects (142). Cells were grown to OD_{600} 0.5 for mid log phase, and OD_{600} 2.0 for stationary phase under standard conditions. Cells were stained with Ethidium Bromide (25 µg/ml) and visualized on an Olympus IX51 Inverted Florescent Microscope using phase contrast. An oil immersion objective lens (100 ×) was used for phase contrast. A DAPI filter was used for

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fluorescence microscopy, excitation wavelength (350), emission wavelength (470 nm).

Images were taken with a Cooke digital camera.

2.2.14 Assays to Determine Transformation Efficiency

Transforming DNA was purified from the following sources. *D. radiodurans* strain LS18 is an uncharacterized streptomycin resistant mutant that was isolated by selection of strain R1 on streptomycin TGY plates. Genomic DNA from strain LS18 for was purified as previously described for other *D. radiodurans* strains. Plasmid pI3 DNA, a shuttle vector known to replicate autonomously in *D. radiodurans*, was purified by minipreparation (Qiagen) from *E. coli* DH5α according to manufacturer protocol.

Transformation experiments were done using two protocols. In the first protocol, wild type strain BAA-816 and strain *recD*::*kan* mutant 3 cells were grown to mid-log phase (OD_{600} 0.5), spun down and resuspended at 6×10^9 CFU/ml in 100 µl of TGY medium with 25 mM $CaCl_2$ and 10% glycerol, and frozen at -80°C. For each experiment, cells were thawed on ice and mixed with transforming DNA (2 µg of LS18 genomic DNA, or pI3 plasmid DNA), and incubated on ice for 10 minutes. Cells were then agitated at 30°C for 30 min. One ml of fresh TGY was added to the cells and transferred to a culture tube. Pre experimental controls for viability were plated, 100µl at a 1:10,000,000 dilution. The remaining transformation mixture was allowed to recover for 18 hrs shaking at 30°C (80). Transformed cells were diluted 1:2,500,000 and 100 µl of cells were spread onto TGY plates to ascertain viable cell numbers from this time point. To determine the number of transformants, 100 µl of the transformation mixture was plated undiluted, or diluted 1:10 onto TGY plates with streptomycin (5, or 50 µg/ml),

or chloramphenicol (3 µg/ml). Plates were kept at 30°C and scored after 2 to 3 days.

Transformation efficiency was calculated as transformants/µg DNA/viable cell number.

Alternatively, competent cells were made by a protocol used by Earl and Battista

(79). Strains, wild type BAA-816 and strain $recD$::kan mutant 3 were grown in 2.0 ml

TGY broth to early log phase (OD_{600} of 0.2), from a 1:20 dilution of an overnight culture.

$CaCl_2$ was added to the culture to a final concentration of 30 mM, and the culture was

agitated at 30°C for 80 min. The culture reached an OD_{600} of 0.4, and 500 µl of cells

were transferred to a new culture tube and placed on ice. The cells were used undiluted

at a concentration of about 1.4 x 10^8 CFU/ml as the high cell density. For the low cell

density they were diluted 1 to 25 for a final cell density of 5.5 x 10^6. Competent cells

were mixed with LS18 genomic DNA (10 µg/ml in the transformation mixture) and kept

on ice for 30 min.   Cells were agitated at 30°C for 18 hours. Controls were plated, 100

µl at a 1:2,500,000 dilution. Transformants were assessed by plating 100 µl of undiluted

transformation mixture, and 100 µl of serial dilutions, 1:5, 1:25, 1:125, and 1:625.

2.3 Results

2.3.1 Generation of the $recD$::kan Mutant

To study the biological function of RecD in *D.radiodurans*, a null mutation was

made in the $recD$ gene by insertional mutagenesis. Disruption of the gene was made in a

plasmid in which the $recD$ gene had been cloned. The purification of this plasmid from

Jianlei Wang's freezer stock of pDr-recD.ptz resulted in 3 µg of purified plasmid. The

PCR to amplify the kan^r sequence produced multiple bands, with a major band at the

expected mobility of approximately 1kb. The gel purified PCR product resulted in 2 µg

DNA. The digestion of pDr-recD.ptz appears to have gone to completion, producing a strong band just below the 6 kb marker. The PCR digestion produced two bands. The more intense band migrated with the 1 kb marker and was predicted to be the *kan*^{r} sequence. The double digests of the plasmid and PCR products were gel purified and ligated together to assemble plasmid pRecDKanDis. Verification of the insertion was done by double digestion using *Sac*II and *Psh*AI (Fig. 2.3 B).

PCR was used to verify the orientation of the insert. PCR using primers KanFor and RecD1 resulted in a DNA fragment of approximately 1.5 kb indicating the insertion occurred as designed (figure 2.3 C, lane 1). PCR using primers RecD1 and RecD2 and template DNA pRecDKanDis was performed to form the linear disruption fragment used to transform wild type *D. radiodurans* to *recD*::*kan* mutant. The reaction produced a weak band of expected mobility (Fig. 2.3 C, lane 2). The transformation of *D. radiodurans* strain R1 to *recD*::*kan* mutant resulted in a few hundred kanamycin resistant colonies from plating 100 µl of the transformation mixture on a kanamycin TGY plate.
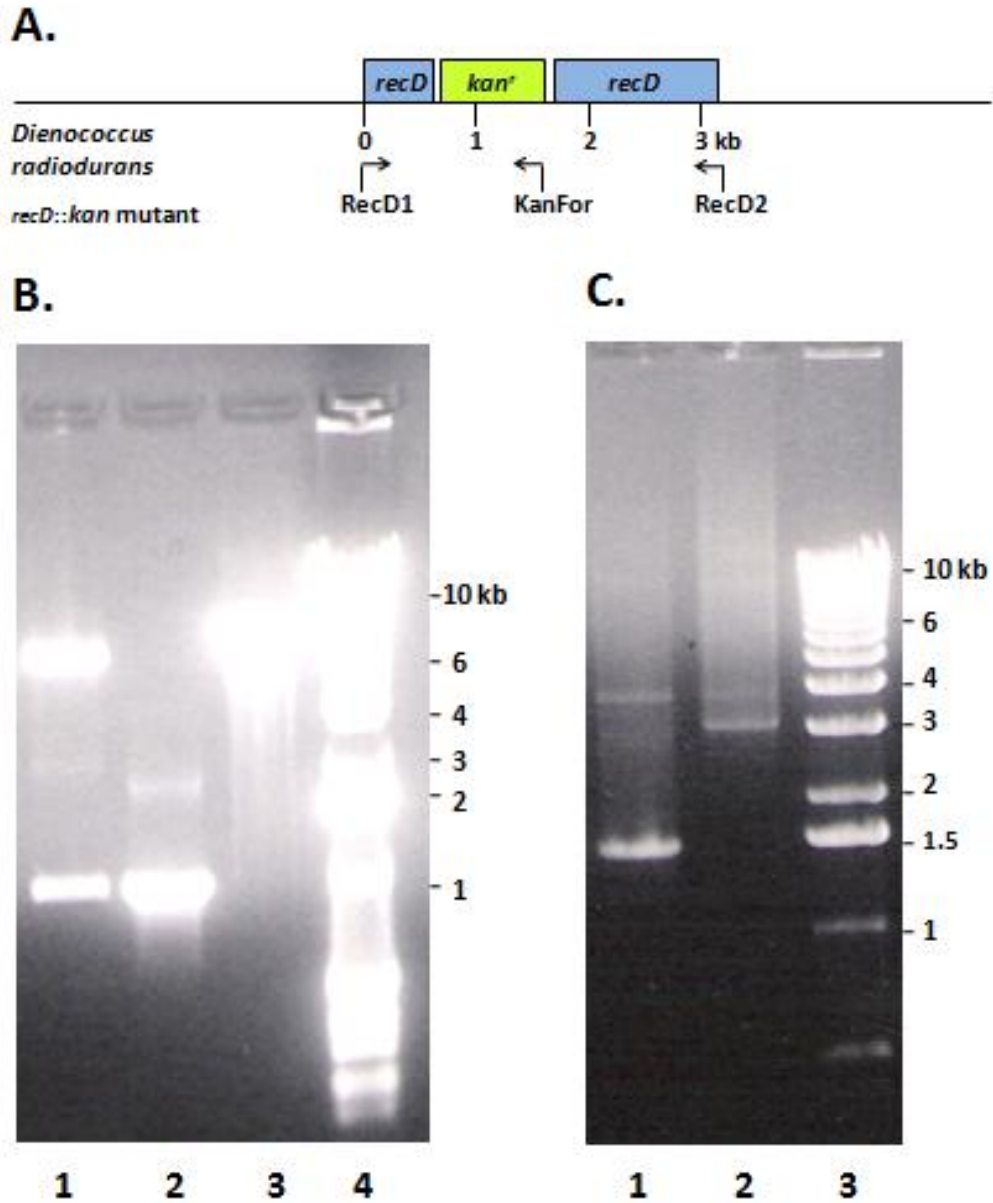
Figure 2.3 Generation and verification of pRecDKanDis, and linear DNA fragment used to make *recD*::*kan* mutant 1. (A) Schematic of the *recD*::*kan* mutation. (B) Image of 0.9% agarose gel of *Sac*II and *Psh*AI double digestions. Plasmid pRecDKanDis (lane 1); *kan*[r] PCR product (lane2); pDr-recD.ptz, (lane3). Fermentas 1 kb ladder (lane4). (C) Image of 0.9% agarose gel. PCR using primers RecD1 and KanFor and template pRecDKanDis (lane 1), PCR using primers RecD1 and RecD2 and template pRecDKanDis (lane 2), Fermentas 1 kb ladder (lane3).

The first *recD*::*kan* mutant was designated *recD*::*kan* mutant 1. Two additional

versions of the *recD*::*kan* mutant were made to determine if the phenotypes found in the

first mutant were not the result of a secondary mutation that could have unintentionally

occurred in the first mutant. The *recD*::*kan* mutant 2 was made by transforming the wild

type strain with genomic DNA from the *recD*::*kan* mutant 1. A few dozen colonies were

obtained from 100 µl of transformation mixture plated on a kanamycin plate. The

recD::*kan* mutant 3 was made by transforming the wild type strain with a PCR product of

the mutation from *recD*::*kan* mutant 1. This transformation resulted in hundreds of

colonies from 100 µl of the transformation mixture plated on kanamycin plates.

2.3.2   Generation of the *D.radiodurans* Δ*recD*::*cam* Mutant

To determine if complete removal of the *D. radiodurans recD* gene produces the

same phenotypes found in the *recD*::*kan* mutants, a complete *recD* deletion mutant was

made. PCR to amplify the *cam*[r] resulted in a DNA fragment of approximately 1 kb and

was successfully ligated into plasmid pCR 2.1, forming plasmid pCR 2.1-cam. PCR

reactions to amplify DNA sequences upstream and downstream of the *D.radiodurans*

recD locus resulted in DNA fragments of about 550 bp, and were digested with

appropriate restriction enzymes, and sequentially cloned into plasmid pCR 2.1-*cam*

successfully. The resulting plasmid pRecDCamDis was verified by performing PCR

using the primers used to assemble it. PCR using primers CamDis1, CamDis4, and

template DNA plasmid pRecDCamDis was used to make the linear disruption fragment

used to generate the Δ*cam*::*recD* mutant (figure 2.4).
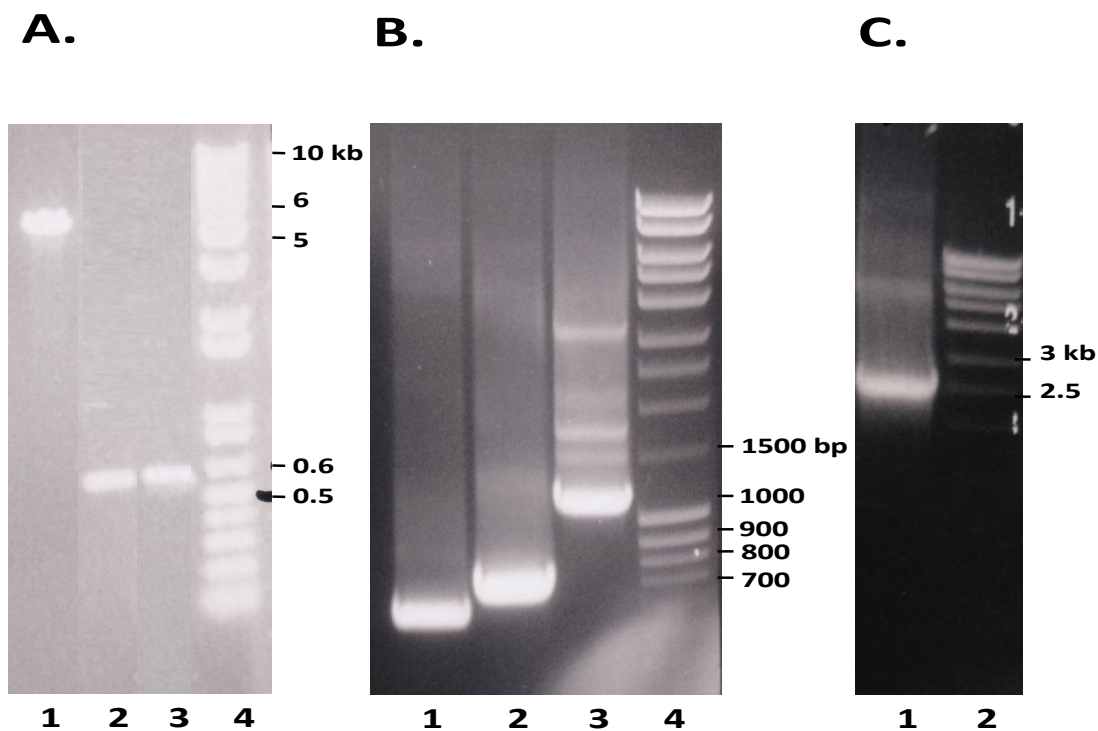
Figure 2.4 Generation and verification of pRecDCamDis and PCR making the DNA fragment used to produce the Δ*cam*::*recD* mutant. (A) Image of 0.9% agarose gel. pCR2.1-*cam* digested with *Hind*III and *Bam*HI (lane 1), PCR reaction amplifying DNA fragment upstream of *recD* gene digested with *Hind*III and *Bam*HI (lane 2), PCR reaction amplifying DNA fragment downstream of *recD* gene digested with *Xho*I and *Nsi*I (lane 3), Fermentas 1 kb ladder. (B) Image of 0.9% agarose gel of PCR reactions using template DNA pRecDCamDis using primers: CamDis1 and CamDis2 (lane 1), CamDis3 and CamDis4 (lane 2), and CamFor and CamRev (lane 3). 1 kb ladder (lane 4). (C) Image of 0.9% agarose gel of PCR reaction using template DNA pRecDCamDis using primers CamDis1 and CamDis4 (lane 1), Invitrogen high mass marker (lane 2).

Despite multiple attempts, it was not possible to make the complete Δcam::*recD* mutant from our wild type strain. The mutant was ultimately made from the *recD*::*kan* mutant 3 strain. The transformation resulted in hundreds of chloramphenicol resistant colonies from 100 µl of transformation mixture plated on chloramphenicol TGY plates.

2.3.3 Genotyping *D.radiodurans recD*::*kan* Mutants by PCR

PCR was used to genotype the *recD*::*kan* mutants as it is convenient way of assessing mutants, as the assay can be performed in one day. DNA concentrations from genomic DNA purifications were typically around 1.0 µg/ml, with $OD_{260/280}$ ratios around 1.75. Primer set Prom1 and RecD2 were used to confirm the presence and completeness of the *recD* mutation. Primer RecD2 is complementary to the 3' end of the *recD* gene, and primer Prom1 is complementary to the sequence upstream of the 5' end of the *recD* gene and is outside of the disruption cassette. The mutant lanes have the expected 3.3 kb fragment. The wild type lane has the expected 2.2 kb fragment. The mutant lanes show no evidence of the wild type product. Primers Prom1 and KanFor were also used. Primer KanFor is complementary to the beginning of the kan^r sequence. Wild type reactions with this primer set produced no product has expected. With this primer set, the mutants have a single product of 1.5 kb (Fig. 2.5). These results suggest the mutations occurred in the correct position in the D. radiodurans genome. The mutation also appears to be complete as no wild type recD gene was detected by the PCR.
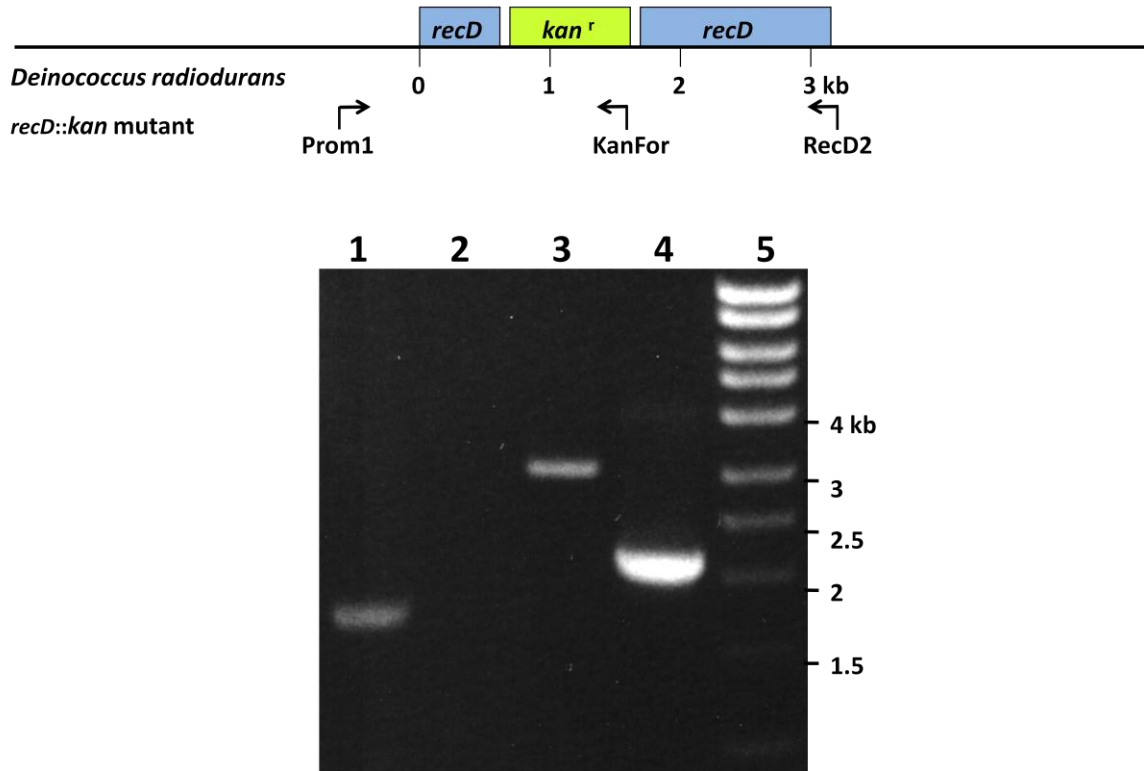
Figure 2.5 PCR genotyping of *D. radiodurans recD*::*kan* mutant 1. (A) Schematic view of the mutation in *recD*::*kan* mutant 1, including the primers used for PCR genotyping. (B) Image of 0.9% agarose gels of PCR using primers KanFor and Prom1 on template DNA from: *recD*::*kan* mutant 1 (lane 1); wild type strain R1 (lane 2). PCR using primers Prom1 and RecD2 on template DNA from: *recD*::*kan* mutant 1 (lane 3); wild type strain R1 (lane 4). Fermentas 1 kb marker (lane 5).

2.3.4 Genotyping *D.radiodurans recD* Mutants by Southern Blotting

Confirmation of mutations in *D. radiodurans* should be done by southern blot.

Due to the polyploidy nature of the organism a method for examining the entire genome

of a mutant needs to be employed to detect residual copies of the wild type gene. The

Southern blot technique is a method of probing the entire genome and is superior to PCR

which amplifies certain sequences of DNA and can be influenced by factors such as copy

number of targeted sequences. According to the protocol from the kit used to perform the

Southern blot in this study, one copy of the wild type gene per cell could be detected.

Three blots were produced to genotype *recD*::*kan* mutant 3, and Δ*cam*::*recD* mutant. The blots contained four separate restriction digests of genomic DNA isolated from wild type strain BAA-816, *recD*::*kan* mutant 3, and Δ*cam*::*recD* mutant. The digestions loaded onto a 0.9% agarose gel as follows: digestion 1 (*Nco*I / *Pst*I double digest), digestion 2 (*Apa*I), digestion 3 (*EcoR*I), digestion 4 (*Eag*I). The gels are shown in figure 2.6 panels A, B, and C.

For genotyping the *recD*::*kan* mutant 3 one probe complementary to the full length *recD* gene (*recD* probe), and one probe complementary to the kanamycin resistance gene (*kan*[r] probe) were generated. The *recD* probe was made using wild type DNA and primers RecD1 and RecD2, while the *kan*[r] probe was made using pCR-Blunt template and primers KanFor and KanRev. For genotyping the Δ*cam*::*recD* mutant, the *kan*[r] probe was used. Additionally, a probe against the chloramphenicol resistance gene (*cam*[r] probe) using primers CamFor and CamRev was used. The results of the reactions are shown in figure 2.6 D and E. The reactions used to make the probes produced some non-specific bands. However, all three test reactions (unlabelled) had major bands corresponding to the expected mobility. The *kan*[r] probe and *cam*[r] probe test reactions gave a strong band at about the 1kb marker respectively. The recD probe test reaction gave a strong band just above the 2 kb marker. The probe synthesis reactions resulted in bands of slightly less mobility as expected due to the incorporation of the DIG label. The *kan*[r] probe synthesis reaction depicted had been previously cleaned up with a gel extraction prior to use. The *recD* and *cam*[r] probes were used without gel purification.

A. *recD*::*kan* mutant 3

B. wild type

C. Δ *recD*::*cam* mutant

D. *kan*ʳ

E. *recD* probe

F. *cam*ʳ probe
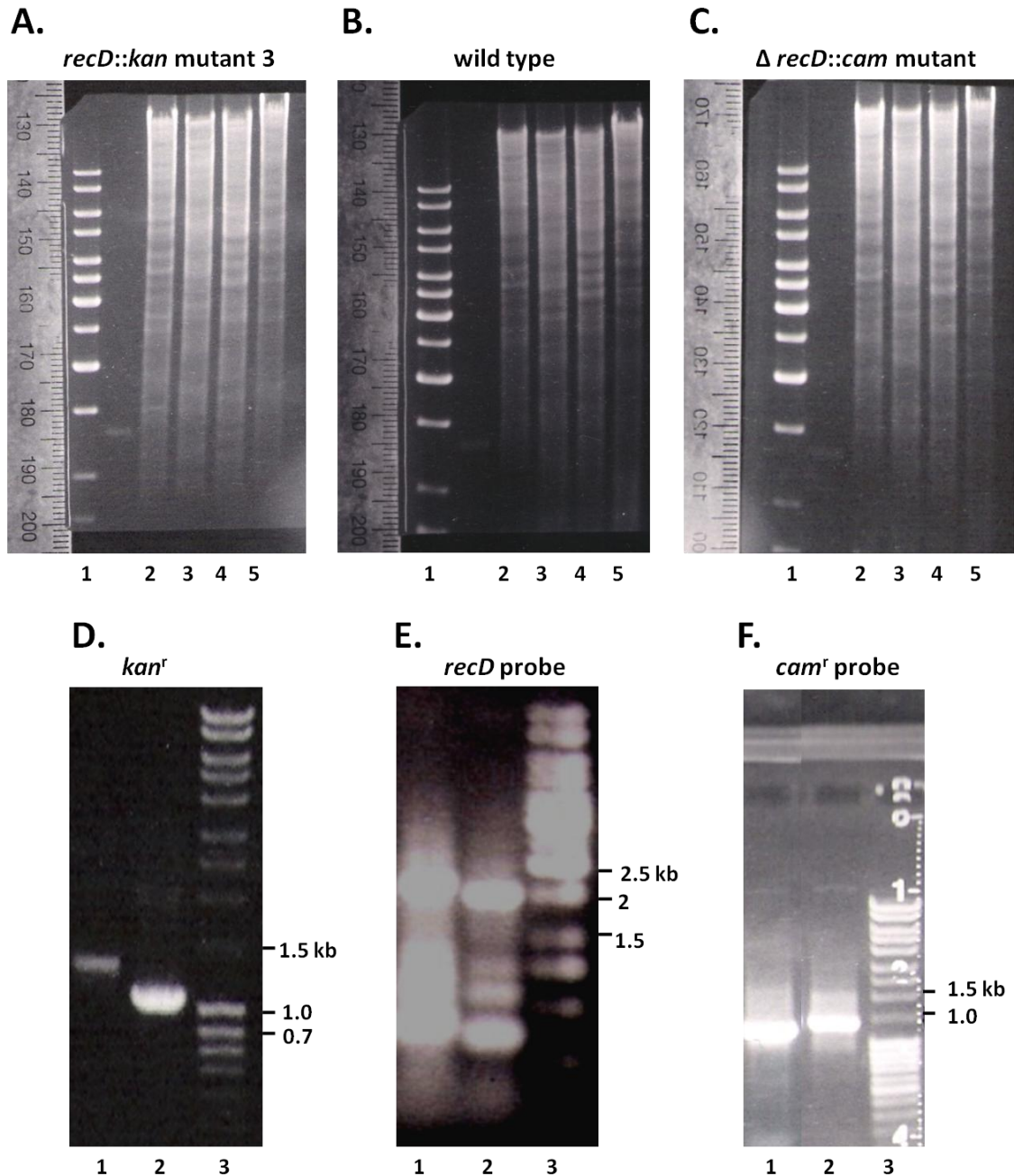
Figure 2.6 Images of 0.9% agarose gels used for transfer to nitrocellulose, and PCR reactions used to generate probes for Southern Blotting. (A, B, C) Images of 0.9% agarose gel of 5ng digested genomic DNA from: *recD*::*kan* mutant 3, wild type, Δ*cam*::*recD* mutant respectively. Fermentas 1 kb ladder (lane 1); *Nco*I/*Pst*I double digest (lane 2); *Apa*I digest (lane 3); *EcoR*I digest (lane 4); *Eag*I digest (lane 5). (D, E, F) Image of 0.9% agarose gels of PCRs used to generate the *kan*ʳ probe, *recD* probe, and *cam*ʳ probes respectively. Control reaction (lane 1); probe synthesis reaction (lane 2); Fermentas 1 kb plus marker (lane 3).

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The Southern blot did not detect any wild type genes in the *recD*::*kan* mutant or any unintended insertions of the kanamycin resistance gene, however they could exist at extremely low frequency. Digestion with the selected restriction enzymes results in different fragments containing *recD* in wt compared with *recD*::*kan* mutant. As a result Southern blotting wild type genomic DNA with a probe specific to the *recD* gene will result in a completely different pattern than blotting *recD*::*kan* mutant genomic DNA. Therefore, a homozygous *recD*::*kan* mutant will not contain any of the bands that are found in the wild type blot. Additionally, we can detect if the *kan*[r] inserted randomly somewhere else in the genome. Bands of the expected mobility were obtained when the *recD*::*kan* mutant 3 blot was probed with the *kan*[r] probe (Fig. 2.7 B). The *Nco*I and *Pst*I digestions resulted in a 6 kb and 2.7 kb band, with a 4.5 kb band when digested with *Apa*I. A 5 kb band was observed upon digestion with *Eag*I, and both a 2.4 kb and a 2.6 kb band were seen when digestion was with *EcoR*I.

The same membrane was then stripped and reblotted with the *recD* gene specific probe. The same size fragments were detected as found using the *kan*[r] probe (Fig. 2.7 C) suggesting that no wild type copies of the gene were present. Also, no bands corresponding to the intact *recD* gene were detected in either blot. Additionally, wild type DNA was digested and blotted in the same manner as the mutant DNA. This blot also resulted in the expected banding patterns. As shown in figure 2.7 E, when probed with the *recD* probe, the wild type blot produced an 8 kb band when digested with *Nco*I and *Pst*I, and a 3.5 kb band when digested with *Apa*I. As well a 4 kb band was observed when DNA was digested with *Eag*I or *EcoR*I. As expected when stripped and reblotted with the *kan*[r] specific probe, no bands were detected (Fig. 2.7 D).
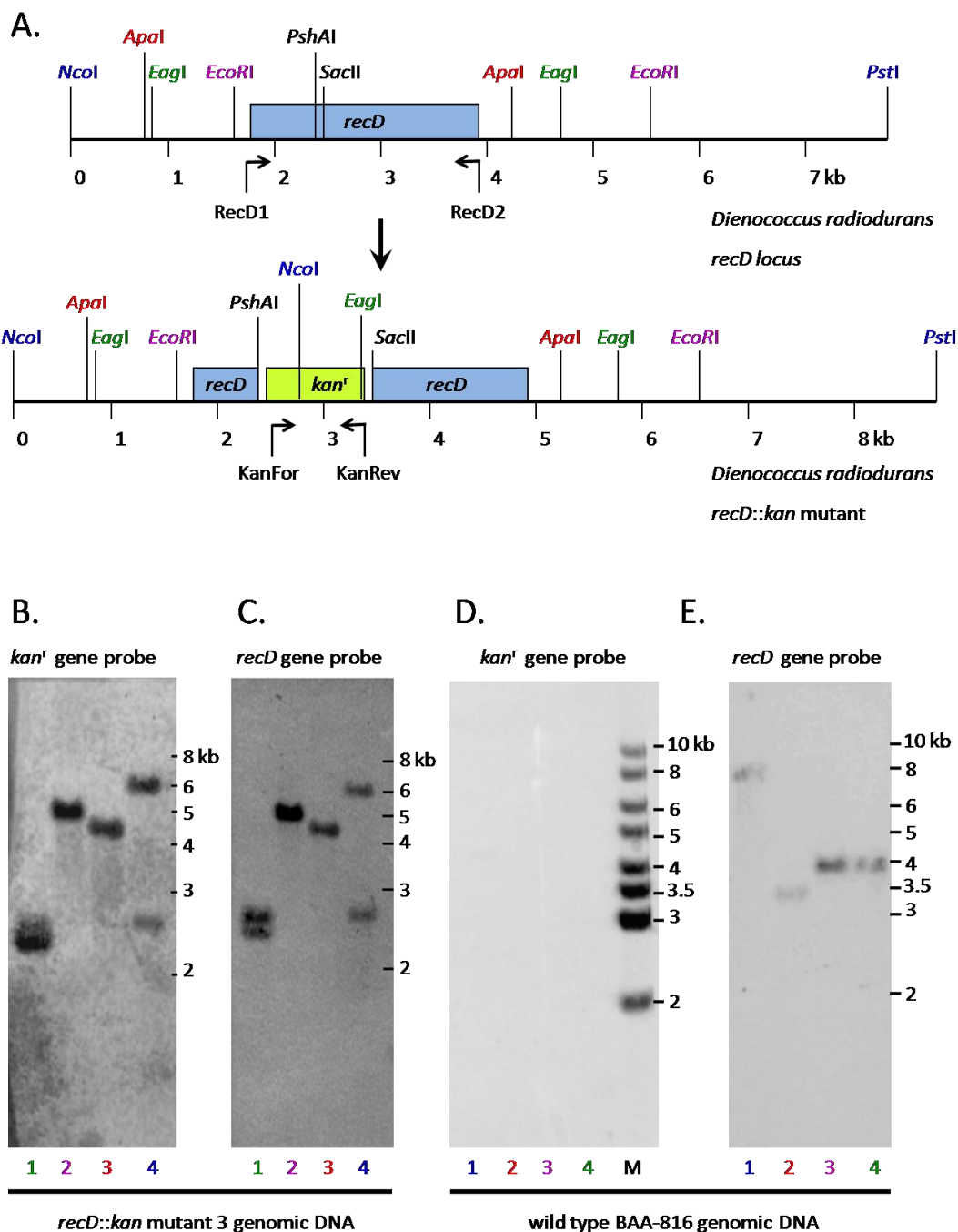
Figure 2.7 Southern blots of wild type *Deinococcus radiodurans*, *recD*::*kan* mutant 3. (A) Schematic of the wild type *Deinococcus radiodurans*, and *recD*::*kan* mutant 3 at the *recD* locus. (B, C) Southern blots of *recD*::*kan* mutant 3 genomic DNA (5ng), probed with *kan*[r] probe (B), and *recD* probe (C). (D, E) Southern blots of wild type BAA-816 genomic DNA (5ng), probed with *kan*[r] probe (D), and *recD* probe (E). *Nco*I/*Pst*I double digest (lane 1); *Apa*I digest (lane 2); *EcoR*I digest (lane 3); *Eag*I digest (lane 4); Fermentas 1 kb ladder (lane M)

Genotyping the Δ*recD*::*cam* mutant DNA was performed with the same restriction enzymes used for the *recD*::*kan* mutant. According to the assay, the mutation was complete and occurred in its intended position. All digestions resulted in bands of the expected mobility with the *cam*^r probe (Fig. 2.8 B). The *Nco*I, *Pst*I double digestion produced bands of 750 bp and 2 kb. The ApaI digest produced a 2.5 kb band. Both the *Eag*I and *EcoR*I digestions produced bands of about 1 kb. When the blot was stripped and re-probed with the *kan*^r probe, no bands were detected (data not shown).
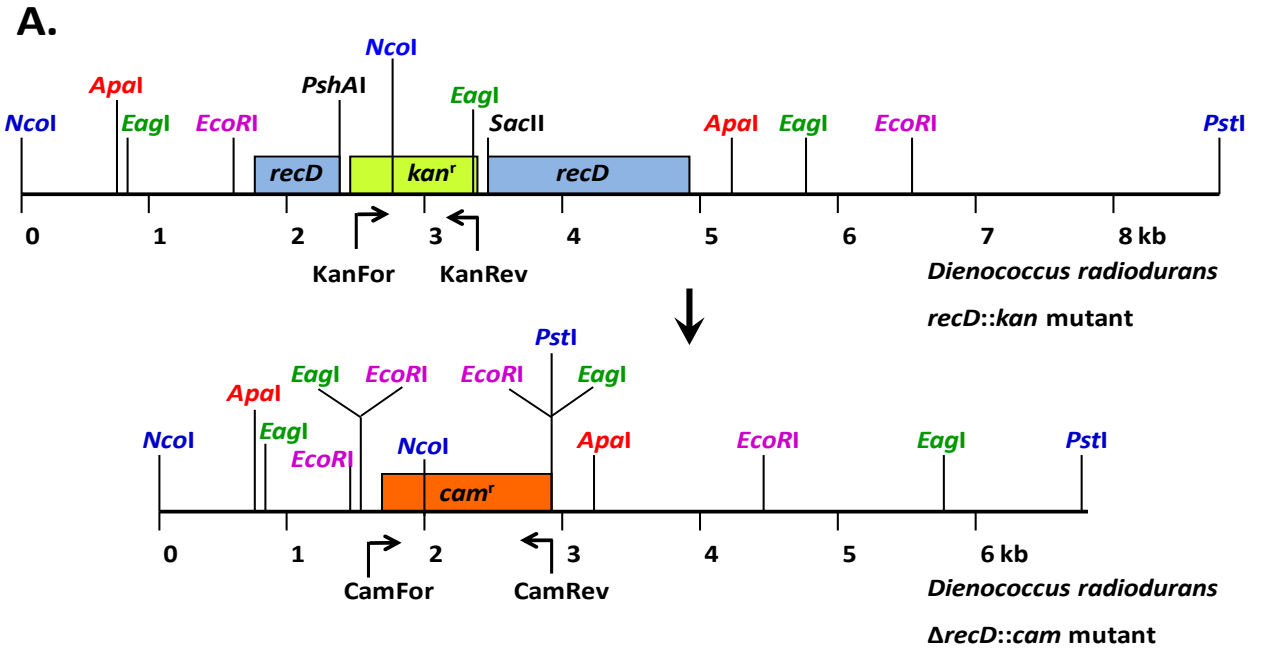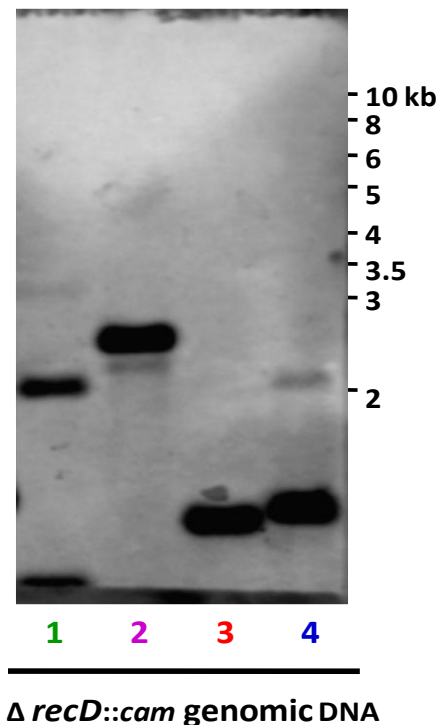
Figure 2.8 Southern blot of wild type *Deinococcus radiodurans* and Δ*recD*::*cam*
mutant. (A) Schematic of *recD*::*kan* mutant 3 and Δ*recD*::*cam* mutant at the *recD*
locus. (B) Southern blot of Δ*recD*::*cam* mutant 3 genomic DNA (5ng), probed with
cam[r] probe. *Nco*I/ *Pst*I double digest (lane 1); *Apa*I digest (lane 2); *Eco*RI digest (lane 3);
*Eag*I digest (lane 4).

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2.3.5 Growth Assay

In *E. coli*, mutations in recombination genes cause growth defects (31-34).
Therefore growth of the mutant was compared to wild type. The *recD*::*kan* mutant 1
strain grew at about the same rate as the wild type *D. radiodurans* R1 strain from which it
was derived, having a doubling time of 100 min in exponential phase. It also reached
approximately the same cell density. The results in figure 2.9 are from one experiment
(n=1), but the experiment was performed independently twice with similar results, (n=1)
for each assay. Plating indicated similar colony counts for both cell lines at matched
densities.
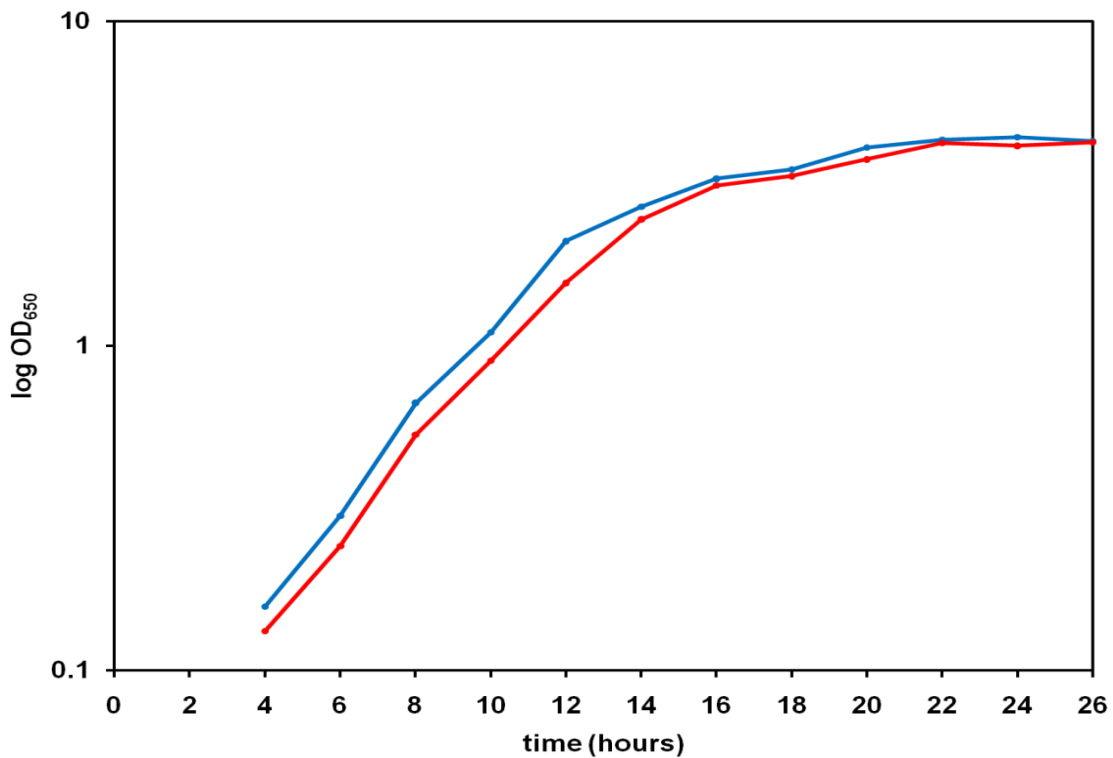
Figure 2.9 Growth of wild-type and *recD*::*kan* mutant *D. radiodurans* strains. Cells
were grown overnight (with kanamycin for the *recD*::*kan* mutant 1), diluted 1:200 into
fresh TGY medium with no antibiotic, and shaken at 30°C. Samples were removed at the
indicated times after dilution, and the OD_{650} was measured. Blue dots, wild-type strain
R1; red dots, *recD*::*kan* mutant 1. Results are from one experiment with single OD_{600}
determinations, (n=1). The experiment was performed independently twice with similar
results.

2.3.6 Mitomycin C Sensitivity

The *recD* mutant was tested for sensitivity to MMC as it alkylates DNA. It is

bifunctional and can cross link DNA. Repair of this damage can result in double strand

breaks in DNA (86, 143). The *recD*::*kan* mutant 3 was as sensitive to MMC as the wild

type *D. radiodurans* strain BAA-816 from which it was derived (figure 2.10). The wild

type and mutant exhibited a shoulder of resistance characteristic of *D.radiodurans*. The

results in figure are from one experiment (n=3), but the experiment was performed

independently twice using *recD*::*kan* mutant 3, and twice using *recD*::*kan* mutant 2, and

once using *recD*::*kan* mutant 1, (n=3) for each individual experiment.
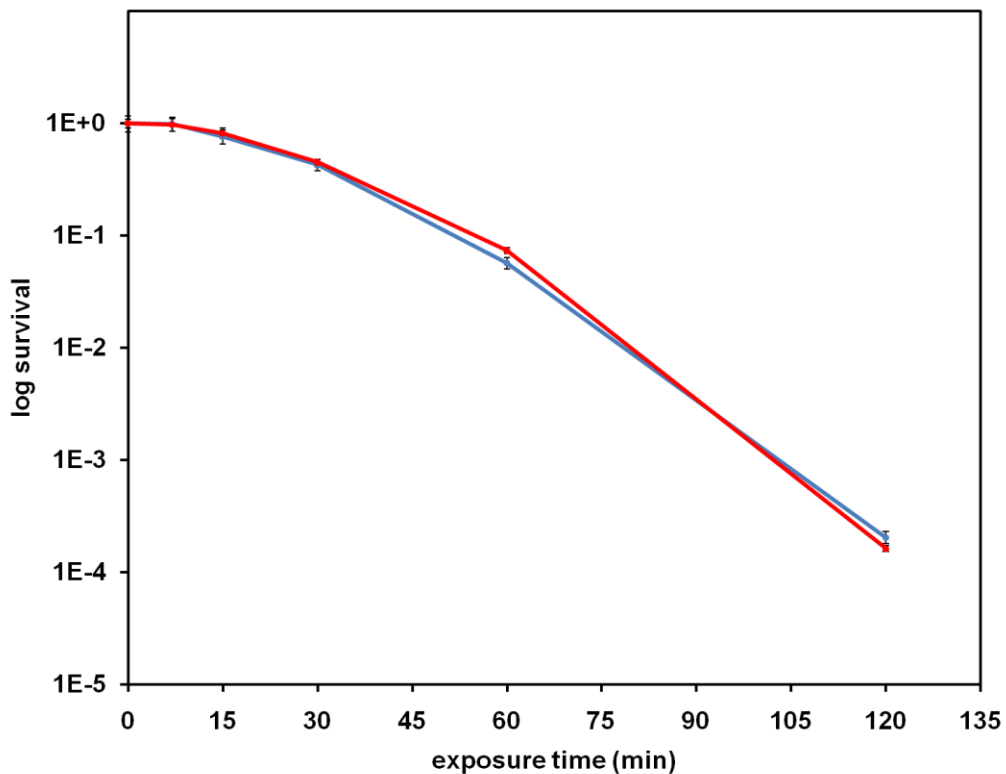
Figure 2.10 Sensitivity of wild-type and *recD*::*kan* mutant *D. radiodurans* strains to MMC. Cells were treated with 10 µg/ml MMC for indicated times, serially diluted and plated on TGY agar plates. Blue dots, wild-type strain BAA-816; red dots, *recD*::*kan* mutant 3. Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed twice with comparable results.

2.3.7 Methyl Methanesulfonate Sensitivity

The *recD* mutant was tested for sensitivity to MMS as it is capable of methylating

DNA, causing double strand breaks when replication forks encounter the altered bases

(140, 143). The *recD*::*kan* mutant 3 was as sensitive to MMS as the wild type *D.*

radiodurans strain BAA-816 from which it was derived. The wild type and mutant

exhibited a shoulder of resistance characteristic of *D.radiodurans* as seen in figure 2.11.

The assay was only performed one time (n=3).
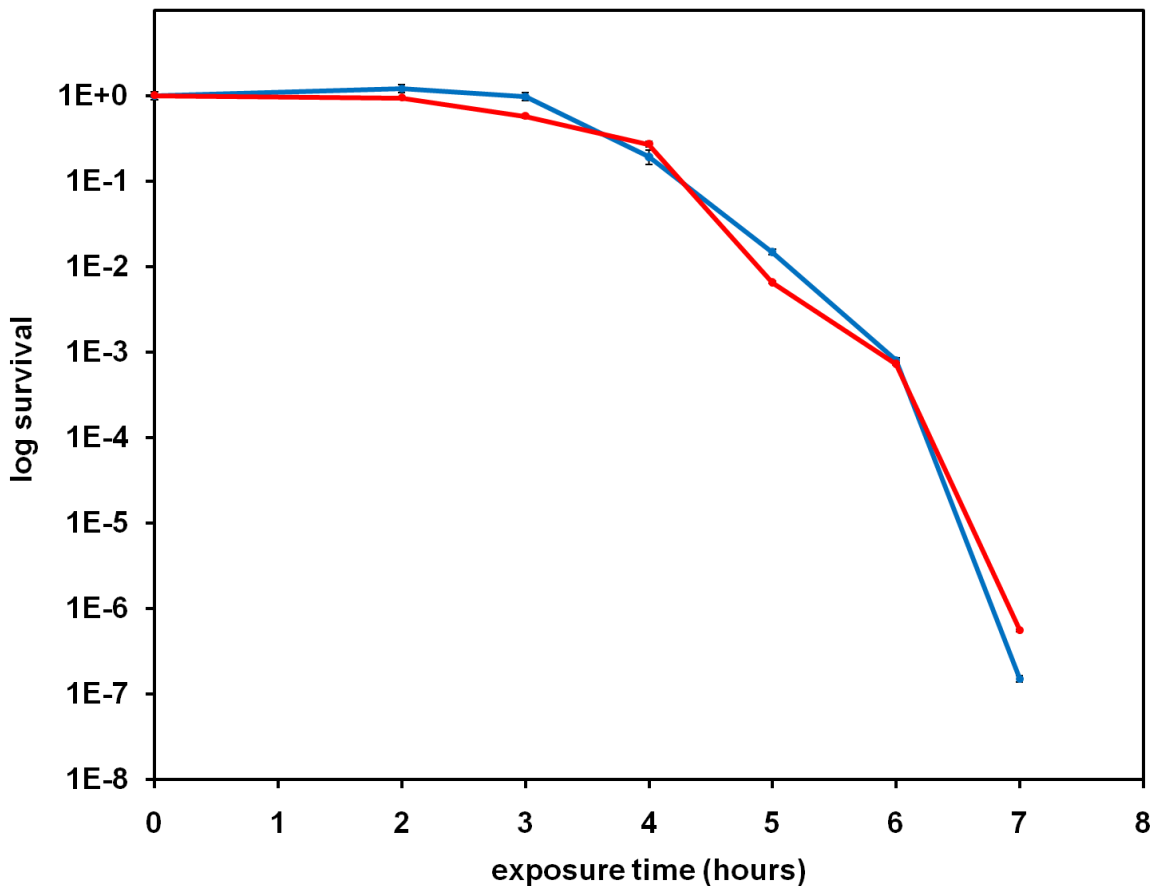
Figure 2.11 Sensitivity of wild-type and *recD*::***kan* mutant *D. radiodurans* strains to
MMS.** Cells were treated with 30mM MMS for indicated times, serially diluted and
plated on TGY agar plates. Blue dots, wild-type strain BAA-816; red dots, *recD*::*kan*
mutant 3. Results are from one experiment, with error bars generated from three
independent determinations (n=3). The experiment was performed once.

2.3.8 Gamma Irradiation Sensitivity

The *recD* mutant was tested for sensitivity to gamma irradiation as it causes many

forms of DNA damage including double strand breaks (132). Both the *recD*::*kan* mutant

3 and the Δr*ecD*::*cam* mutant had increased sensitivity to gamma irradiation compared to

the wild type *D. radiodurans* strain BAA-816 from which they were derived. The wild

type exhibited a broad shoulder of resistance. Both *recD* mutant strains showed an

equivalent drop in survival that increased with the dose of gamma irradiation. The results

in figure 2.12 are from a single experiment (n=3). The experiment was performed

independently; once with the Δ*recD*::*cam* mutant, and multiple times using different

versions of the *recD*::*kan* mutant with reproducible results (n=3) for each independent

experiment.

2.3.9 Hydrogen Peroxide

The *recD* mutant was tested for sensitivity to hydrogen peroxide as it can lead to

DNA damage such as double strand breaks (60). The *recD*::*kan* mutant showed a

significant loss of resistance to hydrogen peroxide when compared with the wild type

strain BAA-816 at 30°C in TGY media. The wild type displayed a marked resistance to

hydrogen peroxide, while the mutant showed an increase in sensitivity in relation to the

wild type that increased except at the final dose tested 250 mM in which the difference

was comparable to 200mM. Additionally, all cells were dead at 300mM hydrogen

peroxide (data not shown). The results in figure 2.13 are from a single experiment (n=3).

The assay was performed independently four different times (n=3 for each) with

comparable results.

When the assay was performed with the cells in PBS rather than TGY on ice, the mutant had the same sensitivity as the wild type. The shoulder of resistance of the two strains was similar to that of the wild type done under the previous conditions. However, the steepness of the drop off was substantially greater when performed on ice, and occurred immediately at the 225 mM dose with the log survival dropping to 10^{-3}. Additionally, the kill curve seemed to level off at that survival range, despite nearly doubling the hydrogen peroxide dose. The results in figure 2.14 are from one experiment (n=3). The assay was performed separately twice, and the results were essentially the same (n=3) for each experiment.
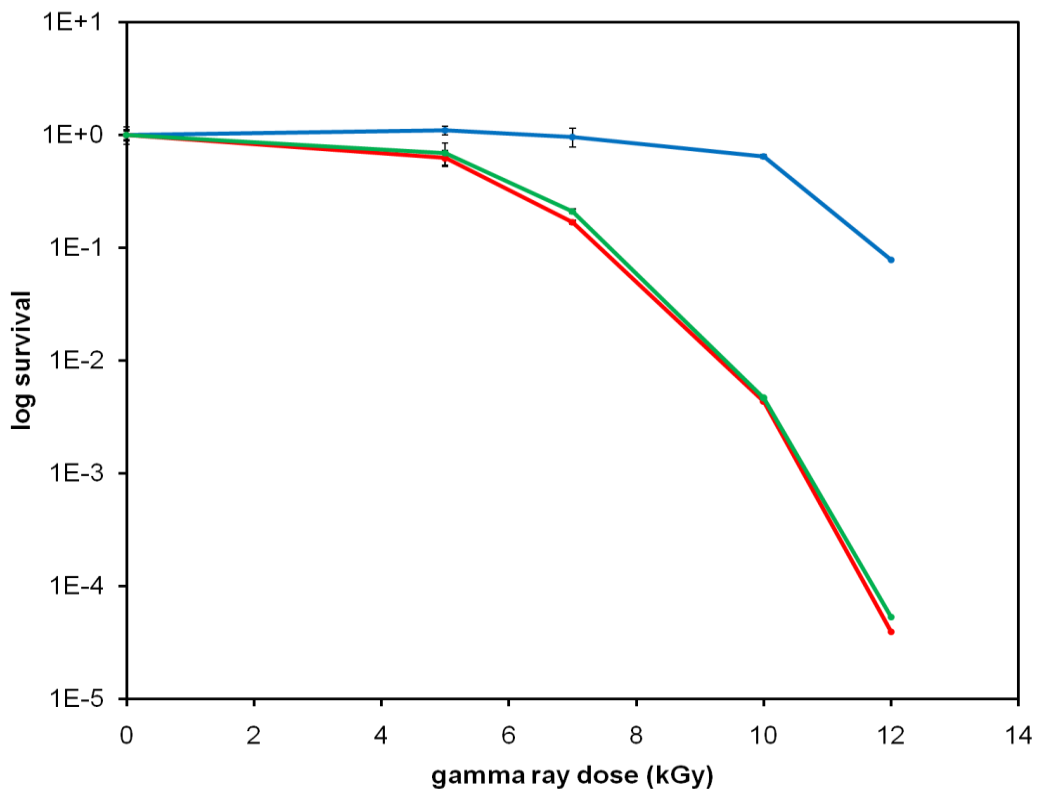
Figure 2.12 Sensitivity of wild-type, *recD*::*kan* mutant, and Δ*recD*::*cam* mutant *D. radiodurans* strains to gamma irradiation. Gamma irradiation was performed on ice, from a [60]Cobalt source at 100 kGy/min, for indicated doses. Blue dots, wild type strain BAA-816; red dots, *recD*::*kan* mutant 3; green dots, Δ*recD*::*cam* mutant. Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed once.

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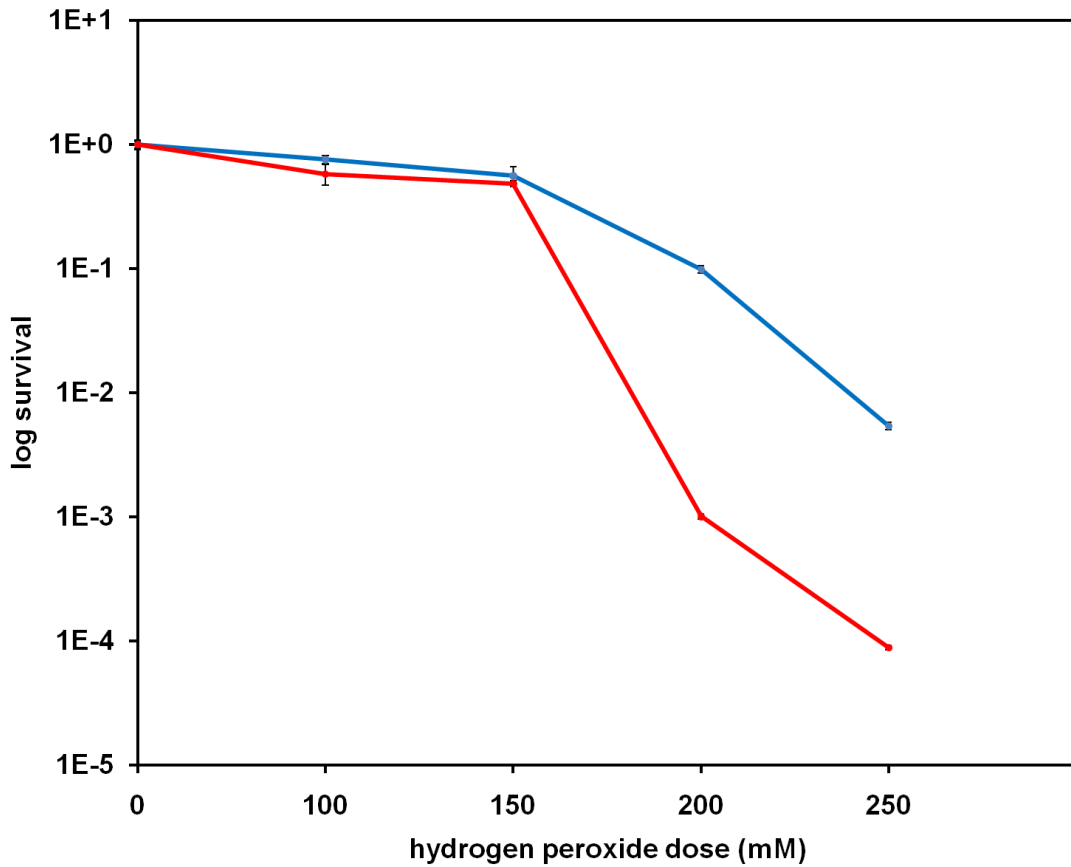
Figure 2.13 Sensitivity of wild-type and *recD*::*kan* mutant *D. radiodurans* strains to hydrogen peroxide at 30°C. Cells were treated with hydrogen peroxide for one hour without shaking, serially diluted and plated on TGY agar plates. Blue dots, wild-type strain BAA-816; red dots, *recD*::*kan* mutant 3. Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed four times with comparable results.
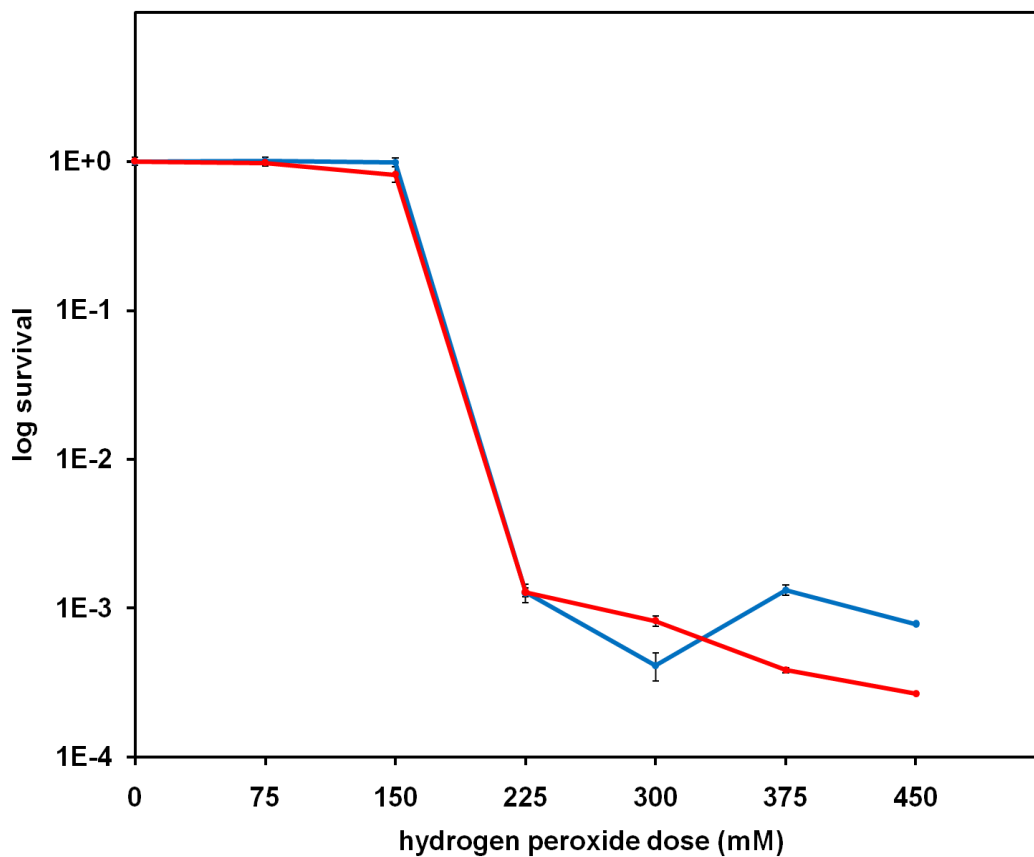
Figure 2.14 Sensitivity of wild-type and *recD*::*kan* mutant *D. radiodurans* strains to hydrogen peroxide on ice in PBS. Cells were treated with hydrogen peroxide for one hour in PBS on ice without shaking, treated with catalase, serially diluted and plated on TGY agar plates. Blue dots, wild-type strain BAA-816; red dots, *recD*::*kan* mutant 3. Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed twice with comparable results.

2.3.10 UV Irradiation Sensitivity

The *recD* mutant was tested for sensitivity to UVC as it is capable of causing

leasions in DNA that creates double strand breaks when replication forks encounter the

leasions (143). Both the *recD*::*kan* mutant 3 and the Δ*recD*::*cam* mutant showed

enhanced sensitivity to UV irradiation compared to the wild type *D. radiodurans* strain

BAA-816 from which they were derived. The wild type exhibited the expected shoulder

of resistance. Both *recD* mutant strains showed an equivalent drop in survival after a

dose of 180 J/m^2, which increased with the dose of UV irradiation. Figure 2.15 shows

the results of one experiment (n=3), but the experiment was performed independently

once with the Δr*ecD*::*cam* mutant, and multiple times with different versions of the

recD::*kan* mutant always with equivalent results, (n=3) individual experiments.

2.3.11 Complementation of UV Sensitivity Using Plasmid pRAD1-*recD*

To strengthen the argument that the sensitivity to UV irradiation in the *D.*

radiodurans recD mutant was a result of a loss of RecD protein, the mutant was

transformed with a plasmid that replicates autonomously in the organism that encodes

recD and its promoter. It should be capable of directing the synthesis of RecD protein in

the mutant. Plasmid pRad1-*recD* was not able to fully complement the *recD*::*kan*

mutation consistently in response to UV irradiation. The results in figures 2.16 to 2.18

are from independent assays using independent complementations of *recD*::*kan* mutant 3

(see materials and methods). When first transformed into the *recD***::***kan* mutant3,

complete recovery of the wild type phenotype was observed under the doses used in

previous UV irradiation assays. (Fig. 2.16). Master plates of the initial complementation

were made. Repeating the assay using clones selected from these plates failed to produce

full recovery of the phenotype (Fig. 2.17). Plasmids were purified from *E. coli* colonies

of a master plate made from the initially selected clones. They were retransformed into

recD::*kan* mutant 3, but they also failed to produce complete recovery (Fig 2.18)
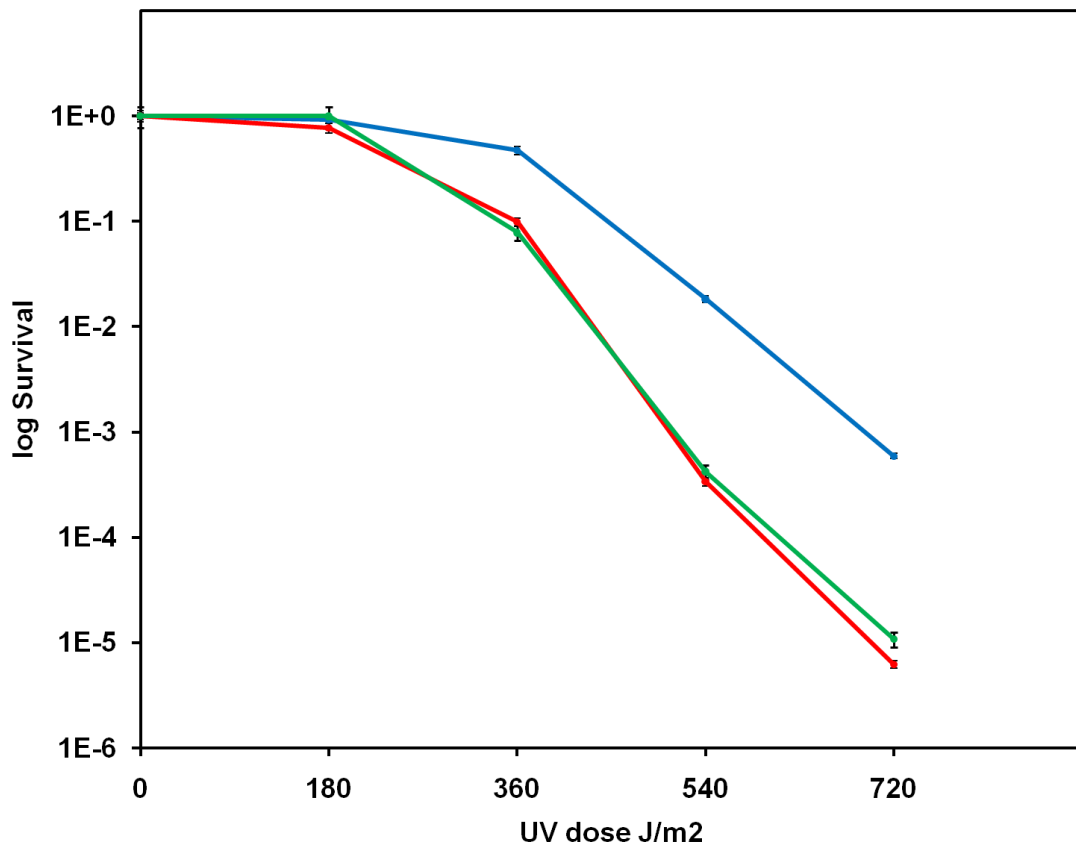
Figure 2.15 Sensitivity of wild-type, *recD*::*kan* **mutant, and Δ*recD*::*cam* **mutant** *D. radiodurans* **strains to UV irradiation.** Cells were spread onto TGY plates and irradiated with UV light from a germicidal lamp at 90 J/m^2/min. Blue dots, wild type strain BAA-816; red dots, *recD*::*kan* mutant 3; green dots, Δ*recD*::*cam* mutant. Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed three times with comparable results.
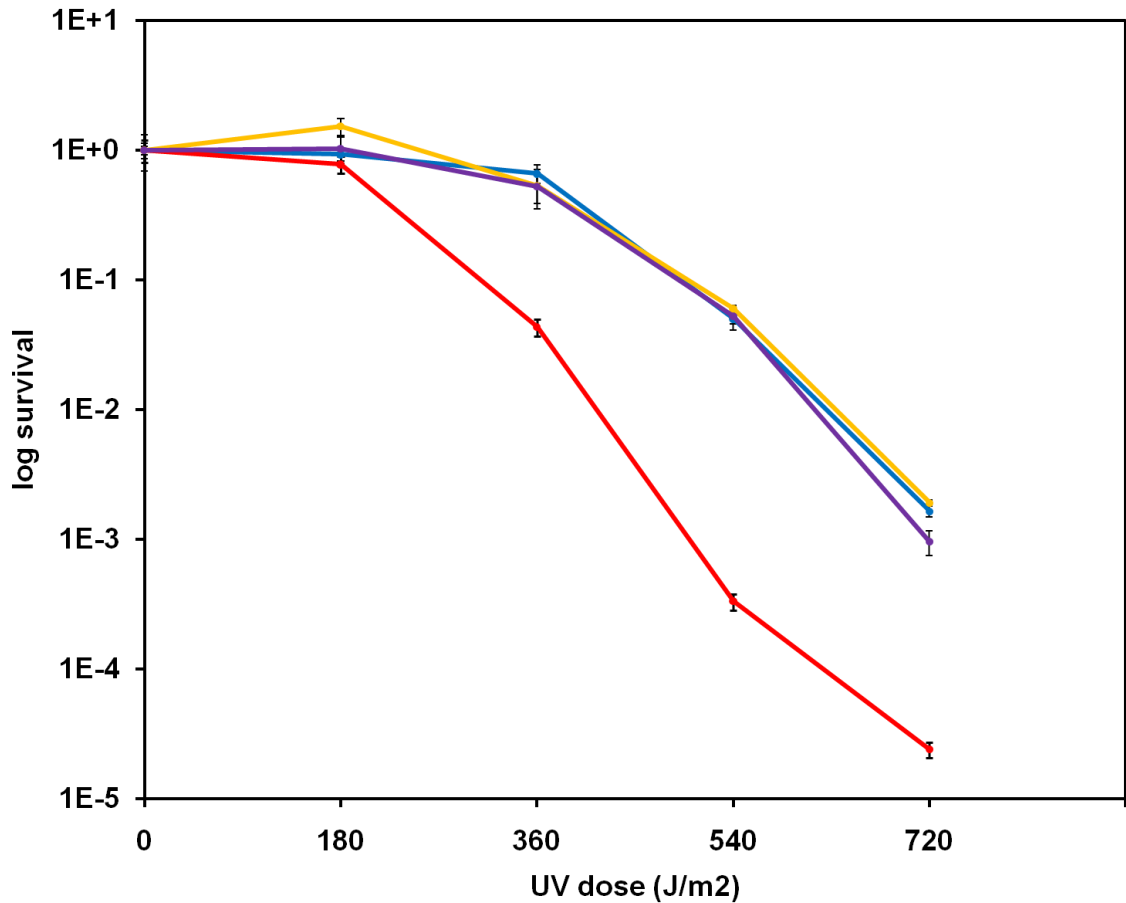
Figure 2.16   UV irradiation test for complementation of the *recD*::*kan* mutatation, using a plasmid containing the *recD* gene and promoter. Cells were spread onto TGY plates and irradiated with UV light from a germicidal lamp at 90 J/m^2/min. Blue dots, wild type strain BAA-816; red dots, *recD*::*kan* mutant 3; orange dots, *recD*::*kan* mutant 3-(pRAD1-*recD*-1); purple dots, *recD*::*kan* mutant 3-(pRAD1-*recD*-3). Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed once.
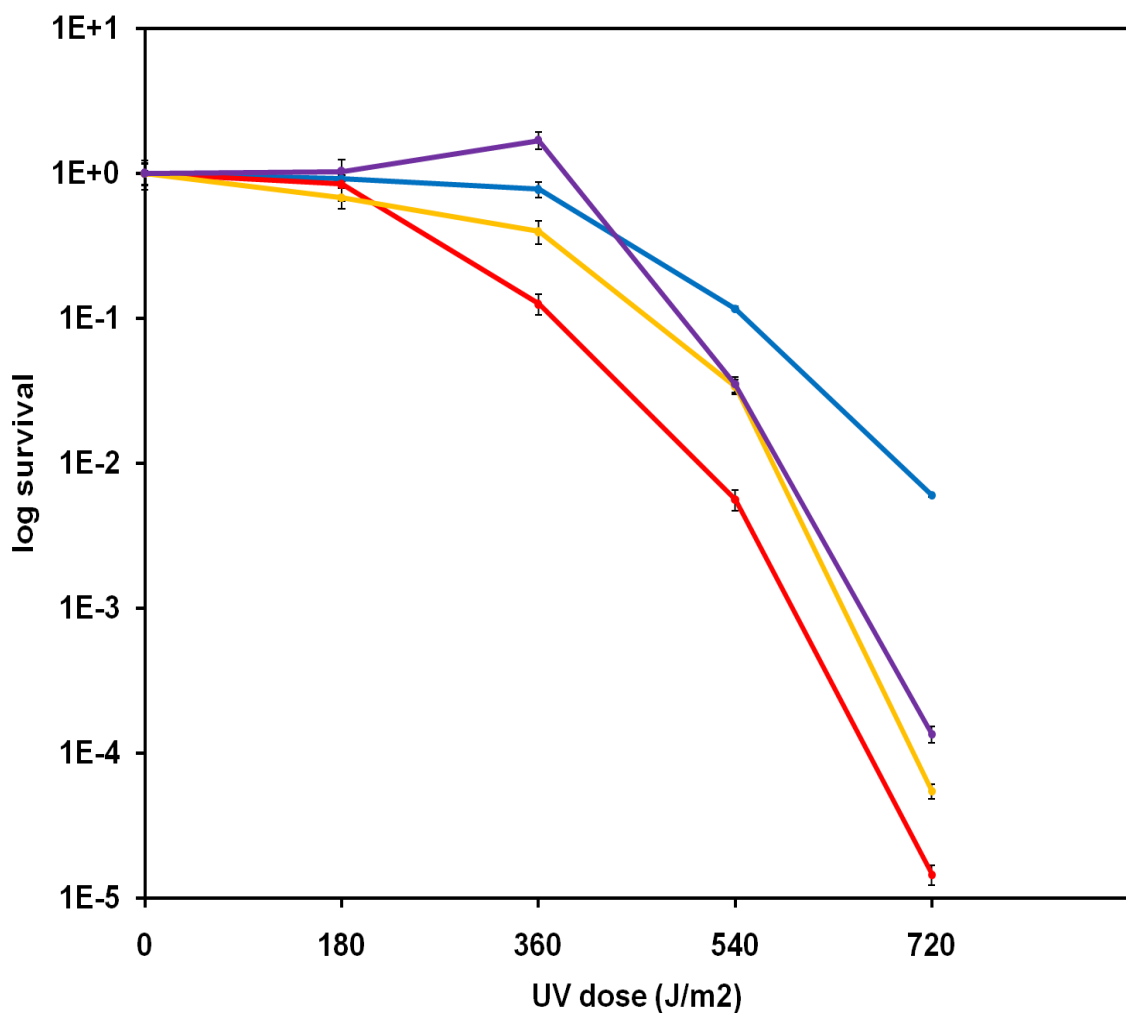
Figure 2.17   UV irradiation test for complementation of the *recD*::*kan* mutatation, using a plasmid containing the *recD* gene and promoter. Cells were spread onto TGY plates and irradiated with UV light from a germicidal lamp at 90 J/m^2/min. Blue dots, wild type strain BAA-816; red dots, *recD*::*kan* mutant 3; orange dots, *recD*::*kan* mutant 3 (pRAD1-*recD*-1)-MP; purple dots, *recD*::*kan* mutant 3-(pRAD1-*recD*-3)-MP. Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed once.
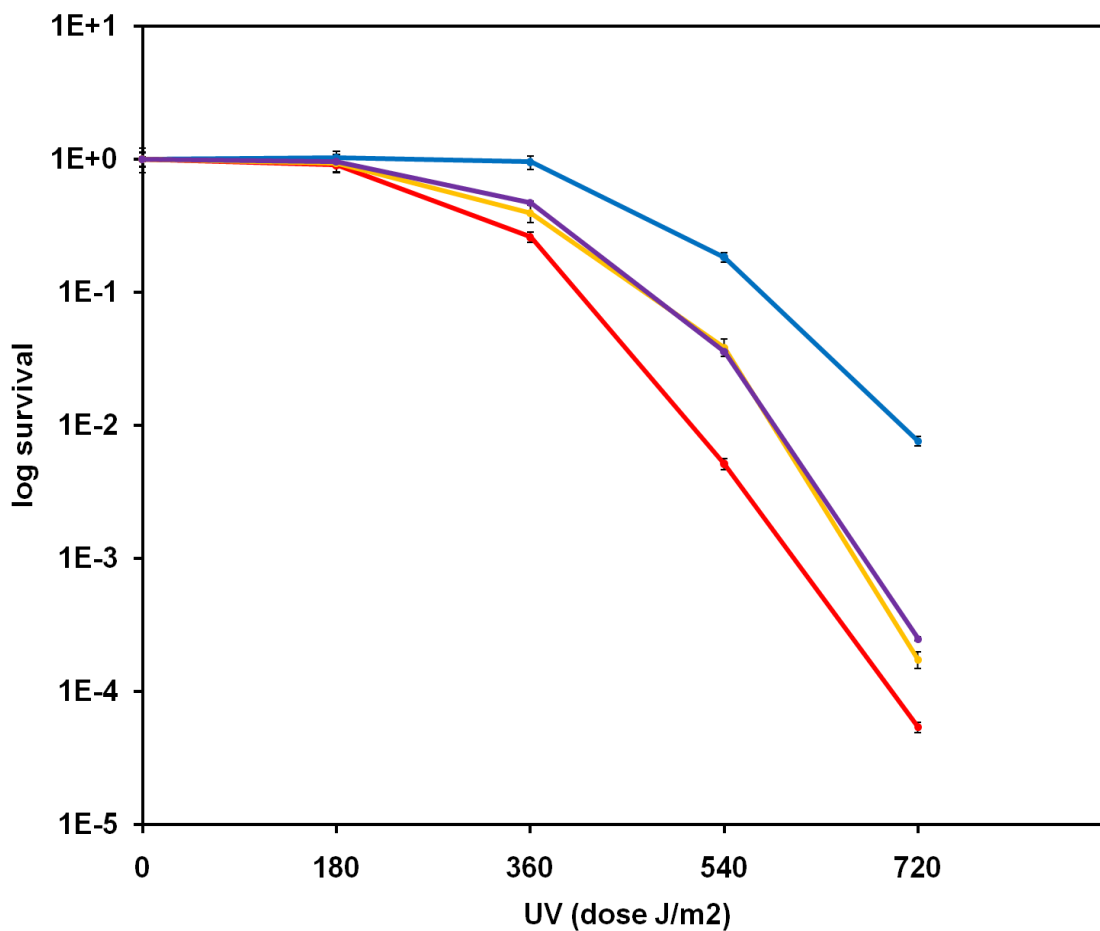
Figure 2.18   UV irradiation test for complementation of the *recD*::*kan* mutatation, using a plasmid containing the *recD* gene and promoter. Cells were spread onto TGY plates and irradiated with UV light from a germicidal lamp at 90 J/m^2/min. Blue dots, wild type strain BAA-816; red dots, *recD*::*kan* mutant 3; orange dots, *recD*::*kan* mutant 3-(pRAD1-*recD*-1.2); purple dots, *recD*::*kan* mutant 3-(pRAD1-*recD*-3.2). Results are from one experiment, with error bars generated from three independent determinations (n=3). The experiment was performed once.

2.3.12 Gross Cellular and Nucleoid Morphology Visualized by Microscopy

Certain DNA repair gene mutants in *E. coli* have cells with unusual morphology, forming filaments. These filamentous cells also have striking defects in chromosome structure, with chromosomes concentrated in some regions, and other regions completely devoid of DNA (142). Therefore *recD*::*kan* mutant 3 was studied by phase contrast and fluorescence microscopy. The *recD* mutant cells appear similar in size and shape to the wild type strain from which they were derived in both growth phases. Both strains exhibit cocci and diplococci as expected for this organism. Tetrads were difficult to observe in phase contrast pictures. The fluorescence images reveal nucleoids of similar size and shape in both strains in both growth phases.

Phase Contrast **EtBr**

wild type Mid Log Phase	A	B
wild type Stationary Phase	C	D
recD:: *kan* mutant 3 Mid Log Phase	E	F
recD:: *kan* mutant 3 Stationary Phase	G	H
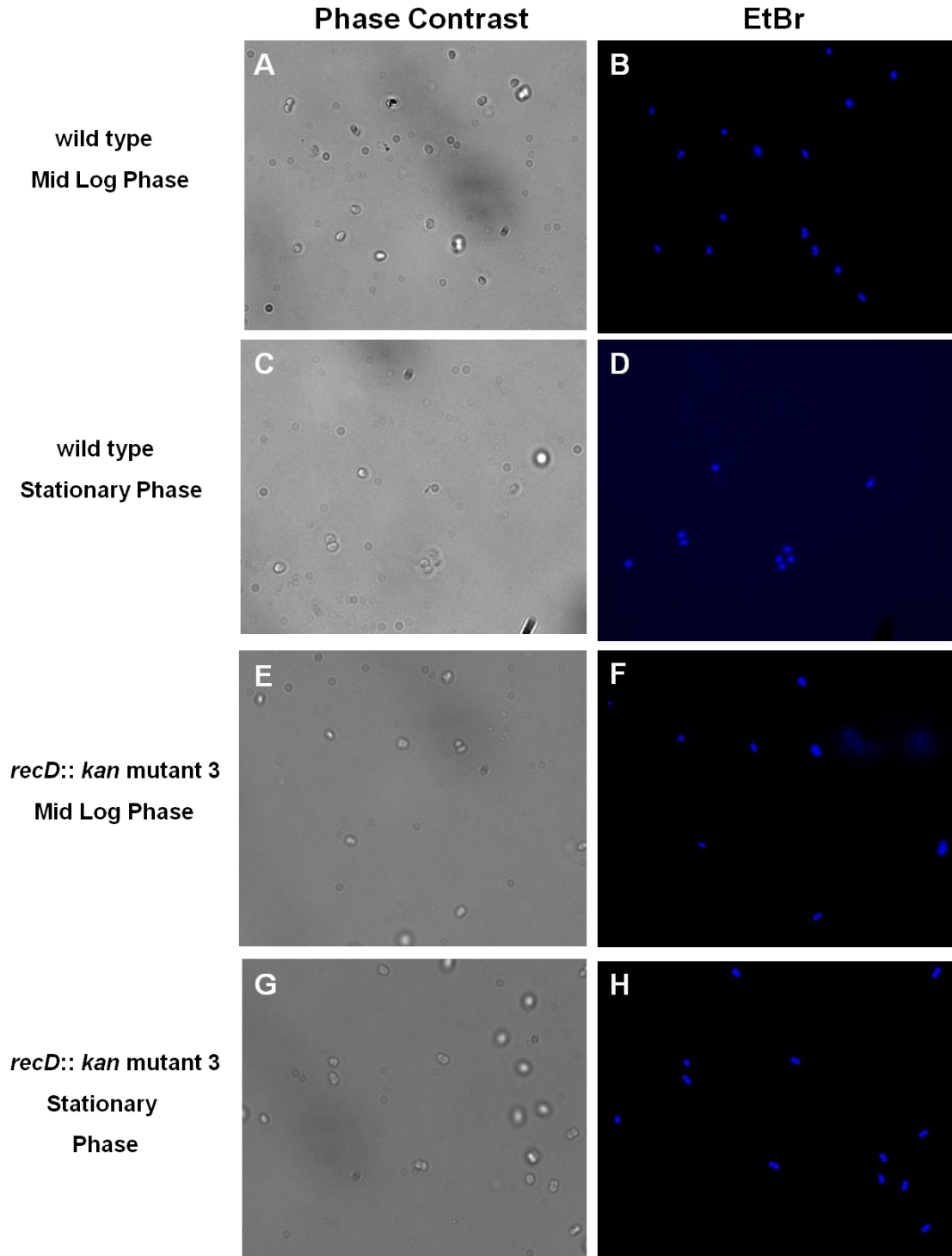
Figure 2.19 Phase contrast and fluorescent microscope images of D. radiodurans wild type strain BAA-816, and *recD*::*kan* mutant 3, at mid log and stationary growth phases. For cell morphology cells were visualized using phase contrast left panels). For visualization of nucleoids, DNA fluoresces blue with Ethidium Bromide (EtBr) in fluorescence images (right panels).

2.3.13 Transformation

D. radiodurans is naturally competent throughout exponential growth. Treatment with $CaCl_2$ leads to increased transformation efficiency (80). *D. radiodurans recD*::*kan* mutant 3 is hyper-transformable compared to the wild type strain. This was tested using procedures from two published sets of conditions (79, 80).

Under the first set of conditions tested, the *recD* mutant showed high transformation efficiency compared to wild type. When plated on 5 μg/ml streptomycin TGY plates, *recD* mutants were between 9 and 200 times more transformable, with a median value of 27 (n=6). The results found on the 5 μg/ml plates were consistent within an experiment but were highly variable between experiments. Increasing the amount of streptomycin on the selective plates resulted in a modest increase in the relative efficiency of the mutant to wild type. The fold increase ranged from 30 to 150 with a median value of 103 (n=4). When transformation experiments were performed using 50 μg/ml streptomycin, extremely high error rates were found even within an experiment. The high error was due to the fact that the number of transformants found on the wild type plates was extremely low, and in some experiments non-existent.

Transformation assays were also performed using plasmid pI3 plasmid DNA (a plasmid known to autonomously replicate in *D. radiodurans*), to determine if the difference in transformation efficiency was caused by an altered capacity to uptake DNA or a change in ability to perform homologous recombination. The relative increase in transformation rate of the mutant was slightly higher than that found using genomic transforming DNA. The experiment was performed only once.

Under the second set of conditions, the *recD* mutant had increased transformation compared to wild type. The transformation efficiency of the wild type was very close to previously published results; however, the increase in transformation efficiency in the mutant compared with the wild type was much less than determined previously. Initially the experiment was performed using 5.5×10^6 cells/ml, as prescribed by Dr. John Battista (80). The fold increase ranged from 6.3 to 2.2, with an average value of 3.8 (n=3). This experiment was performed only twice, but yielded similar results (7.6 and 3.1 fold increase), for an average of 5.4 fold increase in transformation efficiency.

There are some reasons that may have led to the inconsistency in the initial transformation experiments. The cells were stored frozen in a -80^oC freezer. They were moved from ice directly into a freezer box and placed in the freezer rather than being snap frozen in liquid nitrogen first. This results in uneven freezing rates and therefore uneven survival of the freezing process from tube to tube, depending on the relative positions of the tubes in the freezer box. Secondly, there was probably a good deal of variability between the effective concentrations of antibiotic on the selective plates. Care was not taken to make all streptomycin plates in one large batch to ensure continuity. Rather, plates were made as needed using frozen stocks of antibiotics which were subjected to freeze thaw cycles. Additionally, left over plates from older batches were combined with freshly made plates in some instances. Due to the lack of reproducibility and the low transformation efficiency of the wild type even on 5 µg/ml streptomycin, compared with other published results using genomic DNA from LS18 (2×10^{-4} transformants / viable cell/ µg DNA), the second method was employed (79). Under

these conditions the difference in transformation efficiency was less dramatic, but the mutant still had significantly higher transformation efficiency.

While the increase in transformation efficiency in the mutant is significantly higher than the wild type in the first procedure it is unclear what the underlying reason is. In all tests of the two strains capacity for withstanding cellular assault, the *recD* mutant proved to be inferior or equivalent to the wild type. One could postulate that under unfavorable conditions, *D.radiodurans* up regulates its capacity to uptake and use foreign DNA in an attempt to gain genetic material that may aid in surviving the deleterious conditions. In this case the *recD* mutant may be more sensitive to the negative effects of freezing, enhancing its propensity for integrating foreign DNA.

Table III. Transformation Efficiency of the *recD*::*kan* mutant

DNA	Antibiotic/ Concentration	Transformation Conditions	Efficiency of wild type	Efficiency Relative to Wild Type
LS18	5 µg/ml streptomycin	Frozen Cells	$4.7 \pm 0.1 \times 10^{-9}$	21 ± 11 10 ± 2
			$3.0 \pm 1.5 \times 10^{-9}$	9 ± 5 21 ± 11
			$2.3 \pm 1.0 \times 10^{-9}$	208 ± 89 28 ± 12
	50 µg/ml streptomycin	Frozen Cells	$3.8 \pm 1.9 \times 10^{-11}$	97 ± 51 30 ± 16
			$7.8 \pm 14 \times 10^{-11}$	149 ± 262 109 ± 190
pI3	3 µg/ml chloramphenicol	Frozen Cells	$1.5 \pm 2.1 \times 10^{-6}$	114 ± 40
LS18	5 µg/ml streptomycin	Fresh Cells Low Cell Density	$3.4 \pm 0.5 \times 10^{-5}$ $4.5 \pm 0.4 \times 10^{-4}$ $1.5 \pm 0.1 \times 10^{-4}$	6.3 ± 1.0 2.8 ± 0.4 2.2 ± 0.3
	5 µg/ml streptomycin	Fresh Cells High Cell Density	$8.6 \pm 0.8 \times 10^{-5}$ $1.5 \pm 1.3 \times 10^{-4}$	7.6 ± 0.8 3.1 ± 0.5

2.4 Discussion

2.4.1 Generation of the *recD* Mutants

A common method of determining the biological role of a protein is to create a null mutation in the gene. If the genomic sequence of the organism studied is known, a targeted mutation in the gene can be made by inserting an antibiotic resistance gene in place in the gene's sequence. This is known as insertional mutagenesis. The mutant is then tested for abnormal phenotypes. This approach is known as reverse genetics and was employed to study the biological role of the RecD protein in *D. radiodurans*.

The initial *recD*::*kan* mutant 1 and subsequent versions were generated without difficulty. Many colonies were obtained from 100 µl of the transformation using the PCR generated disruption sequence, as well as chromosomal DNA from previously transformed strains. The mutation appeared to be stable and complete after a single passage into liquid culture supplemented with kanamycin.   This was determined by performing PCR on cultures started from plates initially used for selecting transformants. Return of the wild type sequence was never detected by PCR or Southern blotting despite dispensing with kanamycin when growing the cells in liquid media. This does not completely rule out the possibility that there could be low levels of undetected wild type *recD* genes, however such a low frequency would likely not significantly affect the outcomes of the phenotyping of the *recD* mutants. Antibiotics were always used to maintain the mutant strains on solid media.

The Δ*recD*::*cam* mutant was not so easily obtained. Many attempts were made to generate the mutant from the wild type strain BAA-816, but chloramphenicol resistant colonies were never obtained. The Δ*recD*::*cam* mutant was obtained from the *recD*::*kan*

mutant 3 strain. The reason for this is unclear. The resistance genes utilized were of similar size. The lengths of DNA used for homologous recombination were different however. The disruption sequence for the *recD*::*kan* mutant strain had about 200 bp upstream and 2,000 bp downstream of the *recD* gene. The disruption sequence for the Δ*recD*::*cam* mutant strain had approximately 500 bp on either side of the *recD* gene. These differences in the DNA flanking the resistance gene could account for the inability of the wild type to be transformed to the Δ*recD*::*cam* mutant. Ultimately hundreds of colonies were obtained from 100 μl of the transformation.

Another difference is the relative amount of DNA resected from the recipient strain when the disruption sequences were recombined into the target locus. The disruption sequence used to make *recD*::*kan* mutant essentially deletes 50 bp of DNA when recombined into the wild type locus, and adds about 1 Kb of DNA. About 2.2 Kb would be removed when attempting to recombine the Δ*recD*::*cam* mutant disruption sequence into the wild type chromosome, replacing it with only 1000 bp. It does seem plausible that this difference could account for the inability to transform the wild type strain directly to the Δ*recD*::*cam* mutant strain. However, recombining the Δ*recD*::*cam* mutant disruption sequence into the *recD*::*kan* mutant strain results in a loss of over 3.5 kb, replacing only about 1 kb of DNA, with a net loss of a little over 2.5 kb. This is greater than the net loss of making the *recD*::*kan* mutant from wild type.

The most likely explanation is that the *recD*::*kan* mutant is more efficient at taking up and or incorporating foreign DNA. While it is still unknown what the mechanistic reason is for the enhanced competence of the mutant, it is certainly possible that the RecD protein plays a role in preventing non-homologous recombination. If this

is the case, transformation events that would not occur in the wild type strain could be

achievable in *recD* mutants.

2.4.2 Sensitivity to DNA Damaging Agents

The various cellular challenges posed to the *D. radiodurans recD* mutant result in

a variety of types of damage to multiple cellular targets. *D. radiodurans* has shown the

capacity to handle these insults to a greater degree than most any other organism (82).

Most extreme is its ability to handle gamma irradiation, known to damage DNA directly

and indirectly through the generation of reactive oxygen species (82). The organism has

shown an amazing capacity to repair DNA damage such as double strand-breaks (83).

The RecD protein of *D. radiodurans s*hares homology with DNA repair proteins involved

in double strand break repair in other organisms. The *recD* mutant was tested against a

panel of DNA damaging agents known to directly or indirectly cause double strand

breaks in DNA.

D. radiodurans recD mutants are sensitive to gamma and UV irradiation as well

as hydrogen peroxide. Gamma and UV radiation, and hydrogen peroxide are reported to

damage DNA in a variety of ways. Gamma and hydrogen peroxide directly cause DNA

breaks, and lead to the formation of reactive oxygen species which can alter DNA bases

through oxidation (84). UV irradiation causes cyclobutyl pyrimidine dimers and

pyrimidine (6-4) pyrimidone photoproducts (13). All three of these DNA damaging

agents have been shown to form double strand breaks, either directly or indirectly as a

result of the repair process (143). The phenotype is consistent with *recBCD* mutations in

E. coli (99), which show sensitivity to these and other DNA damaging agents. While

D.radiodurans has no obvious *recB* or *recC* homologs, however these results suggest that RecD participates in some form of DNA repair pathway, possibly through recombinational repair.

Inconsistent with this hypothesis is the apparent lack of sensitivity to the methylating agent MMC and alkylating agent MMS in *recD* mutants. Sensitivity experiments performed using the two agents ultimately reduced the surviving fraction of the initial wild type culture by at least four orders of magnitude. The mutant had the same sensitivity as the wild type to both agents. Nucleotide excision repair of single strand adducts and double strand cross links caused by reaction of DNA with MMC are known to form double strand-breaks in *E. coli* (86), and *D. radiodurans* (87). Furthermore, *D. radiodurans* recA, uvrD, polA, and mutants are all sensitive to MMC, indicating that DNA repair processes are responsible for tolerance of the drug (88-90). Interestingly though, Earl and Battista reported that *D. radiodurans* strain 302, which has a 144 bp deletion resulting in a loss of 34 bp from the beginning of the uvrA1 gene and 110 bp of the upstream sequence is sensitive to MMC but not gamma or UV irradiation (79). This suggests that there may be pathways for repairing damage from hydrogen peroxide, gamma and UV irradiation that are independent of repair pathways for MMC and MMS.

There is precedence for this peculiar resistance to certain DNA damaging agents and not others in *D. radiodurans*. Certain pentose phosphate shunt mutants have been found to be sensitive to gamma and UV irradiation, and hydrogen peroxide, but have not found to be MMC or MMS sensitive (91). Zhang *et al*. have demonstrated that mutants of genes in the pentose phosphate shunt pathway have a reduced pool of intermediates in

the pathway such as NADPH, which are important precursors to DNA biosynthesis and a source of reducing power. It is well known that *D. radiodurans* undergoes extensive DNA synthesis following exposure to gamma irradiation (92). They suggest that the pentose phosphate shunt aids in resistance to gamma and UV irradiation by providing precursors for DNA repair and reducing power for combating oxidative stress.

Hydrogen peroxide treatment and gamma irradiation are known to cause the formation of reactive oxygen species (84). It is therefore possible that RecD plays a role in an antioxidant pathway. This has in fact been postulated by a group which reported that *D. radiodurans* R1 *recD* mutants are twofold deficient in catalase B activity (104).

2.4.3 Effects on Gross Cellular and Nucleoid Morphology Assessed by Microscopy

Certain DNA repair gene mutants in *E. coli* have cells of variable length, including exceptionally long filaments. These filamentous cells also have striking defects in chromosome organization, with chromosomes stacked into aggregates in some regions, and other regions completely devoid of DNA (142). The experiments performed to assess differences in cellular and nucleoid morphology in *D. radiodurans* strains were not pursued at the resolution necessary to make in depth analysis. To be accurate, transmission electron microscopy should be employed (49). Nonetheless, the *recD* mutant cells appear similar in size and shape to the wild type strain from which they were derived in both growth phases tested. Both strains exhibit cocci and diplococci as expected for this organism. Tetrads were difficult to observe in phase contrast pictures, likely an issue of resolution limits. The fluorescence images reveal nucleoids of similar size and shape in both strains in both growth phases tested.

2.4.4 Effect on Transformation Efficiency

According to these experiments, *D. radiodurans recD* mutants are hyper-transformable with respect to the wild type strain. Therefore it seems likely that the protein plays a role in suppressing transformation. The difference in transformation between the wild type and mutant strain varies depending on the conditions used. The second set of conditions produced a more modest difference in efficiency but the results were more consistent, and the conditions used were more controlled.

Natural transformation has three main steps. First is the binding and internalization of the exogenous DNA. Secondly the DNA may be identified as foreign and degraded by exonuclease activity. Last is the integration of the DNA into the host's genome by homologous recombination or the formation of an autonomously replicating unit if plasmid DNA is used. The relative difference in transformation efficiency using genomic DNA, which must integrate into the host's genome was similar to that found using plasmid DNA known to replicate autonomously in *D. radiodurans*. Additionally if RecD is involved in degrading foreign DNA, then transformation of the LS18 DNA would likely be the same in mutant and wild type as LS18 has the same parental strain as the wild type used in this study. This suggests that the effect is a result of an enhancement of the organism's ability to uptake transforming DNA rather than an increase in homologous recombination, or effects on DNA degradation. However, this conclusion has not been confirmed.

The *recD*::*kan* mutants capacity to be transformed by the *recD*::*cam*^{r} linear disruption fragment and the wild types inability to do so, suggests a discrepancy in the way homologous recombination is performed in the two strains. It is possible that the

plasmid is erroneously recombined into the mutant's genome rather than maintained as an autonomous replicating unit, supporting the model that RecD functions in a recombination capacity. Unfortunately plasmids are not efficiently recovered from *D. radiodurans* strains, so testing for free plasmid in cell extracts is not possible. Southern Blotting using a DIG labeled version of plasmid pI3 as a probe could be performed to further suggest one mechanism over the other.

In appearance, this phenotype is similar to that seen in *E. coli* RecD mutants, which have demonstrated three to six fold higher levels of recombination (94-99). Another *E. coli* helicase that has been implicated in preventing homologous recombination is UvrD. It has been shown to inhibit homologous recombination by displacing RecA from RecA coated nucleofilaments (100). Contrastingly, a *uvrD* helicase mutant from *D. radiodurans* is less transformable than wild type (101). The means by which *D. radiodurans* RecD helicase suppresses transformation is undetermined.

Chapter 3 Catalase Activity from *Deinococcus radiodurans recD* mutant

3.1 Introduction

Many of the effects of ionizing radiation are a result of the production of oxidative stress in the form of super oxide ($O_2^{\cdot-}$) and hydroxyl radicals ($HO^{\cdot}$) produced by the radiolytic splitting of water (131, 132), detailed in chapter 1. Super oxide dismutase (SOD) converts super oxide to hydrogen peroxide (H_2O_2) and oxygen (O_2) gas (114, 61). Hydrogen peroxide generates $HO^{\cdot}$ and $HOO^{\cdot}$ using iron as a catalyst through a process known as Fenton chemistry. Hydroxyl radicals are damaging to a number of cellular components, such as DNA, proteins, and lipids (60, 61). Hydrogen peroxide is effectively degraded by catalase (115). *D. radiodurans* is 100 times more resistant to hydrogen peroxide than *E. coli*, and reportedly has 50 times higher baseline catalase activity (112, 38). Catalase activity has been demonstrated to be essential for normal resistance to gamma irradiation in *D. radiodurans* (116). Additionally, radioresistance increases with advanced growth phase in *D. radiodurans*. Coincidentally, catalase activity also increases in these later growth stages (112).

D. radiodurans RecD has been implicated in resistance to hydrogen peroxide (109, 111), and gamma irradiation (109). Proteomics experiments detect RecD protein only in later growth phases (mid log and later), concurrent with increased catalase activity (112, 67). Zhou et al. reported that *D. radiodurans recD* mutants have 2-3 fold reduction in catalase B activity (111).   They suggested that this could account for the sensitivity of the *recD* mutant to hydrogen peroxide. We decided to investigate this further in our *recD* mutant strain. Also, to test if RecD protein expression modulates catalase activity in response to growth phase, *in vitro* catalase assays were performed.

In response to hydrogen peroxide, *D. radiodurans* induces catalase activity. *D. radiodurans* expresses three catalase enzymes, A (I), A (II), and B, that can be detected in activity-stained gels (Fig. 3.1). Catalase B activity appears to be responsible for the increased catalase activity observed after treatment with hydrogen peroxide (112). Interestingly, sub lethal doses of hydrogen peroxide induce resistance to a subsequent lethal dose of hydrogen peroxide, gamma radiation, or UV radiation. This indicates a relationship in resistance to these forms of cellular insult. Supporting this relationship, *recD*::*kan* and Δ*recD*::*cam* mutants are sensitive to hydrogen peroxide, gamma radiation and UV radiation (109). Chloramphenicol, an inhibitor of protein synthesis, was shown to be capable of eliminating this inductive effect (112). This suggests that *D. radiodurans* needs protein synthesis to achieve an induced resistance to hydrogen peroxide and ionizing radiation. When the lethal dose of hydrogen peroxide was applied to cells at 4°C rather than at 30°C (normal growth temperature), the result was equivalent survivability for wild type and the *recD* mutant (results shown in Chapter 2, Fig. 2.14). Active processes such as protein synthesis would presumably be slowed considerably in cells incubated at 4°C. This suggests that the RecD protein confers resistance in a manner that requires active cellular processes such as modulation of protein expression. It seems possible that the RecD protein is a component of the catalase B regulation machinery. *In vivo* and *in vitro* experiments were performed to determine if the RecD protein plays a role in modulation of catalase B activity in response to a sub lethal dose of hydrogen peroxide.
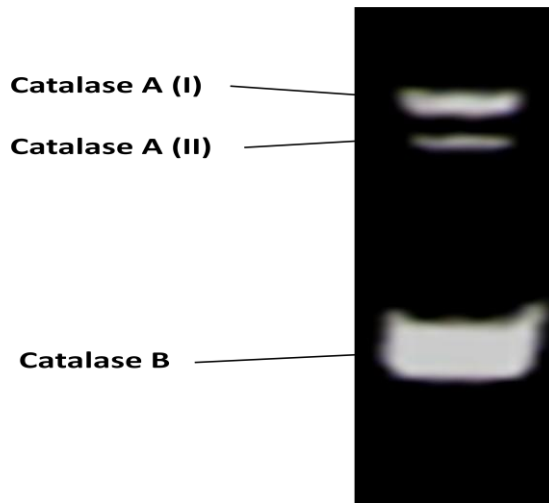
Figure 3.1 Zymogram of *D. radiodurans* catalase activity from Wang and Schellhorn, 1995. Native SDS-PAGE gels of untreated *D. radiodurans* cell lysates were incubated in a dilute solution of hydrogen peroxide. The gels were then stained with ferric chloride, and potassium ferricyanide, which darkens the gel wherever hydrogen peroxide is present. This reveals bands of catalase activity as unstained regions of the gel (112).

Recycling of damaged proteins has been shown to be important for recovery from irradiation in *D. radiodurans* (38). Some researchers have postulated that radiation resistance in highly radiation resistant bacteria is due to the antioxidative effects of manganese providing a protective effect to proteins. Additionally, Daly and others have suggested that protein damage, not DNA damage is the limiting factor for surviving the effects of radiation resistance. Supporting this assertion, DNA damage is detectable in radiation sensitive (such as *E. coli*) and resistant bacteria (such as *D. radiodurans*) at low doses of irradiation, and does not correlate with loss of viability in radiation resistant bacteria. However, protein damage detected as carbonylation of proteins by the Oxyblot Kit™ (Millipore) occurs at low doses of irradiation in radiation sensitive bacteria and only at very high doses in radiation resistant bacteria, correlating with loss of viability in both radiation resistant and radiation sensitive bacteria (58, 61).

It is therefore possible that a *D. radiodurans* mutant deficient in an antioxidant pathway would incur radiation induced oxidative protein damage at a lower dose of irradiation than the wild type. Conversely, both strains would have similar accumulation of DNA damage at irradiation doses below which protein damage begins to negatively impact DNA repair enzymes. If radiation induced lethality is indeed due to protein damage resulting from excessive accumulation of hydroxyl radicals generated from gamma radiation induced hydrogen peroxide interacting with iron, incubating treating cells with hydrogen peroxide should produce similar effects. To test this hypothesis *recD*::*kan* mutant 3 and the wild type strain from which it was derived were exposed to increasing doses of hydrogen peroxide. Protein damage was assessed with the Oxyblot kit™, and DNA damage was assessed by Pulsed Field Gel Electrophoresis (PFGE).

3.2 Materials and Methods

3.2.1 Preparation of cell lysates for *in vitro* Catalase Assays

To prepare lysates for comparison of catalase activities at various growth stages, overnight cultures were diluted 1:10 into 100 ml of fresh TGY. Cells were agitated using an orbital shaker at 30°C until an OD_{600} of 0.5 for mid log phase cultures, and OD_{600} of 2.0 for stationary phase cultures. Cells were then placed on ice for 10 min. before being pelleted in an ultra centrifuge (5,000 $\times$ G) at 4 °C. The supernatant was decanted and cells were stored at -80°C. The next day cells were thawed on ice and resuspended in 10 ml of 50mM potassium phosphate buffer, pH 7.4. The cells were then passed through a French press five times at 1,000 PSI. Lysates were then transferred to microcentrifuge tubes and the insoluble fraction was removed by centrifugation at 16,000 $\times$ G for 10 min

at 4°C. Cleared lysates were transferred to fresh tubes and kept on ice. Total protein concentration was determined using the Bradford assay with BSA as the protein standard. Lysates were then diluted to approximately 600 µg/ml.

To prepare lysates for induction of catalase assays, cultures were prepared as described for different growth phases with the following exceptions. Cells were grown to OD_{600} 0.4. After the cells reached the desired growth phase, a non-lethal inducing dose of hydrogen peroxide (10 mM) was added directly to the culture in the induced group. Nothing was added to the uninduced control cells. Cells were then agitated for another hour at 30°C. The cells were then pelleted, frozen, and lysed as described above.

3.2.2 Catalase Activity Determined by Spectrophotometric Assay

Determination of total catalase activity was performed spectrophotometrically. Assays were performed in 50 mM potassium phosphate buffer, pH-7.4. Hydrogen peroxide was diluted in the buffer to a concentration of 0.072% (~ 0.2 mM). Lysates were diluted approximately 1:15 yielding a final concentration of about 40 µg/ml. To a quartz cuvette, 250 µl of the hydrogen peroxide solution was added. Next, 250 µl of the diluted lysate was added, and the cuvette was immediately covered with parafilm, inverted four times, and inserted into the UV spectrophotometer (Cary 50 Bio, Varian Inc.). The absorbance at 240 nM was monitored every 0.000208 min using kinetics software. The slope was converted from absorbance units/min to µmoles hydrogen peroxide/min using the extinction coefficient for hydrogen peroxide at 240 nm which is $43.6 \text{ M}^{-1} \text{ cm}^{-1}$ (13). This slope was divided by the total protein in the assay, giving µmoles/min/µg protein. Finally this was converted to units of catalase activity/µg protein

using the standard of one catalase activity unit equals the amount of activity needed to break down one μmole of hydrogen peroxide per min.

3.2.3 Catalase Activity Determined by Zymogram Quantification

Catalase activity was quantified using zymograms. Two native (lacking SDS) 7.5% PAGE gels were run for 3 hr at 100 mV(constant) per experiment. Lysates were mixed with 6× native loading dye (0.5 M Tris pH 7.0, 50% glycerol) and kept on ice without boiling. One gel was loaded with 26 μg total protein per sample and the other was loaded with 0.5μg total protein per sample. The gels were rinsed after electrophoresis in 200 ml water with shaking for one hr. They were then soaked in 200 ml of a 0.003% hydrogen peroxide solution shaking for 10 min. The gels were rinsed with water as briefly as possible. The gels were then stained with 1% ferric chloride, and 1% potassium ferricyanide. The stain colors the gel green wherever hydrogen peroxide is present. Catalase destroys the hydrogen peroxide in the gel, in the area where the enzyme is present producing bands of less staining, a process termed reverse staining. The lack of the band in the gel is representative of the total catalase activity in that area of the gel. Gels were analyzed using UN-SCAN-IT software (Silk Scientific). A box was drawn around the largest band for a set of bands to be analyzed. The box was copied and pasted over the other bands to be analyzed. Additionally a box of the same size was pasted over a section of the gel where no bands were present to serve as background. The intensity of the band was expressed as the total pixels from its boxed area minus the total pixels from the background box.

3.2.4 Induction of Resistance to Hydrogen Peroxide *in vivo* Study

Twenty ml cultures of wild type and *recD*::*kan* mutant 3 were started from 1:10 dilutions of overnight cultures. Once the cells reached an OD_{600} 0.4, the cells were exposed to two different non lethal doses of hydrogen peroxide (5 mM or 10 mM) for one hour with shaking at 30°C. The media was exchanged in all cases and replaced with fresh media. All cells were then treated with 175 mM (final concentration) hydrogen peroxide for one hour at room temperature with no shaking. The tubes were then treated with 3,000 units of catalase/ml (Sigma based on 2,500 units/mg) for 30 minutes, to destroy the remaining hydrogen peroxide, then serially diluted and plated. Dilutions were the same as previous hydrogen peroxide assays.

3.2.5 Protein Damage in *D. radiodurans* Strains as Assessed by the Oxyblot Kit™

D. radiodurans wild type and *recD*::*kan* mutant 3 were grown to OD_{600} 0.5, in 20 ml of TGY under standard conditions. They were incubated with 0, 25, 50, 100, 150, 200, and 250 mM concentrations of hydrogen peroxide at 30°C for one hour, with shaking. The tubes were treated with 3,000 units of catalase/ml (Sigma based on 2,500 units/mg) for 30 min, to destroy the remaining hydrogen peroxide. The cells were then pelleted by centrifugation ($5,000 \times G$) for 10 min on ice. The pellets were lysed in 500 µl of lysis buffer provided by the kit using a Minibead Beater homogenizer (BioSpec) and silica beads. The samples were homogenized at 4,800 RPM for 60 seconds, then placed on ice for 5 min. The homogenation was repeated five times. Briefly, 10 µg of protein from the lysate extract were derivatized with 2,4-dinitrophenylhydrazine (DNPH) provided in the kit for 15 min, prior to stopping the reaction using the neutralization

buffer included in the kit. A sample derived from a *Halobacterium sp.* NRC-1exposed to

7.5 kGy gamma irradiation was processed in parallel to the *Deinococcus* samples and

included on the same blot as a positive control for the procedure. The samples were run

on a 4 to 20% SDS-PAGE gradient gel. The gel was transferred to immobilon-P

(Millipore) PVDF membranes overnight at constant amperage 30 mA for 12 hours. After

transfer, blots were blocked with blocking buffer, 4% BSA/TTBS (0.3% Tween 20, 20

mM Tris, 137 mM NaCl, pH 7.6), shaking for one hour at room temperature. The blot

was incubated for one hour, at room temperature, with agitation containing the rabbit

anti-DNPH primary antibody (diluted 1:150) provided by the kit in blocking buffer. Next

the blot was incubated with the kit's goat anti-rabbit HRP linked secondary antibody

(diluted 1:300) under the same procedure as the primary antibody. Bands were visualized

using ECL (Pierce), and Kodak MXB film+, Kodak GBX developer and replenisher,

using a Kodak film processor.

3.2.6 Pulsed Field Gel Electrophoresis of *D. radiodurans* Strains Exposed to Hydrogen
Peroxide

     D. radiodurans strains were grown and treated with hydrogen peroxide as

described for oxyblotting. Ten ml of cells were harvested by centrifugation ($5,000 \times G$)

for ten min. The cells were washed twice in 0.9% NaCl. The pellet was resuspended in

500 μl of 125 mM EDTA, pH 8.0. The cell suspension was mixed with 500 μl of 1.5%

Incert® Agarose (Lonza). The Incert® Agarose was melted in 125 mM EDTA, pH 8.0,

by microwaving, and cooled to 60°C prior to mixing with the cell suspension. The two

were mixed by inversion and 100 μl aliquots were pipetted into individual wells of

BioRad plug molds. Ten plugs per treatment were allowed to polymerize (10 min at -

20°C) then stored at 4°C. Plugs were to transferred to 40 ml of a 1 mg/ml lysozyme

solution (50 mM EDTA, pH 7.5), and incubated overnight at 37°C. The plugs were then

incubated in 10 ml of a 1 mg/ml proteinase K solution (0.5M EDTA, 1%

lauroylsarcosine, pH 9.3), for 6 hours at 50°C. The plugs were then washed three times

with 10 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and one time with 10 ml

restriction Buffer O (Fermentas). Plugs were subsequently digested in 2.5 ml restriction

Buffer O, supplemented with 50 units of *Not*I (Fermentas). The plugs were run on 1%

agarose (Pulsed Field Certified, BioRad) in $0.5 \times$ TBE (TBE buffer 25 mM Tris, 25 mM

boric acid, 0.25 mM EDTA), using a CHEF (BioRad) system. The conditions were as

follows, 12°C, 6 V/cm^2, linear pulse ramp (10-60 seconds), 120° switching angle for 22

hours. The gel was then stained with a 0.5 mg/ml EtBr solution, destained and

visualized.

3.3 Results

3.3.1 Catalase activity at Mid Log and Stationary Phase

Kinetics measurements on lysates from wild type and *recD*::*kan* mutants in both

mid log and stationary phase cultures suggest that the mutant has less catalase activity, at

either growth phase (Fig 3.2). These experiments indicate that the mutant has roughly

67% of the catalase activity found in the wild type in stationary phase, and 71% in mid

log. However, statistical analysis of the data suggests that the results are not significant.

An unpaired T-test comparing the wild type and mutant in exponential phase cultures

gave a p-value of 0.15, the same analysis on the data comparing the stationary phase

cultures was 0.064. Both wild type and mutant show the capacity to up-regulate catalase

activity somewhat in response to later growth phases. Wild type shows a 15% increase at

stationary phase compared to mid log, and the mutant has a 10% increase. Again

statistical analysis of the data suggests the result is not significant. A paired T-test

comparing the wild type data for both growth conditions gave a p-value of 0.066, while

the same test of the mutant data for both growth conditions gave a p-value of 0.22.

Paired T-tests were used when comparing cultures generated from a single colony,

otherwise an unpaired T-test was used. Statistical analysis was performed using

Graphpad found at:

(http://www.graphpad.com/quickcalcs/ttest1.cfm)

A.
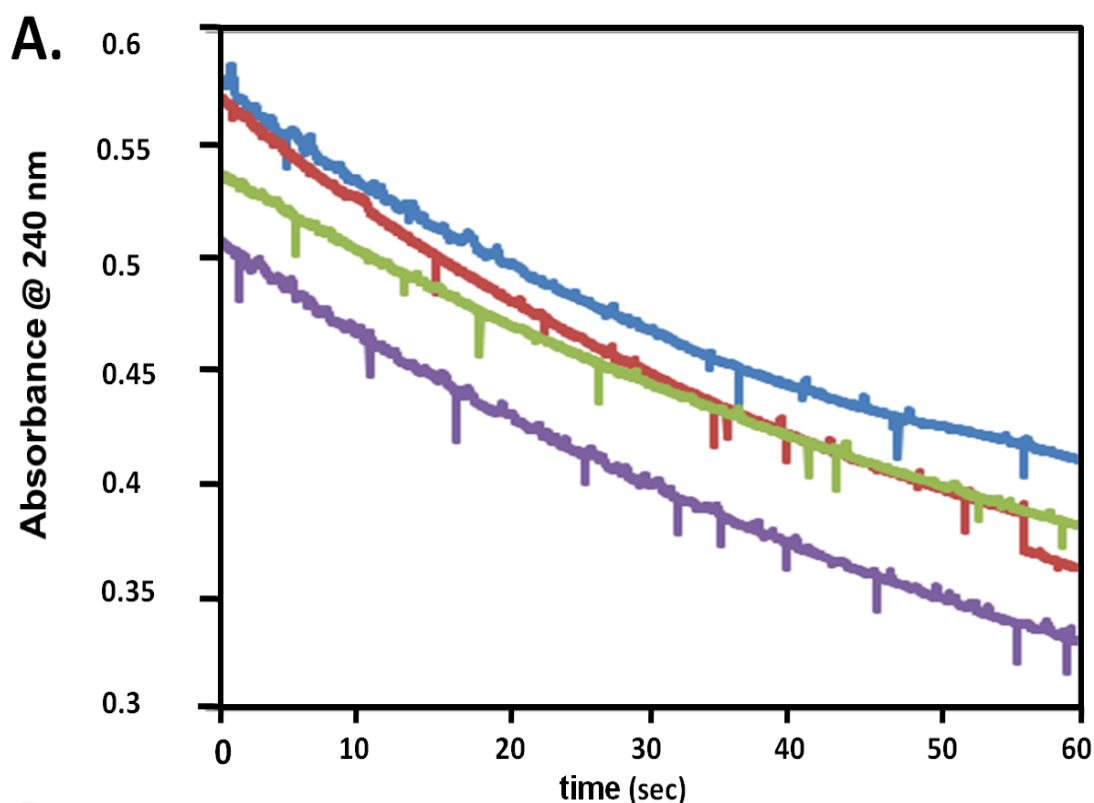
B.

Strain	Growth Phase	Units/mg protein	Determinations
Wild Type	Mid log	239 ± 43	4
	Stationary	331 ± 43	4
recD::kan	Mid log	191 ± 18	4
	Stationary	234 ± 50	4

Figure 3.2 Catalase activity of wild type *D. radiodurans* and *recD*::*kan* mutant 3. Cells were grown to OD$_{600}$ 0.5 for mid log, and OD$_{600}$ 2.0 for stationary. (A) Catalase reaction time courses from whole cell lysates (75 μg/ml final concentration) of; wild type mid log (blue line), wild type stationary (red line), *recD*::*kan* mutant mid log (green line), *recD*::*kan* mutant stationary (purple line) catalyzing the breakdown of 0.2 mM hydrogen peroxide measured by absorbance at 240nm. (B) Quantitation of the kinetics data, from two independent experiments with three determinations per experiment, (n=6). One unit of catalase activity is defined as the amount of enzyme needed to breakdown 1 μmole of H_2O_2 in one minute.

Zymograms of cell lysates from *D.radiodurans* strains (Fig. 3.3, and 3.6)

produced four bands in this study, rather than three as found in figure 3.2(112).

Therefore the bands in the zymograms generated in this study will be referred to as bands

1 through 4 counting from the top of the gel to the bottom. Estimations as to which

bands in the zymograms generated in this study correlate with those in figure 3.2 were

based on the relative intensities of the bands. It is likely that band 2 and 3 correlate to the

catalase A(I) and A(II) respectively, in figure 3.2 (112). In figure 3.2 the band at the top

of the gel which is catalase A(I) is more intense than the one immediately below it which

is A(II). Of the three bands in the top half of the zymograms generated in this study, the

middle band is the most intense. The best explanation of the banding pattern in the top of

the zymograms from this study would be that band 2 the most intense of the upper three

bands would be catalase A(I), and band 3 would correspond to catalase A(II) the less

intense band just below the band for catalase A(I). Of the three forms of catalase, B has

the most activity under these conditions (Fig. 3.2), and almost certainly correlates with

band 4 in the zymograms generated in this study. The origin of band 1 is unclear, as

genome sequencing of *D. radiodurans* revealed three potential catalase genes (12).

Figure 3.3A and B show there is no significant difference in the wild type and

mutant in band 4 catalase activity. Quantification of the bands in figure 3.3C, which have

5% as much loaded protein reveals that band 2 has less activity relative to band 4 than

found in figure 3.3A. It seems that while both cell lines are able to increase band 4

activity in response to higher cell concentration, wild type has a much greater capacity to

do so. Both cell lines have comparable levels of band 1 and 2 at matched growth phases.

Interestingly, both the wild type and mutant reduce band 2 activity in stationary phase

compared to mid log phase. Again, band 3 activity is different between the strains. At

mid log phase the difference is 2 fold, and about 3 fold in stationary phase. Additionally,

both cell lines are able to induce catalase activity in response to a non lethal dose of

hydrogen peroxide.
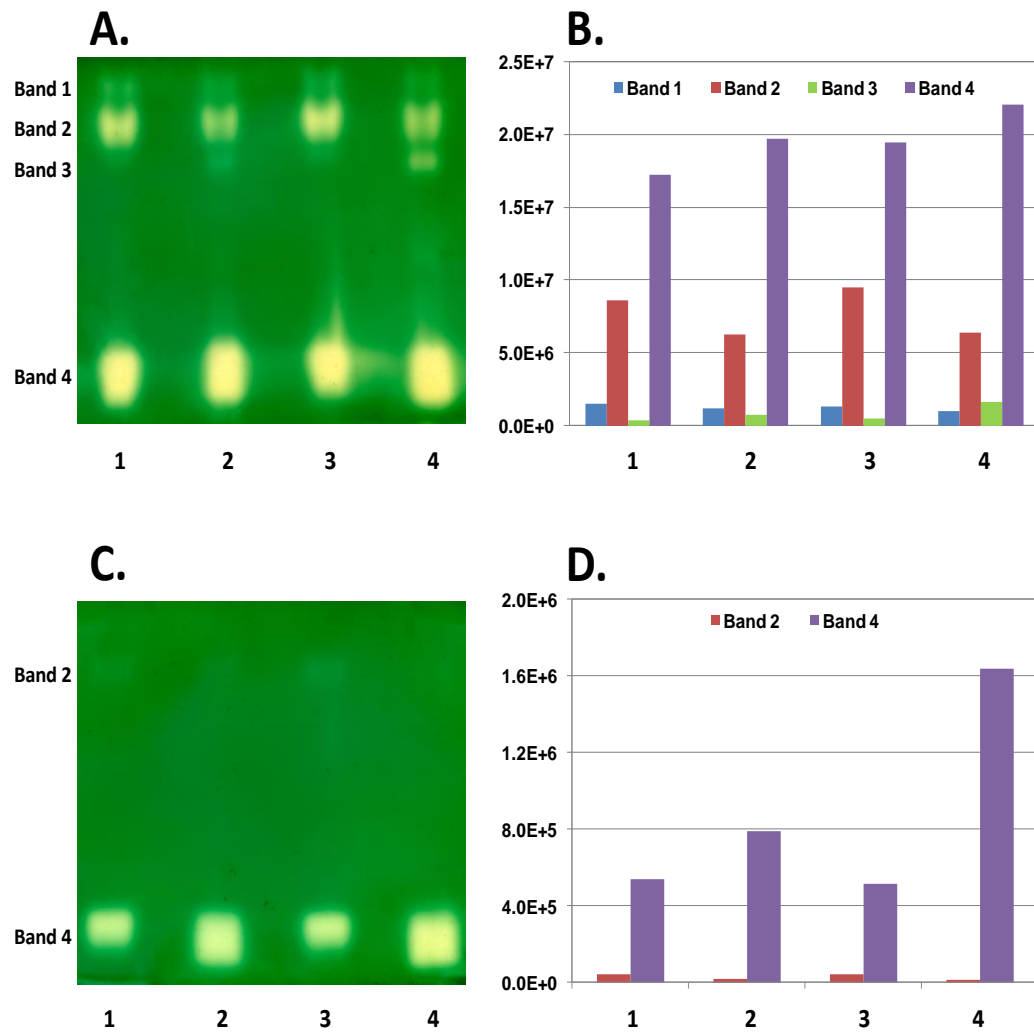
Figure 3.3 Zymograms of wild type *D. radiodurans* and *recD*::*kan* mutant stained for catalase activity. Cells were grown to OD_{600} 0.5 mid log, and stationary OD_{600} 2.0. (A) 37.5 µg total protein. Lane 1 *recD*::*kan* mutant mid log phase, lane 2 *recD*::*kan* mutant stationary phase, lane 3 wild type mid log phase, lane 4 wild type stationary phase. (B) Histogram of gel in Panel A, units in total pixels. (C) 1.9 µg total protein, same lane assignment as in A. (D) Histogram of gel in Panel C, units in total pixels. The experiment was performed twice with comparable results.

3.3.2 Induction of Resistance to Hydrogen Peroxide After Pretreatment With Non-Lethal Doses of Hydrogen Peroxide

In response to 175 mM hydrogen peroxide for one hour, *recD* mutants exhibited one tenth the viability of wild type cells. In wild type cells, the pretreatment with 10 mM hydrogen peroxide resulted in a 10 fold increase in resistance to the second (lethal) dose, while the pretreatment with 5mM hydrogen peroxide resulted in an 8 fold increase in resistance. In *recD*::*kan* mutant 3 cells, the pretreatment with 10 mM hydrogen peroxide resulted in a 17 fold increase in resistance to the second (lethal) dose, while the pretreatment with 5 mM hydrogen peroxide resulted in a 12 fold increase in resistance (Fig 3.4). However, this experiment has been difficult to reproduce, as frequently little or no induction was obtained for either strain. These results suggest that RecD is not responsible for induction of resistance to hydrogen peroxide.
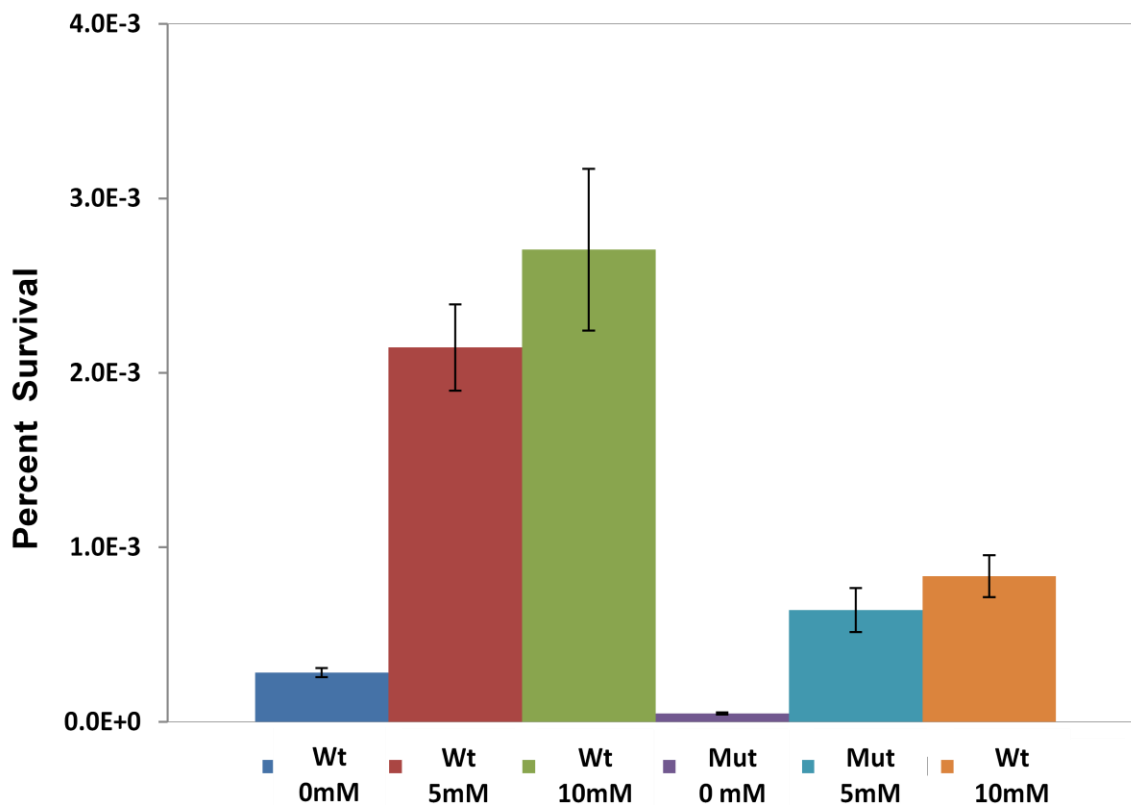
Figure 3.4 Survival of wild type *D. radiodurans* and *recD*::*kan* mutant to 175 mM H_2O_2 after pretreatment with a non-lethal dose of H_2O_2. Cells were grown to OD_{600} = 0.4, then induced with 0, 5 or 10 mM H_2O_2 for 1 hour 30°C. Cells were then treated with 175 mM H_2O_2 for one hour at room temperature. Results are from one experiment (n=3).

3.3.3 Catalase Activity After Pretreatment With 10 mM Hydrogen Peroxide

Table I shows that *recD*::*kan* mutant 3 has 67% of the total catalase activity of wild type *D.radiodurans* under the control conditions. An unpaired T-test gave a p-value of 0.047. This is at the threshold of what is generally accepted as significant. Additionally, wild type cells have a 10% increase in total catalase activity after the pretreatment with a non-lethal dose of hydrogen peroxide. A paired T-test gave a p-value of 0.12 suggesting it is not a significant result. The *recD* mutant also shows an increase in catalase activity after pretreatment, roughly 15%. A paired T-test gave a value of 0.0015 suggesting the *recD* mutant can significantly increase its catalase activity in response to pretreatment. Paired T-tests were used when comparing cultures generated from a single colony, otherwise an unpaired T-test was used. Statistical analysis was performed using Graphpad found at:

(http://www.graphpad.com/quickcalcs/ttest1.cfm)

A.
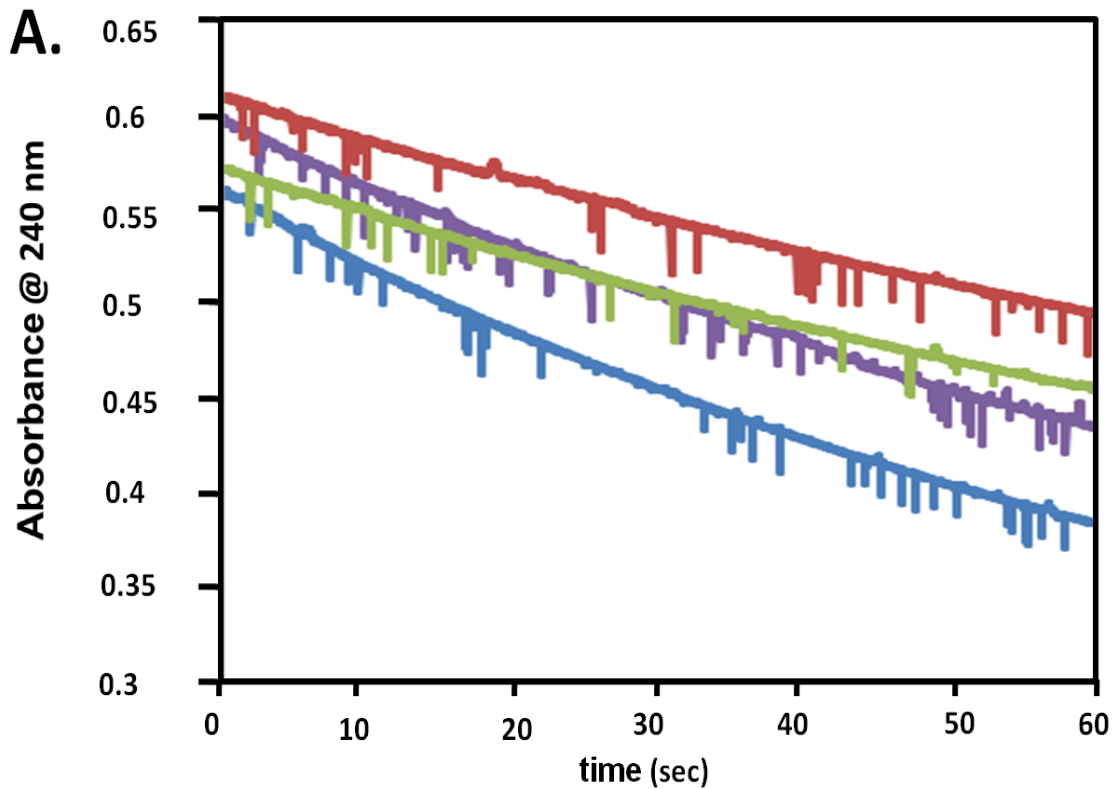
B.

Strain	Pretreatment	Units/mg protein	Determinations
Wild Type	Induced	302 ± 37	4
	Control	276 ± 7	4
recD::*kan*	Induced	220 ± 21	4
	Control	185 ± 55	4

Figure 3.5 Catalase activity of wild type *D. radiodurans* and *recD*::*kan* mutant 3.
Cells were grown to OD_{600} 0.4, then induced with 10mM H_2O_2 for 1 hour at 30°C. (A) Catalase reaction time courses from whole cell lysates (52 µg/ml) final concentration of; wild type mid log (blue line), wild type stationary (red line), *recD*::*kan* mutant mid log (green line), *recD*::*kan* mutant stationary (purple line), catalyzing the breakdown of 0.2 mM H_2O_2 measured by absorbance at 240 nm. (B) Quantitation of the kinetics data, from two independent experiments with three determinations per experiment, (n=6). One unit of catalase activity is defined as the amount of enzyme needed to breakdown 1 µmole of H_2O_2 in one min.
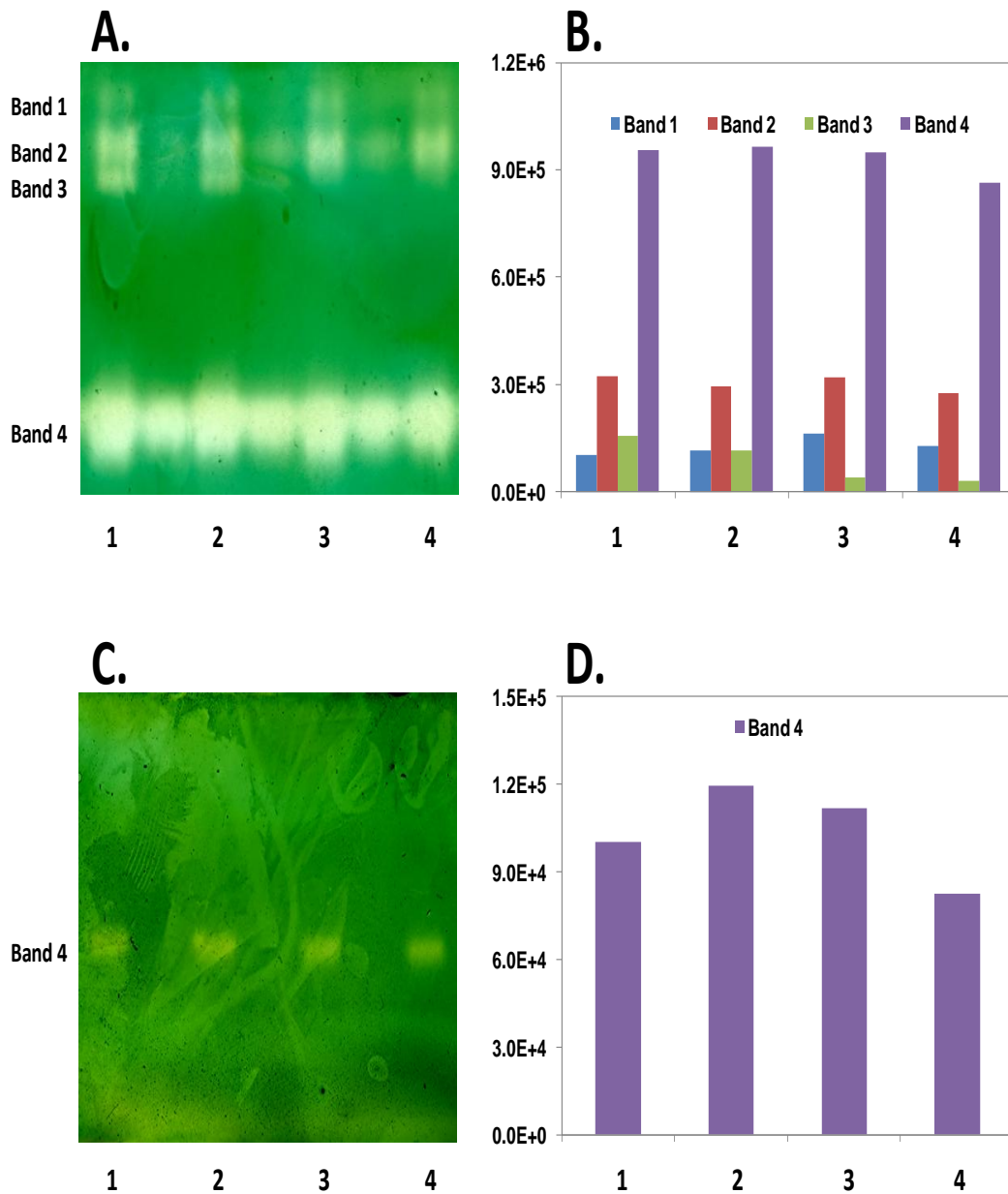
Figure 3.6 Zymograms of wild type *D. radiodurans* and *recD*::*kan* mutant 3 stained for catalase activity. Cells were grown to OD_{600} 0.4, then induced with 10 mM H_2O_2 for 1 hour 30°C. (A) 26.0 μg total protein. Lane 1 wild type induced, lane 2 wild type uninduced, lane 3 *recD*::*kan* mutant induced, lane 4 *recD*::*kan* mutant uninduced. (B) Histogram of gel A, in panel units in total pixels. (C) 0.5 μg total protein, same lane assignment. (D) Histogram of gel in panel C, units in total pixels. The experiment was performed twice with comparable results.

Zymograms from lysates of cells pretreated with hydrogen peroxide versus controls show very little difference between cell type or treatment group in band 4 catalase activity (Fig. 3.6D). Also, there is very little difference between all four examples of band 1 and 2 activity. However, the mutant seems to have a 4 fold reduction in band 3 catalase activity under both conditions tested compared with wild type. The wild type cells seem to have a modest increase in band 3 activity (roughly 25%), while the mutant shows an increase of approximately 18% (Fig. 3.6B). It is possible that the *recD* mutant is incapable of wild type control of the catalase activity found in band 4. It is unlikely that it has physiological relevance to the resistance of the *recD* mutant to hydrogen peroxides, as the catalase activity found in band 4 is a very minor contributor of overall catalase activity as determined by these experiments.

3.3.4 Oxyblot of Cell Lysates Exposed to Hydrogen Peroxide

Figure 3.7 is an image generated from an Oxyblot of *D. radiodurans* strains wild type BAA-816 , *recD*::*kan* mutant strain 3, and an *Halobacterium sp.* NRC-1 The *Halobacterium sp.* NRC-1, exposed to 7.5 kGy of gamma irradiation was included in the blot as a positive control for the procedure produced bands of strong reactivity to the anti DNPH antibody. It was not the best choice for a positive control. Ideally a sample made from *D. radiodurans* that had been damaged by a form of oxidative damage known to generate a signal in oxyblots such as a high dose of gamma irradiation should have been used. Unfortunately we were unable to have access to the gamma source to generated this control. Both the wild type *D. radiodurans* and *recD* mutant strain failed to react with the anti-DNPH antibody, while the highest dose of hydrogen peroxide treated cell

lysates (250 mM) produced similarly faint bands of immunoreactivity with the anti-

DNPH antibody. It is important to note that this experiment was only performed once

and would need to be repeated at least twice to confirm the result.

1   2   3   4   5
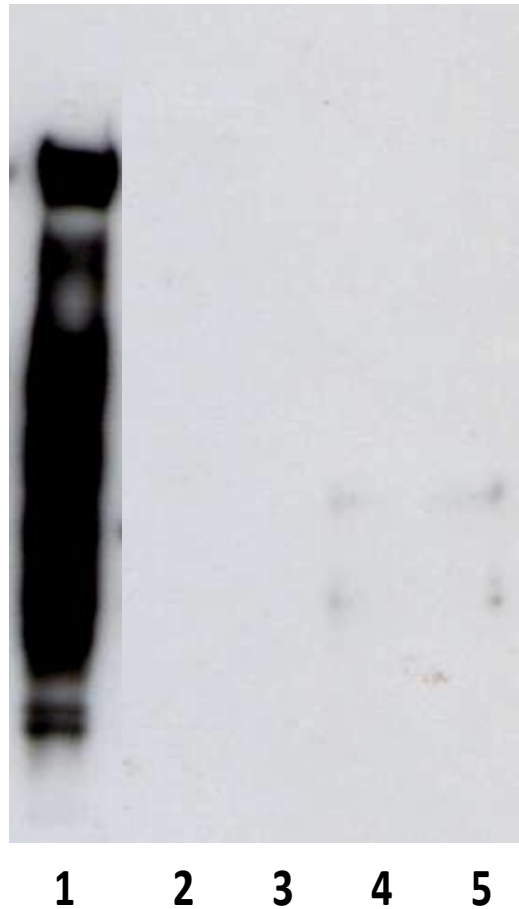
**Figure 3.7 Oxyblot of *D. radiodurans* wild type strain, and *recD*::*kan* mutant 3
exposed to hydrogen peroxide.** Lane 1, *Halobacterium sp.* NRC-1 exposed to 7.5 kGy
of gamma irradiation, which was prepared and blotted in parallel with the D. radiodurans
samples as a positive control. Lanes 2 and 4: wild type, untreated (lane 2); 250 mM
hydrogen peroxide (lane 4). Lanes 3 and 5: *recD*::*kan* mutant 3, untreated (lane 3); 250
mM hydrogen peroxide (lane 5). The experiment was performed once.

3.3.5 Pulsed Field Gel Electrophoresis of Hydrogen Peroxide Treated *D. radiodurans*

The PFGE image reveals a banding pattern characteristic of *Not*I digested *D. radiodurans* genomic DNA in the untreated controls lane 1 in figure 3.8 A and B (83). The *recD*::*kan* mutant 3 (Fig 3.8 B) had bands of less intensity compared to the wild type lanes (Fig. 3.8 A). In both wild type and mutant, the banding pattern is blurred in lanes with DNA from cells treated with hydrogen peroxide. The wild type maintains discrete bands at the lowest dose of hydrogen peroxide tested (25 mM) shown in figure 3.8A lane 2, although the bands appears to have fainted slightly. Figure 3.8B lane 2 indicates a loss of banding in the *recD* mutant. All lanes containing DNA from cells exposed to hydrogen peroxide doses at or above 50 mM show a complete loss of the *Not*I DNA digestion banding pattern seen in the untreated groups.
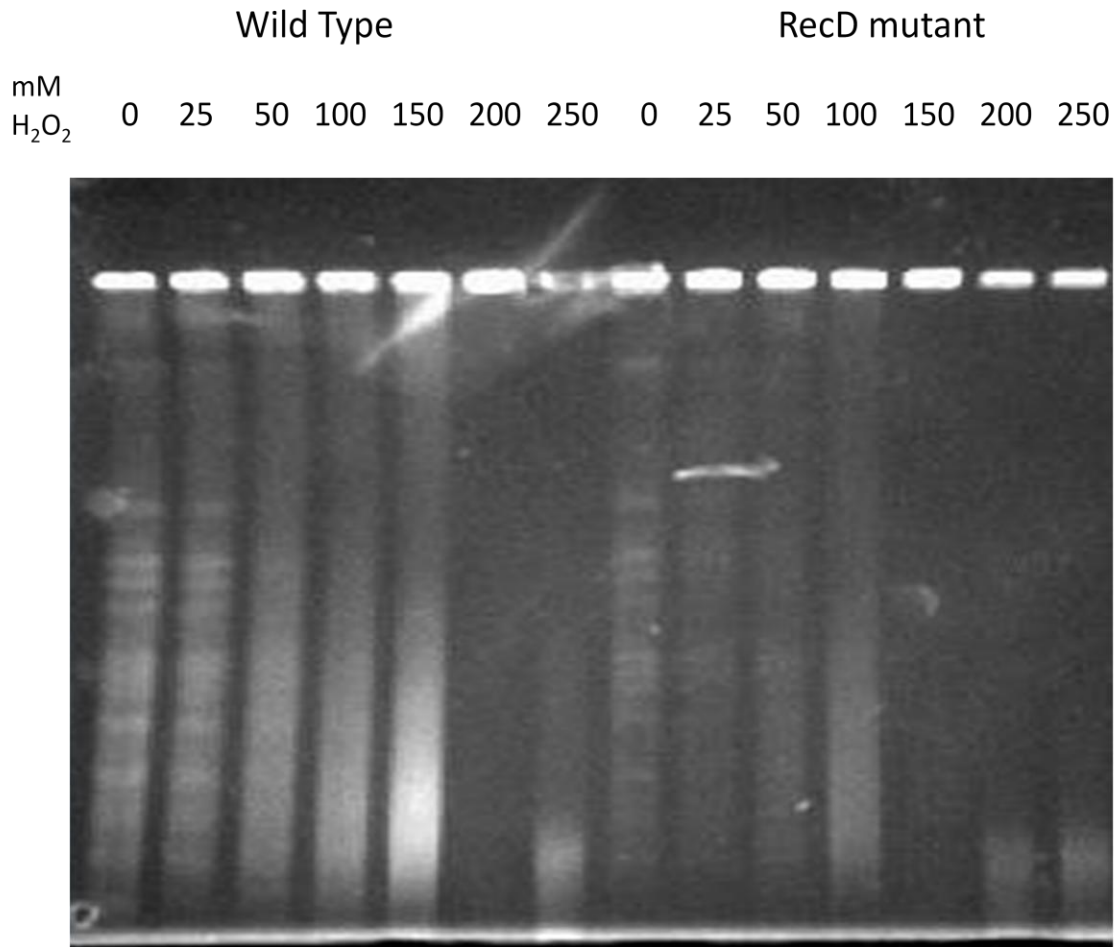
Figure 3.8 Image of Pulsed Field Gel Electrophoresis of wild type *D. radiodurans* recD::*kan* mutant 3 Exposed to Hydrogen Peroxide. (A) *Not*I digested chromosomal DNA from wild type strain BAA-816 cells exposed to indicated doses of hydrogen peroxide. (B) *Not*I digested chromosomal DNA from *recD*::*kan* mutant 3 cells exposed to indicated doses of hydrogen peroxide. The experiment was performed once.

3.4 Discussion

3.4.1 Effects on Catalase Activity

The purpose of this study was to determine if *recD* mutants have reduced catalase

activity, and if this contributes to the phenotypes described in chapter 2. The *D.*

radiodurans recD::*kan* mutant has demonstrated a reduced capacity to endure cellular

damage in response to hydrogen peroxide, and gamma irradiation. Both of these agents

have been shown to generate reactive oxygen species (59-61, 118). The *recD* mutant was found not to be sensitive to the DNA alkylating agents MMC or MMS. It also shows a small but significant reduction in baseline catalase activity in these studies (figure 2, 5). These findings contrast with the results presented by Zou et al who noted a twofold loss of catalase B activity in *recD* mutants in exponentially phase cells (111). These results suggest that the RecD protein may be involved somehow in an antioxidant pathway. However, the mutant is hypertransformable, suggesting a role in DNA metabolism.

The loss of approximately 30% of total catalase activity determined by the spectrophotometric assay could be enough to explain the observed phenotypes. However, this seems unlikely as knocking out the major catalase (catalase B) resulted in little sensitivity to gamma irradiation and hydrogen peroxide (113). The zymograms seem to indicate the main difference between the catalase activities of the two strains lies in band 3. According to figure 4b, band 3 accounts for roughly 5% of the total catalase activity for the wild type strain in stationary phase. This is severely over estimating the percent activity for band 3 as band 4 is too intense to be accurately quantified by this gel. For example, the zymogram in figure 4b shows that band 2 is roughly 30% as intense as band 4 in the wild type stationary phase lane. However in figure 4D, band 2 is only 0.75% of band 4 in lane 4. This would mean that band 3 in the wild type stationary phase is about 0.15% of the total catalase activity. It is possible that the zymogram doesn't give an accurate representation of the physiologic activities of the catalase enzymes. Under severe oxidative stress, the pH in the cytoplasm could change. Perhaps under these conditions another catalase, such as the one responsible for band three's activity could be the major contributor of catalase activity. This possibility could be explored by

performing the zymograms at various pH levels. Again, it could be that the kinetics

experiments are performed under conditions that don't accurately reflect the *in vivo* levels

of catalase activity under extreme oxidative stress. Lastly, it could be that the protein

responsible for the activity detected in band 3 is located in a critical cellular location,

making its total activity secondary to where it performs its function.

It is possible that the RecD protein participates in a regulon for handling oxidative

stress, and that it has an additional role in preventing foreign DNA from being

incorporated into the genome.   Additional experiments that could be done would be to

knock out the protein responsible for the catalase activity in band 3 and compare the

phenotype to the *recD*::*kan* mutant. This could be followed up by quantitative RT-PCR

on the mRNA of the catalase responsible for the activity of band 3 in wild type versus

recD::*kan* mutant. If the transcript is found in lower concentrations in the mutant we

would be able to postulate that RecD modulates catalase activity at the transcriptional

level. If not we could investigate the role of RecD post transcriptionally. Unfortunately

only one of the bands in the catalase zymograms has been correlated with a *D.*

radiodurans gene (*katA*) (113).   This gene encodes the catalase responsible for band 4,

the major band in the assays presented here. Also, only three catalase genes have been

annotated for *D.radiodurans*. It may be that band one is not from a unique gene but

rather a transcriptional variant, or posttranscriptional modification of one of the other

catalase genes.

3.4.2 DNA and Protein Damage in *D. radiodurans recD* mutant Exposed to Hydrogen

Peroxide

Figure 3.7 suggests that proteins in both the mutant and wild type are minimally

damaged by incubation with hydrogen peroxide as assessed by Oxyblotting. Exposure to

a dose of hydrogen peroxide that results in a loss of viability of two and four orders of

magnitude in the wild type and mutant respectively, failed to offer evidence of significant

protein damage in either strain. Additionally, the difference in protein damage detected

did not vary significantly at a dose known to cause a difference of viability of over two

orders of magnitude.

Similarly, the results of the PFGE experiments are inconclusive. While the

mutant appears to lose distinguishable bands as early as the 25 mM hydrogen peroxide

dose in figure 3.8B, viewing of the actual gel on a UV light box suggested that the loss of

distinct bands is not different in the wild type and mutant. The overall DNA load in the

control lane and in other lanes insinuates that the DNA concentration present in the gel is

much lower in the mutant lanes (Fig. 3.8 A and B). The disappearance of the banding

pattern found in the untreated control disappeared in cells treated with 50 mM hydrogen

peroxide, which was the second to lowest dose tested. This implies that breaks in the

backbone of chromosomal DNA do occur in *D. radiodurans* treated with hydrogen

peroxide treatment. Treatment with 50 mM hydrogen peroxide under the conditions

employed for this experiment does not result in any loss of viability in either the wild

type or the mutant. Furthermore, wild type *D. radiodurans* exhibits no significant loss of

viability under these treatment conditions up to doses of 150 mM hydrogen peroxide, at

which point the *Not*I digested chromosomal DNA in these cells produces a smear at the

bottom of the gel in the PFGE experiment (Fig. 3.8A). This result reverberates the notion that repair of extensive breakage of *D. radiodurans'* chromosomes is easily repairable by the organism.

The PFGE experiments suggest that extensive chromosomal breakage occurs in wild type *D. radiodurans* at the same rate as in the *recD* mutant in response to hydrogen peroxide. The oxyblot experiment suggests that protein damage as assessed by carbonylation is not significantly different between the wild type and *recD* mutant. These results suggest that the increased sensitivity of the *D. radiodurans recD* mutant to hydrogen peroxide is not due to a lack of ability to reduce the cellular damage incurred from hydrogen peroxide. It is a most likely the result of a reduced capacity to repair this damage. The results presented in figures 3.7 and 3.8 were pilot studies intended to determine if DNA or protein were differentially damaged in *D. radiodurans recD* mutants compared to the wild type strain.

Chapter 4 Production and Use of Anti-RecD Monoclonal Antibody

4.1   Introduction

Monoclonal antibody technology was developed in the nineteen seventies. The procedure was initially developed somewhat by accident. In 1973, Schwaber, J and Cohen fused human and mouse myelomas, tumor cells of plasma cells (immune system cells which secrete antibodies) attempting to understand the genetic control of antibody diversity. The cells resulting from the fusion (hybridomas) were found to secrete antibodies from both parental cell lines (119). In 1975, the technique was advanced by Kohler and Milstein (120) who along with Niels Kaj Jerne received the Nobel Prize for physiology or medicine in 1984. They fused myeloma cell line P3X63Ag8 with splenocytes from a mouse which had been immunized with sheep red blood cells. They then cloned the hybridomas (isolated individual clones by dilution into 96 well plates) and discovered that 10% of the clones secreted antibodies against sheep red blood cells into the culture media. As the clones are derived from myelomas, they are immortal and can be stored indefinitely in liquid nitrogen. This demonstrated that mono-specific antibodies recognizing a specific antigen could be generated. It was soon realized that this procedure could be used to make antibodies against a variety of antigens.

Before the development of monoclonal antibody technology, antibodies were made by injecting animals such as rabbits, goats, or horses with the antigen of interest. Blood was subsequently drawn and the serum was isolated by spinning out the insolubles and cells. The serum obtained was rich in antibodies against the injected antigen. The antibodies were polyclonal in nature, as they were derived from a number of different plasma cells, producing antibodies binding to different places (epitopes) on the antigen.

113

This provides advantages and disadvantages compared to monoclonal antibodies. Polyclonal antibodies react with many portions of an antigen and are therefore able to recognize different regions of an antigen. This may prove useful, as sometimes epitopes of an antigen of interest in an immunoassay are masked by other factors. A complication arises when antigens have epitopes that are similar to portions of other molecules such that the antibody binds to them with strong affinity, a phenomenon known as cross reactivity. Monoclonal antibodies are less subject to cross reactivity and are therefore much more specific in their recognition of antigen.

Today, monoclonal antibodies are still made primarily by immunizing mice with a selected antigen, and harvesting their splenocytes. A variety of myeloma cell lines now exist for use as fusion partners. These cell lines have been engineered so that they no longer secrete antibodies of their own, so that the derived hybridoma line will excrete only the intended antibody. These myeloma lines are hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) deficient. This greatly aids in the selection of hybridomas as it provides a means of removing the unfused myelomas. HGPRT is an enzyme critical in an alternate biochemical pathway used for purine synthesis used when cellular hypoxanthine is limited. Normal leukocytes have another pathway for purine synthesis that predominates under normal conditions. A folic acid analog, aminopterin inhibits the HGPRT pathway, allowing only the hybridomas to proliferate. Unfused splenocytes are selected against naturally due to their short lifespan.

A monoclonal antibody directed against *D. radiodurans* RecD was generated and designated Dr-RecD mAb. Monoclonal antibodies are a powerful tool for studying proteins. They can be used to detect, quantify, and purify proteins from cell extracts.

Additionally, they can be used to identify proteins that physically associate with the protein that the antibody was generated against, a technique known as co-immunoprecipitation.

4.2 Materials and Methods:

4.2.1 Purification of *Deinococcus radiodurans* RecD Protein

The protocol for purifying *D. radiodurans* RecD protein was worked out previously by Dr. Jianlei Wang (15). The *recD* gene was cloned into plasmid pET21a (Novagen Corp) forming plasmid pDr-RecD-pET21a, which expresses the RecD protein with a six-histidine tag on its C-terminus, in an IPTG inducible manner. The protein is purified by a two step process. A culture of *E. coli* expression strain BL21 (Invitrogen Corp) containing plasmid pDr-RecD-pET21a (which confers ampicillin resistance) is grown in a 2 L culture to OD_{600} 0.5 under conditions described in chapter 2. The culture is then induced to synthesize the recombinant protein with 0.5 mM IPTG (final concentration) at 30°C for three hours. The cells were harvested by centrifugation (5,000 $\times$ G) for 5 min at 4°C. The cells are resuspended in native binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, 10 mM PMSF, pH 7.8) supplemented with protease inhibitors for His-tagged proteins according to manufacturer's instructions (Sigma Aldrich P8849). The cells are lysed and nucleic acids are sheared by sonification on ice (Branson Sonifier 450). Insolubles are removed by centrifugation (16,000 $\times$ G) for 30 min, at 4°C. The lysate is passed over a 1 ml Ni^{2+}-NTA column made using ProBond™ Resin (Invitrogen corp). The column was washed with 100 column volumes of washing buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8). The

column was eluted using 10 column volumes of elution buffer (500 mM imidazole, 20 mM sodium phosphate, pH 7.8), 500 mM sodium chloride).

The first purification was dialyzed against 4 L of buffer A (20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol). The dialyzed protein was then passed over a 5 ml single stranded DNA-cellulose column (Sigma). The column was washed with 10 column volumes of buffer A with 150 mM NaCl. The column was then eluted with 20 ml buffer A containing 1.5 M NaCl. The elution was concentrated using a 50 ml centricon (Amicon) to 1 ml. The concentrated sample was dialyzed against 1 L of buffer A with 50% glycerol and stored in a -80°C degree freezer in 100 µl aliquots.

4.2.2   Immunization of Mice, and Harvesting of Splenocytes

The following procedures used to generate monoclonal antibodies were performed in the laboratory of Dr. David Pierce, at the Johns Hopkins School of Medicine, department of pulmonary and critical care. BALB/c mice (6-8 weeks old) were obtained from Jackson laboratories and maintained by animal services, Johns Hopkins University School of Medicine. On day one, 3 mice were immunized subcutaneously using a 22 gauge needle with 33 ng (at 3.5ng/µl) of purified RecD protein combined with 100 µl of Freund's complete adjuvant (Sigma-Aldrich F-5881). On day 21, the mice were immunized subcutaneously a second time, each mouse received 25 ng of purified RecD protein and 100µl Freund's incomplete adjuvant (Sigma-Aldrich F-5506). On day 25 the mice were euthanized by cervical dislocation. Procedures following euthanasia of the mice were done using sterile tissue culture techniques.

Spleens were removed and thoroughly teased apart using sterile forceps. The suspension

was then passed through a cell strainer separating splenocytes from the connective tissue.

The splenocytes were then spun down ($200 \times G$) for 10 min using a table top centrifuge.

Splenocytes were then resuspended in 5 ml ice cold DMEM (Gibco) and were kept on ice

till the cell fusion. Viable cell number was calculated using a hemocytometer and the dye

trypan blue (Fisher). Viable cells continuously pump the trypan blue from their

cytoplasm and appear translucent on the light blue background from the trypan blue stain.

Dead cells appear dark blue as they accumulate the blue stain. The concentration of

viable cells was calculated by the following formula:

$$\text{\# of viable cells/ml} \quad = \quad \frac{\text{total cells counted} \quad \times \quad 10 \text{ (dilution factor)} \quad x \quad 10^4}{\text{\# of large squares counted}}$$

The concentration of viable cells was 2×10^7 cells/ml in 5 ml, ultimately yielding 1×10^8

viable cells.

4.2.3   Preparation of Myeloma Cell line and Cell Fusion

The myeloma cell line SP2/0 German Collection of Microorganisms and cell

culture (DSMZ) was obtained two weeks earlier from Dr. Norman Peterson, Johns

Hopkins School of Medicine Department of Comparative Medicine. The cells arrived on

dry ice and were immediately placed in a -80°C freezer. Cells were thawed in a 37°C

water bath and spun down ($900 \times G$) for 10 min. The freezer media was removed and the

cells were resuspended in 10 ml complete media, DMEM 20% fetal bovine serum (FBS)-

(Hyclone #SV 30014.03), with antibiotic (Gibco #15240-096). The cells and media were

transferred to T-75 tissue culture flasks (BD-Falcon), and incubated in a 37°C incubator

with 5% CO_2. Four days prior to harvesting the splenocytes, the SP 2/0 cells were sub-cultured into three T-175 flasks at a density of 5×10^4 cells/ml in 30 ml complete media per flask.

On the day of the cell fusion, the number of viable myeloma cells was determined via the same method as for the splenocytes. The cell fusion is performed using a 2:1 ratio of splenocytes to myeloma cells. Therefore 5×10^7 cells were spun down ($200 \times$ G) for 10 minutes and resuspended in five ml of ice cold DMEM without serum or antibiotics and kept on ice. The two cell lines were then combined in a 50 ml centrifuge tube, and 10 ml of ice cold HBSS was added. The cells were then spun down at $200 \times$ G for 4 min to form a loose pellet. The supernatant was carefully removed using an aspirator. The tube was recapped and flicked several times until the pellet could be observed to move when the tube was tilted. Next, 1 ml of prewarmed (37°C) 50% PEG 1500 (Roche #783 641), 50% DMEM solution was added to the cell pellet, slowly over a one minute time period. While the PEG was added the cells were gently stirred. The mix was then incubated in a 37°C water bath for one minute. Then one ml of prewarmed DMEM was added in the same manner as the PEG/DMEM mixture, with gentle stirring over the course of one minute. The cells were then incubated in the 37°C water bath for one minute. Ten ml of prewarmed DMEM was added to the cells slowly with gentle mixing over the course of two minutes. Again the cells are incubated for one minute in a 37°C water bath. The cells were then spun down ($200 \times$ G) for 3 minutes, and the media aspirated. The cell pellet was gently resuspended in 50 ml HAT media, DMEM, plus 20% Fetal Bovine Serum, antibiotics, and HAT supplement (Sigma #H0262). Aliquots (180 µl) of the cell suspension were dispensed into two 96 well tissue culture plates (BD-

Falcon), these plates were designated high dilution. The remaining 16 ml were diluted back to 50 ml with fresh HAT media, and mixed by inversion. This cell suspension was then dispensed into three 96 well plates, 180 µl per well, and were designated low dilution. Plates were kept in a tissue culture incubator, 5% CO_2 at 37°C.

Four days later 150 µl of media was removed from each well of the five plates. Afterword, 150 µl of HT media composed of DMEM, FBS, antibiotics, and HT supplement (Sigma #H0137) was added to each well. Care was taken when removing and adding media, not to disturb the cells on the bottom of the plate. This was done so that wells containing colonies arising from a single cell were not confused with wells containing multiple colonies arising from independent hybridomas. Subsequently, half the media was changed with HT media as previously described every Monday, Tuesday, and Wednesday. Every day that the media was changed, the growth of colonies was monitored, using an inverted microscope (Olympus). Once colonies were well established, wells containing single colonies were selected for expansion. Cells were expanded by flushing a selected well with the media contained within, by trituration using a P200 pipetter. The suspended cells were transferred to one well of a 24 well tissue culture plate (BD-Falcon) and one ml of complete media was added. A total of 24 wells from the 96 well plates were expanded to a 24 well plate. Four days later the wells were confluent, and all the media was changed with fresh complete media. The old media was saved in 1.5 ml microcentrifuge tubes and tested for antibodies reactive to purified C-terminal His-tagged RecD.

4.2.4 Selection of Positive Clones, Subcloning and Cryopreservation

Selection of positive clones was simplified as the hybridoma cells secrete the antibody they are producing, allowing screening assays to be performed directly using the media in which the cells are growing. Initial screens were performed by Enzyme-Linked ImmunoSorbent Assay (ELISA). The procedure involves coating the surface of 96 well plates with a target protein. Next the wells are incubated with an antibody against the target protein (primary antibody), followed by an antibody specific to the primary antibody (secondary antibody) which has been conjugated to an enzyme for detection. An interaction between target protein and primary antibody is detected using a substrate appropriate to the detection enzyme and quantified using a 96 well plate reader.

The ELISA plates (BD-Falcon) were coated with purified RecD protein (4.5 ng/µl) in coating buffer (0.1 M $NaHCO_3$), 4°C overnight. The wells were then washed 4 times with distilled water. The wells were then blocked with a 3% BSA in coating buffer for one hour, 37°C. 50 µl of the tissue culture medium from each well in which cells were growing was transferred from the 24 well tissue culture plates to the appropriately labeled 96 well ELISA plates in triplicate, and incubated at 37°C for 1 hour. Three wells were incubated with unused tissue culture medium as a control. The tissue culture media was removed and the wells were washed 10 times with PBS (Gibco) with 0.05% Tween 20 (Sigma). A secondary antibody which is anti-mouse conjugated to alkaline phosphatase (#A5781) was diluted 1:1000 in 1% BSA/PBS and 25 µl was added to the wells followed by incubation for 1 hr at 37°C. The secondary antibody was removed and the wells were washed 10 times with PBS with 0.05% Tween 20. The interaction between the RecD protein and an antibody in the tissue culture media was visualized by

adding 25 µl of a solution made by dissolving 1 tablet of Sigma phosphatase substrate (#104-105). The plates were analyzed using a 96 well plate reader at 405 nM (BioRad).

Western blotting was performed to determine if the antibodies they secreted react with the purified protein in its denatured form. Purified C-terminal His-tagged RecD (100 ng/lane) was run on 7.5% SDS PAGE gels, and transferred to immobilon-P (Millipore) PVDF membranes using the mini protean system (BioRad). Kaleidoscope prestained protein ladder (BioRad) was used to as a molecular weight standard and a means of detecting completion of transfer. Blots were transferred overnight at constant amperage 30 mA for 12 hours. After transfer, blots were blocked with blocking buffer, 4% BSA/TTBS (0.3% Tween 20, 20 mM Tris, 137 mM NaCl, pH 7.6), shaking for one hour at room temperature. The blocking buffer was removed and was replaced with the tissue culture supernatant to be tested with shaking, at room temperature. The blots were then washed three times with TTBS. The secondary antibody was either anti-mouse alkaline phosphatase (Novagen #71045), or anti-mouse horse radish peroxidase (RDI #715035151). Blots were visualized with alkaline phosphatase linked secondary antibody using a STORM phosphorimager at a wavelength of 477 nM, and substrate ECF (Amersham). Blots visualized with horseradish peroxidase linked secondary were developed using ECL (Pierce), and Kodak MXB film+, Kodak GBX developer and replenisher, using a Kodak film processor.

Cells determined by ELISA to have antibodies recognizing C-terminal His-tagged RecD were expanded to tissue culture flasks (T-25 BD-Falcon). Once the cells reached confluence, samples were taken for cryopreservation. Hybridoma cells were collected by centrifuging the media at (900 × G) for 10 min. Five ml of supernatant from a confluent

culture was spun down. The supernatant was removed and the cell pellet was resuspended in 50% FBS, 40% DMEM, 10% DMSO (Sigma), and transferred to 1.5 ml cryovials (BD Falcon). The cells were then frozen at -80°C for up to one month before being transferred to liquid nitrogen for permanent storage. Once analyzed for specific antibody production by ELISA and Western blot, a clone P3/D10 was selected and subcloned by diluting the cells to approximately 1 cell per 100 µl and plated at 200 µl per well in 96 well plates. The plates were analyzed as before for colonies that appeared to arise from a single cell. Twenty four subclones were selected for expansion into 24 well plates. The clones were again tested by ELISA and Western blot. Two of these clones were selected for cryopreservation.

4.2.5 Detection of RecD Protein in *D. radiodurans* Whole Cell Lysates

Dr-RecD mAb was used to determine the level of RecD found in wild type strain *D. radiodurans* BAA-816, and to ascertain if *recD*::*kan* mutants were still able to express RecD. Western blots were run on whole cell lysates of the two strains as described for screening of the hybridomas. Purified RecD, or whole cell lysates of induced BL21 harboring plasmid pDr-RecD-pET21a, (undetermined protein concentration) were used a positive controls. The SDS PAGE gels for Western blotting *D. radiodurans* lysates were loaded with 50 µg of total protein as determined by Bradford assay.

4.2.6 Detection of C-terminal His-tagged RecD from *D. radiodurans*

To detect RecD expression in *D. radiodurans*, a RecD mutant was produced which had its *recD* gene fused to the 6×His sequence. It was designated C-terminal His-

tagged RecD mutant. This was accomplished by cloning the *recD* gene and 6×His tag

from pDr.-RecD-pET21a into pCR2.1 using Taq polymerase, and primers RecD1 (from

chapter 2), and Dr.RecDHis sequence below.

Dr.RecDHis   **5'-GGATCCTTTGTTAGCAGCCGGATCTCAGTG-3'**

The resulting plasmid (pCR2.1-RecD-HisTag) was transformed into competent *D.*

radiodurans BAA-816. The entire plasmid is inserted by homologous recombination by

a single cross over event. This integrates the entire plasmid at the end of the *recD* locus,

adding the codons for six histidine residues in frame with the *recD* gene.

Construction of His-tagged RecD
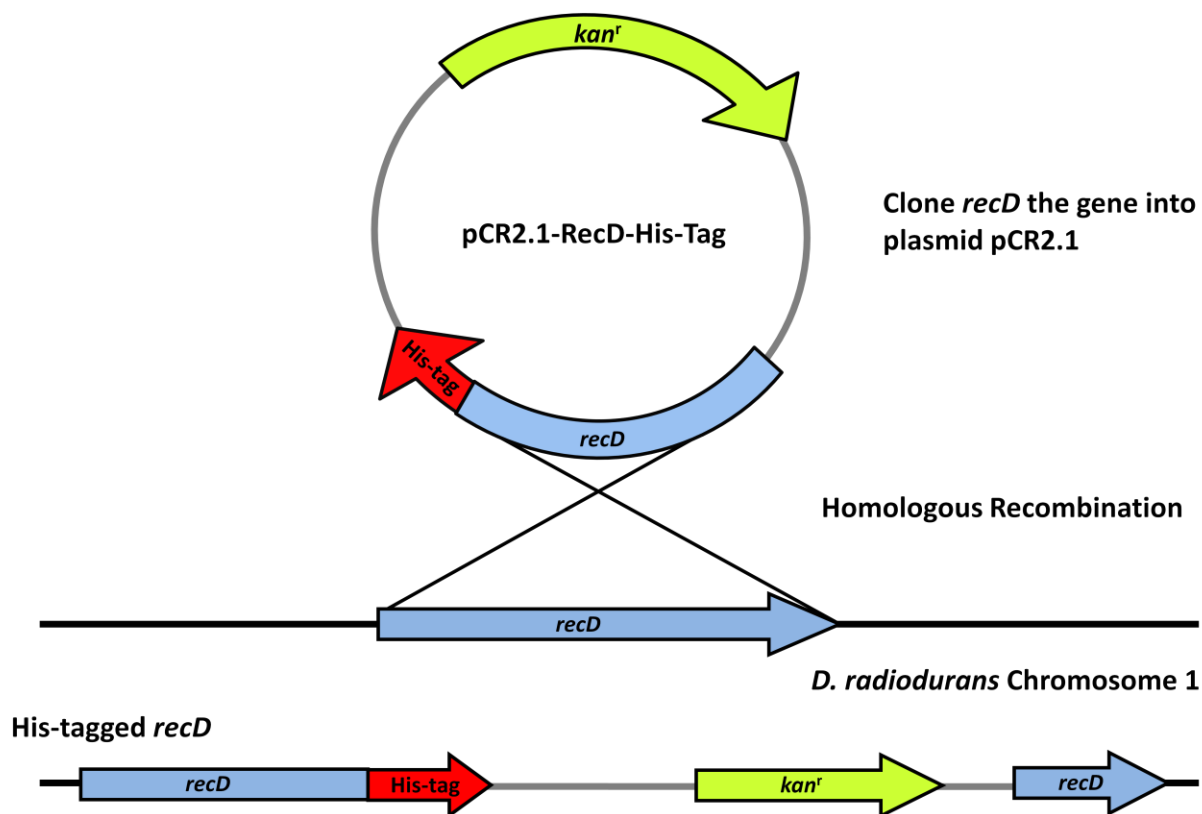
Figure 4.1 Schematic representation of the construction of C-terminal His-tagged *recD* in *D. radiodurans*. The full length *D. radiodurans recD* gene was cloned into plasmid pCR2.1 using primers RecD1 and Dr.RecDHis. The plasmid was transformed into wild type strain BAA-816 and selected for on kanamycin. The insertion occurs by homologous recombination as a single cross over event.

A 200 ml culture of *D. radiodurans* strain C-terminal His-tagged RecD mutant was grown to $OD_{600} = 0.7$ under standard conditions described in chapter 2. The cells were harvested by centrifugation ($5,000 \times G$) for 10 min, and lysed in approximately one ml of lysis buffer from the Profound Pull Down kit (Pierce) supplemented with 3 mM PMSF and protease inhibitor cocktail (for use with His-tagged proteins-Sigma). The

cells were lysed by vortexing at 4°C for 2 hr with approximately one ml of 0.1 mM glass beads (Bio Spec Products) in a 2.0 ml cryovial (BD-Falcon). The lysate was incubated with 50 µl of Ni^{2+} NTA (Probond™ Resin) slurry that had been (washed 5 times with lysis buffer), at 4°C for 30 min shaking. The Ni^{2+} NTA was collected by centrifugation (1,200 × G) for 1 min, and washed 5 times in wash buffer provided in the pull down kit. The bound proteins were eluted in 50 µl of the pull down kit's elution buffer containing 1M imidazole. As a control, the same procedure was applied to wild type *D. radiodurans*. Thirty µl of the elution was run on a 7.5% SDS PAGE gel, transferred to a PVDF membrane, and blotted.

4.2.7 Detection of Recombinant *D. radiodurans* C-terminal His-tagged RecD Protein

Will Shadrick, a PhD. Candidate in our lab made three additional constructs of the *D. radiodurans* recD gene for expression in *E. coli*. One version pDr.RecD-untagged-pET21a, expresses an untagged version of the RecD protein. Plasmid pDr.RecDNterm-pET21a expresses 289 amino acids of the amino terminal portion of RecD with a C-terminal 6×His tag. Plasmid pDr.RecD290-pET21a expresses amino acids 290 to 715 of the carboxy terminal end of RecD with a C-terminal 6×His tag. Whole cell lysates of IPTG induced BL21 harboring plasmid Dr.RecD-untagged-pET21a were tested for the anti-RecD antibody's capacity to detect untagged RecD. Both N and C terminal portions of RecD were expressed in BL21 and purified over a Ni^{2+}-NTA column. These constructs were employed to test if Dr-RecD mAb recognizes the 6×His tag or a portion of the RecD protein.

4.2.8 Immunoprecipitation of RecD Protein from *D. radiodurans* Whole Cell Lysates

A one liter culture of wild type *D. radiodurans* was grown to $OD_{600} = 1.0$. The cells were harvested by centrifugation ($5,000 \times G$) for 10 min, and were lysed in 15 ml of lysis buffer (40 mM Tris, 150 mM NaCl, 0.1% NP-40, 0.5 mM PMSF, 1.0 mM EDTA, 1.0 mM $MgCl_2$, 1.0 mM DTT, pH 7.4), supplemented with protease inhibitor cocktail for use with His-tagged proteins. The cells were lysed by passing through a French Press five times at 1,000 Psi. The lysate was cleared by centrifugation ($10,000 \times G$) for 30 min 4°C. The lysate was split into three 5 ml aliquots.

One aliquot was incubated with 60 ng of purified C-terminal His-tagged RecD protein, 5 ng and 50 µl of protein G beads (Pierce), prewashed $5 \times$ with lysis buffer, shaking at 4°C for 1.5 hours. The second aliquot was incubated under the same conditions without exogenous RecD protein. The third aliquot was the same as the second without antibody (mock immunoprecipitation) or addition of RecD protein as a negative control. The resin was collected by centrifugation ($1,200 \times G$) for 1 min, and washed 6 times with ice cold lysis buffer, using the mini-spin columns from the Profound Pull Down kit. The washes were retained for gel analysis. The bound proteins were released from the resin by dissolving the collected resin in 100 µl of SDS loading buffer. The samples were boiled, and the resin and insolubles were removed by centrifugation ($16,000 \times G$) for one min. 50 µl of sample was loaded on a 7.5% SDS PAGE gel. Gels were then silver stained.

4.3 Results

4.3.1 Clonal Selection

96 well tissue culture plates from the low dilution cell fusion had many wells without any colonies, some wells that appeared to have colonies arising from a single clone (one symmetrical clump of cells), and a few wells with multiple colonies. The high dilution plates had a few wells that appeared to have single colonies, but had many wells with multiple colonies. For this reason, 24 of the wells with single colonies from the low dilution plates were chosen for expansion, and tested for anti-RecD antibody production. All but a few of the initial clones grew well with characteristics similar to the SP2/0 myeloma line. Clone P3/D10 subclones also grew well, however a few subclones did not survive expansion into 24 well plates.

4.3.2 Tests of Clones for Anti-RecD Antibody Production

Cell culture supernatants from the initial twenty clones selected for expansion had absorbances at least 5 fold higher, and no more than 8 fold higher than control wells which were (95.3 ± 7.2) in the ELISA screen (data not shown).

Western analysis on the supernatants from the twenty expanded clones which grew well reacted strongly with RecD blotted onto PVDF. Clone P3/D10 gave the strongest reaction and was chosen for subcloning (Fig. 4.2).
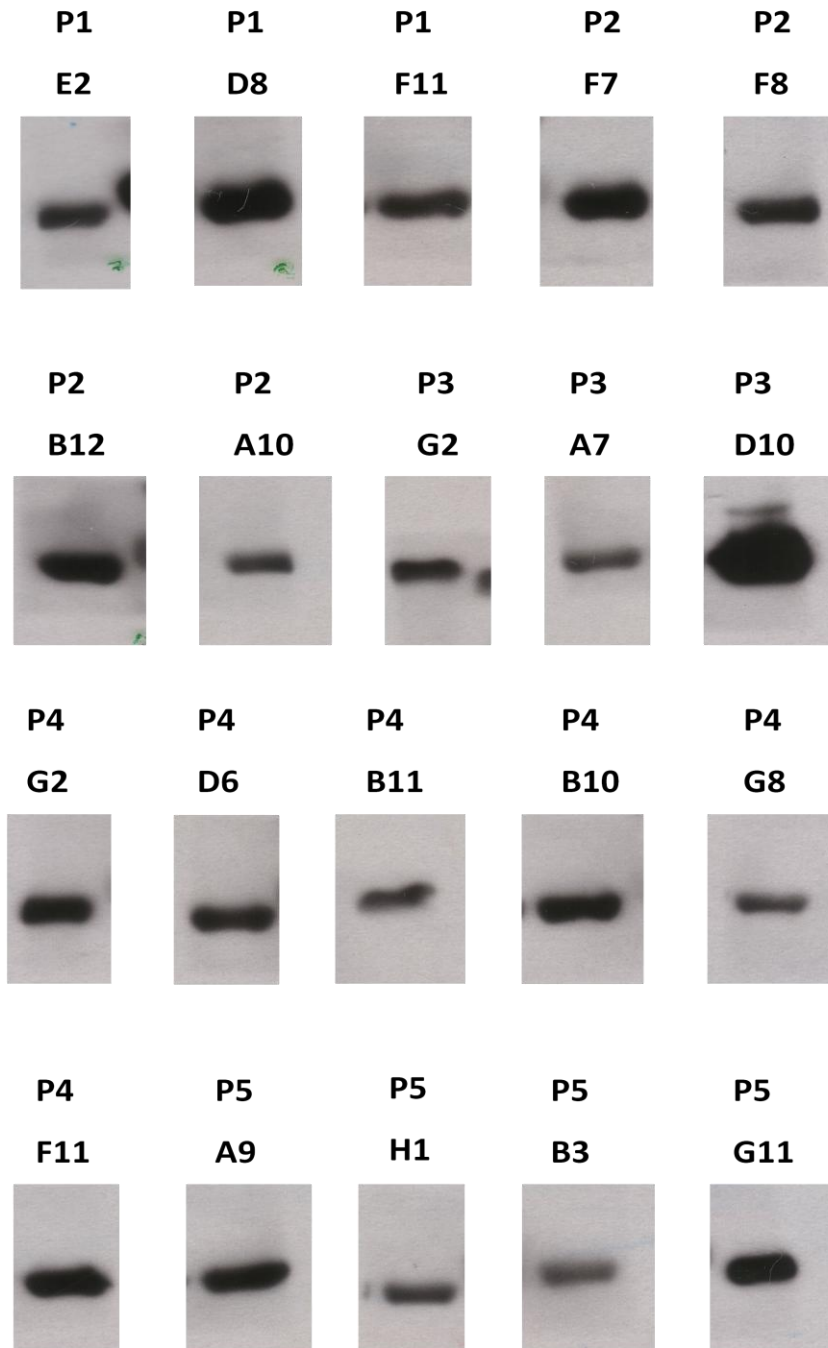
Figure 4.2 Western Blots of purified C-terminal His-tagged RecD blotted with supernatants from expanded hybridoma clones.   SDS PAGE gels containing purified C-terminal His-tagged RecD protein were transferred onto PVDF membranes and blotted with supernatants from indicated clones. Labels are as follows; the upper of the two numbers designates the 96 well plate from which the clone was originally isolated. The lower number designates the clone's position in the plate.

All subclones of P3/D10 performed well in ELISA assays, with absorbances above control similar to the initial selected clones. Western analysis showed that all expanded subclones were immunoreactive with purified C-terminal His-tagged RecD protein (Fig. 4.3).
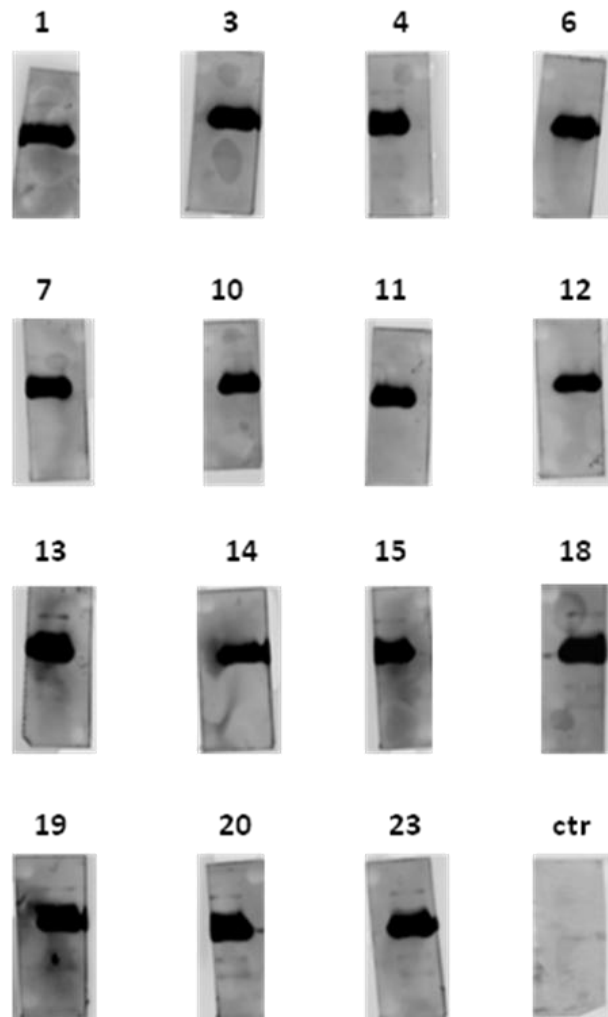
Figure 4.3 Western Blots of purified C-terminal His-tagged RecD blotted with supernatants from expanded hybridoma subclones of clone P3/D10. SDS PAGE gels containing purified C-terminal His-tagged RecD protein were transferred onto PVDF membranes and blotted with supernatants from indicated subclones of clone P3/D10.

4.3.3 Immunodetection of RecD Protein in *D. radiodurans*

Antibody mAB clone P3/D10-19 reacts readily with purified C-terminal His-tagged RecD and unpurified, untagged RecD expressed in *E. coli* BL21, and does not react strongly with any BL21 proteins (figure 4.4 A). The antibody's reactivity can be mapped to the N-terminal 289 amino acids of RecD (figure 4.4 C). Multiple efforts to detect RecD in wild type *D. radiodurans* in Western blots of whole cell lysates were unsuccessful. Increasing the protein load to the upper limit of SDS PAGE gels 50 µg/lane (figure 4.4 A and B) failed to produce bands of the correct apparent mobility in Western blots. An experiment was performed to determine if RecD expression could be induced by UV irradiation. Samples of wild type strain BAA-816 was grown to OD_{600} 0.7. Six ml of the culture was evenly distributed in an empty, uncovered 100 mm petri dish. The cells were subjected to 450 J/m^2 or 1350 J/m^2 as described in Chapter 2. The cells were allowed to recover for 30 or 90 minutes post irradiation at 30°C, shaking prior to Western blotting. No conditions tested produced bands of expected mobility. However, whole cell lysates of a D. radiodurans RecD mutant containing a C-terminal His-tagged RecD fusion protein purified over a Ni^{2+} NTA column did produce a band of correct mobility (figure 4.4C). Whole cell lysates of wild type D. radiodurans passed over Ni^{2+} NTA column did not result in a band of cross reactivity of RecD's mobility in this control.

4.3.4 Detection of *D. radiodurans recD* Constructs in *E. coli*

Western blot using antibody mAB clone P3/D10-19: detects purified C-terminal His-taggedRecD expressed in BL21, figures 4.4A lane 1, and B lane 1 respectively. It also detects recombinant untagged RecD from whole cell lysates of induced BL21 harboring pDr.RecD-untagged-pET21a, and does not detect any proteins from uninduced whole cell lysates of BL21 harboring pDr.RecD-untagged-pET21a, figures 4.4A lanes 4 and 5 respectively. Western blotting of Ni^{2+} NTA purified N-terminal peptide (amino acids 1-289) expressed in BL21 did produce a band of expected mobility, while the C-terminal peptide (amino acids 290-725) purified under the same conditions did not; figure 4.4D lanes 2 and 3 respectively.
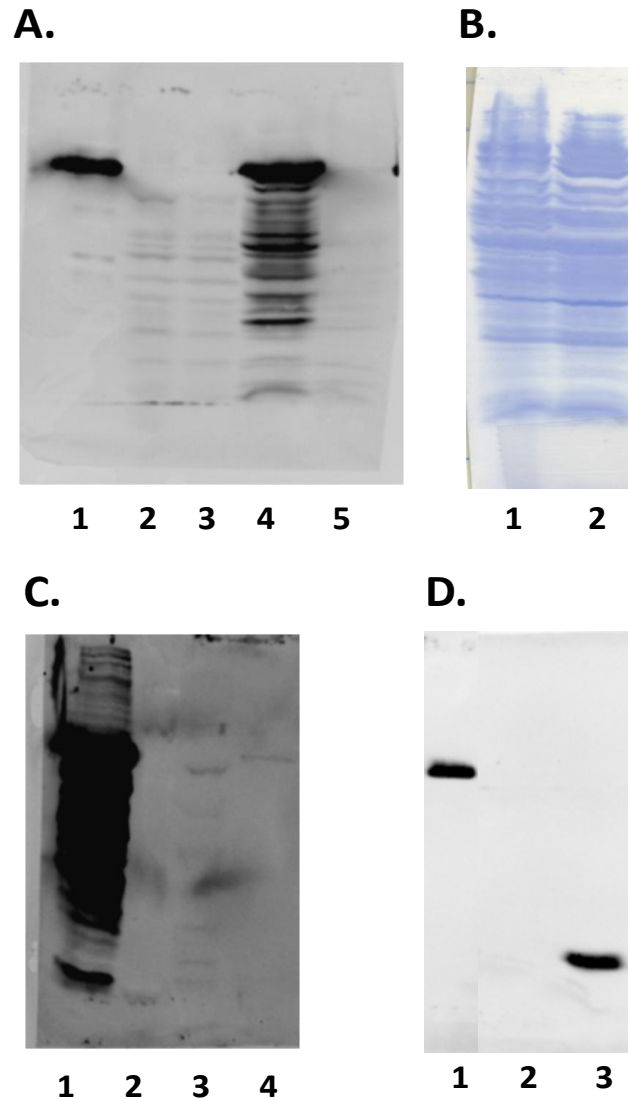
Figure 4.4 Blots to detect *D. radiodurans* RecD protein using antibody Dr-RecD mAb clone P3/D10-19. (A) Western blot using antibody mAB clone P3/D10-19: purified C-terminal His-taggedRecD (lane1), whole cell lysates of wild type *D. radiodurans* strain BAA-816 (lane 2), and strain *recD*::*kan* mutant 3 (lane 3), untagged RecD from whole cell lysates of induced BL21 harboring pDr.RecD-untagged-pEt21a (lane4) uninduced whole cell lysates of BL21 harboring pDr.RecD-untagged-pEt21a (lane 5). (B) Coomassie stained blot reflecting the protein load from panel (A). Lanes 1 and 2 in are identical in load and order to lanes 2 and 3 from panel A, and were cut from redundant lanes from the same transfer. (C) Western blots of: whole cell lysates of induced BL21 harboring pDr.-RecD-pET21a (lane 1), Ni^{2+} NTA mock purification of wild type *D. radiodurans* lysate (lane 2), prestained protein ladder (lane 3) Ni^{2+} NTA purification of C-terminal His-tagged RecD *D. radiodurans* mutant lysate (lane 4). (D) Western blot of: Ni^{2+} NTA purified C-terminal, amino acids 290-725 (lane 2), and Ni^{2+} NTA purified N-terminal amino acids 1-189 (lane 3) peptides of D. radiodurans RecD protein.

4.3.5 Immunoprecipitation of RecD Protein From *D. radiodurans* Whole Cell Lysates

It had previously been determined that *D. radiodurans* expresses very little RecD protein. Therefore immunoprecipitations were performed on whole cell lysates with or without purified C-terminal His-tagged RecD as bait to increase the chance of detecting associated proteins. The results were the same in both cases except for the absence of a band corresponding to the mobility of the RecD protein in the immunoprecipitation without added exogenous RecD. Both Gels resulted in additional bands. The additional bands were similar in mobility and intensity in all lanes of both immunoprecipitations as well as the mock immunoprecipitation. (Fig. 4.5).
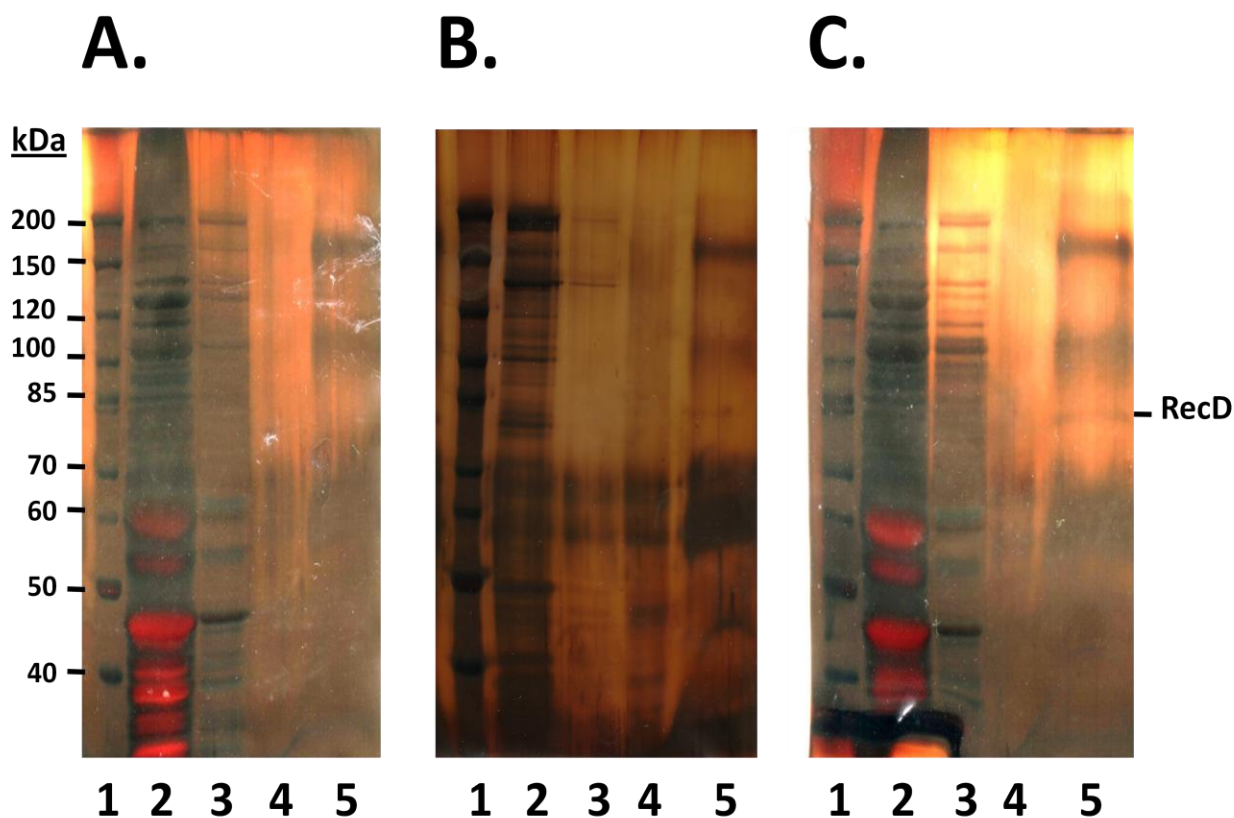
Figure 4.5 Silver Stained SDS PAGE gels. (A) Immunoprecipitation of *D. radiodurans* whole cell lysates using antibody mAB clone P3/D10-19, prestained marker (lane 1), flow through (lane 2), first wash (lane 3), last wash (lane 4), elution (lane 5). (B) Mock Immunoprecipitation (no antibody) of *D. radiodurans* whole cell lysates using antibody mAB clone P3/D10-19, prestained marker (lane 1), flow through (lane 2), first wash (lane 3), last wash (lane 4), elution (lane 5). (C) Immunoprecipitation of *D. radiodurans* whole cell lysates spiked with purified C-terminal His-tagged RecD protein using antibody mAB clone P3/D10-19, prestained marker (lane 1), flow through (lane 2), first wash (lane 3), last wash (lane 4), elution (lane 5).

4.4 Discussion

Antibody Dr-RecD mAb clone P3/D10-19 reacted with C-terminal His-

taggedRecD in both Western blots and ELISAs. This indicates that all clones secrete an

antibody against RecD that can bind to the protein in both its denatured and native

conformations. The antibody recognizes the first 289 amino acids in the N-terminal region of RecD as shown using BL21 expressing plasmid pDr.RecDNterm-pET21a.

Antibody Dr-RecD mAb clone P3/D10-19 was useful in suggesting that *D. radiodurans* maintains RecD protein at a very low copy number. This was demonstrated by inability to detect the protein whole cell lysates of wild type *D. radiodurans* by Western blot. It was necessary to first concentrate a His-tagged version of the protein by Ni^{2+} NTA purification from a large culture of *D. radiodurans* to detect it via Western blot. This notion is supported by preliminary results demonstrating that *D. radiodurans* RecD protein is toxic when over expressed in *E. coli* (work done by Eric Chao, an undergraduate in our lab). It has also been reported that *E. coli* RecD protein is maintained at very low copy number, around ten copies per cell (26).

Immunoprecipitations to determine functional binding partners of RecD (coimmunoprecipitation) were not successful. Proteins were pulled down with protein G beads; however, they are likely non-specific interactions with one of the other components of the immunoprecipitation such as the antibody or the protein G beads themselves. This is most likely the case as both immunoprecipitations and the mock immunoprecipitation resulted in bands of the same mobility and intensity. It is possible that antibody Dr-RecD mAb clone P3/D10-19 is not able to pull down binding partners of RecD. It was shown to bind to the N-terminal portion of the protein which is likely involved in associating with other proteins, as the C-terminus contains the helicase motifs of the protein.

Chapter 5 Conclusion

5.1 Importance of Studying Helicase Enzymes

The importance of DNA helicases is evident by their ubiquitous presence in all organisms from prokaryotes to eukaryotes.   They are vitally involved in almost every facet of DNA metabolism, including DNA repair, replication, and recombination (121). Due to the fundamental nature of their functions, mutations in helicases are often very deleterious to the organism. In prokaryotes, helicase defects can result in impaired growth or death due to defects in replication or unresolved DNA damage (31-34). Aberrant helicase activity also accounts for a number of clinical disorders in humans. Cockayne's syndrome, xeroderma pigmentosom, trichothiodystrophy, Bloom's syndrome, and Werner's syndrome are all a result of abnormal helicase activity (122).

5.2 Importance of Studying *Deinococcus radiodurans*

The extremely radiation resistant bacterium *D. radiodurans* could prove to be a precious resource for manipulating and understanding the world we live in. Much interest in genetically engineering a radiation resistant bacterium to recover radionuclides and heavy metals from radioactive waste has been demonstrated in the last decade. *Deinococcus radiodurans* strains harboring genes for precipitating uranium have already been developed. Specifically, the *phoN* a gene which encodes a nonspecific acid phosphatase, from *Salmonella enterica serovar Typhi*, has been expressed in *D. radiodurans*.   The engineered strain can bioprecipitate uranium, and retains this capacity even after exposure to 6 kGy of ^{60}Co gamma irradiation. The strain presents an intriguing potential use for the organism (123-127).

5.3 Possible Role of RecD Helicase in *Deinococcus radiodurans*

Regardless of whether the organism is ever effectively used for the bioremediation of nuclear waste, it will almost certainly advance our understanding of DNA and cellular resistance to gamma irradiation and other cellular assaults. Despite evidence suggesting that a means of retarding protein damage is the key to surviving massive doses of irradiation, it must be appreciated that a repair DNA repair mechanism equal to the task of handling the collateral DNA damage must have also evolved.

In the absence of recBC homologs in *D. radiodurans*, a number of recombinational repair models have been put forth to explain the organism's capacity to repair double strand breaks. The most current iteration was called ESDSA (57). In this model, the first step in homologous recombination (Fig. 1.4, step1), the role of RecBCD enzyme in many bacteria, is provided by endonuclease activity from a yet unidentified enzyme. Presumably the nuclease would produce the 3' overhang necessary for the second step. This exonuclease may function in conjunction with a helicase as does the nuclease RecB in RecBCD. The next step involves strand invasion of homologous double strand sequence by the 3' single stranded overhang formed in step 1 (Fig. 1.4, step 2). This step is catalyzed by a RecA, or RecA homolg in all branches of life. Zahdradka et al. claim that this step is RecA independent however (57). If this is the case, another unidentified enzyme is almost certainly necessary to facilitate this energetically unfavorable event.

I propose that RecD helicase could participate in either or both of steps 1 and 2 in the ESDSA model. It seems plausible in light of the enzymes *in vitro* helicase activity and homology to *E. coli* RecD, a component of one of the enzymes (RecBCD)

responsible for catalyzing strand invasion in most gram positive bacteria. The enzyme was shown to unwind linear double strand DNA with 5' overhangs *in vivo* (15). It is possible that the enzyme facilitates the endonuclease activity described for step 1 of the ESDSA model. It could also function in the strand separation necessary for step 2.

The lower amount of catalase activity found in the *D. radiodurans recD* mutant is likely insignificant. There is evidence suggesting that this is a physiology irrelevant phenomenon (or at most a minor contributor) for resistance to gamma irradiation and other cellular insults.   First, the mutant is sensitive to UV light. The type of UV light (UVC) used in this study does not invoke oxidative stress, and would not likely require catalase avtivity for resistance. Secondly, the mutant has an enhanced ability to take up transforming DNA, suggesting the enzyme plays a role in some form of DNA metabolism. Lastly, null mutants of the *katA* gene in *D. radiodurans*, responsible for the major band of catalase activity detected in zymograms (Fig 3.1) are less sensitive to gamma radiation than the *recD* mutant (113). Given the above line of reasoning I think it is fair to claim that *D. radiodurans* RecD does not play a role in an anti-oxidant pathway as suggested by Zhou et al. (103), but rather has a function in DNA repair.

APPENDICES

Transformation phenotype of *Bacillus subtilis yrrC* mutant

Introduction

     Bacillus subtilis is a gram negative bacterium whose proteome and transcriptome has been well characterized. For homologous recombination the organism uses the AddAB set of enzymes to process double strand DNA breaks prior to RecA loading, in contrast to and other gram positive bacteria which employ the RecBCD enzyme. *Bacillus subtilis* also encodes a putative helicase with high homology to RecD from RecBCD holoenzymes, designated *yrrC*. The region of homology is most significant in the C-terminal domain of the proteins where the helicase motifs are located. The N- terminus of the YrrC gene is much longer than that seen in RecD genes that are known to be members of RecBCD complexes. This groups the gene with other *recD*-like genes which have extended N-termini. This group includes the *recD* gene in *D. radiodurans*. The *recD* mutants in *D. radiodurans* have been shown to be more transformable that the wild type strain (109). A null mutant in B. subtilis was constructed by insertional mutagenesis conferring erythromycin resistance. The mutant had previously shown to be insensitive to DNA damaging agents compared to wild type. Transformation efficiency was compared to wild type by transforming with genomic DNA from *B. subtilis* strain RH2077 which is resistant to chloramphenicol. *B. subtilis* is naturally transformable, but achieves maximal transformation efficiency after being cultured to stationary phase.

Materials and Methods

Both strains were grown to stationary phase, OD_{600} 1.0 in PAB media (Difco). At this point the cells were frozen in 300 µl aliquots at -80°C in 15% glycerol. On the day of transformation the cells were thawed on ice and incubated with shaking at 37°C. Ten µg of transforming DNA was added to the cultures and they were incubated for another 30 min with shaking at 37°C. One ml of 4× PAB was added to culture and it was incubated for another 90 min with shaking at 37°C. One hundred µl of the transformation mixture was plated on 3 µg/ml chloramphenicol PAB agar plates in triplicate. The remaining transformation mixture was diluted 1:200,000 by serial dilution in triplicate and 100 µl was plated on PAB Agar plates without antibiotics to asses viable cell numbers.

Results

The assay was performed twice with similar results, the *yrrC* mutant was less transformable than wild type by 2 to 3 fold.

Transformation *yrcC* Mutants

Strain	Transforming DNA	Viable Cells	Transformants/CFU/ug DNA	Relative Transformability
10/5/05			Chloramphenicol (3ug/ml)	
Wild type	2088 (10 μg) (cam^r)	2.47($\pm$0.147)$\times10^9$	2.47($\pm$0.17)$\times10^{-5}$	1.0
yrcC		2.2($\pm$0.1)$\times10^9$	4.28($\pm$0.72)$\times10^{-6}$	.197($\pm$.036)
10/17/05			Chloramphenicol (3ug/ml)	
Wild type	2088 (10 μg) (cam^r)	2.5($\pm$0.39)$\times10^9$	1.3($\pm$0.38)$\times10^{-6}$	1.0
yrcC		1.43($\pm$0.35)$\times10^9$	5.73($\pm$1.17)$\times10^{-7}$	.31($\pm$.11)

Discussion

These results implicate the YrrC protein as a player in the natural transformation of B. subtilis. Contrastingly to its RecD homolog from D. radiodurans, it appears to perform in a pro-transformation capacity. Although the effect is opposite to that found in *D. radiodurans recD* mutants, these results suggest a role in transformation for this *recD-*like family of helicases.

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