ABSTRACT

Title of Document: EXPRESSING *DEINOCOCCUS RADIODURANS* RECD IN *ESCHERICHIA COLI*: PHENOTYPIC EFFECTS IN RECBCD(-) AND RECD(-) CELLS

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The RecD helicase is a member of the RecBCD complex, which is essential for repair of double-stranded breaks (DSBs) in DNA via homologous recombination in *Escherichia coli*. The microbe *Deinococcus radiodurans* is capable of fixing high amounts of DSBs, owing to an efficient repair system. However, *Deinococcus* does not contain any *recB* or *recC* genes; only a RecD-like helicase has been observed.

In *Escherichia coli* strains mutant for the native RecD subunit, the *D. radiodurans* RecD cannot restore nuclease activity when infected with T4 gene2$^{-}$ bacteriophage. Cell viability tests and mitomycin C exposure of RecBC(+)D(-) strains show no adverse effects from the Dr RecD. A negative phenotype was encountered with strains lacking RecBCD and expressing *D. radiodurans* RecD protein. Microscopy studies of RecBCD(-) *E. coli* expressing the *D. radiodurans* RecD helicase show long cellular structures termed filaments. The Dr RecD protein may be binding to and disrupting the replication fork.

# EXPRESSING *DEINOCOCCUS RADIODURANS* RECD IN *ESCHERICHIA COLI*: PHENOTYPIC EFFECTS IN RECBCD(-) AND RECD(-) CELLS

By

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# Dedication

1. Mom and Dad!

2. Melissa, my sister and one of my best friends.

3. To my past boss Dr. Walter Patton, who trusted my protein preps.

4. To my current boss, Dr. Julin, thanks for keeping me around proteins. They're fun!

5. My graduate school committee – You've been more helpful than I ever could have imagined.

6. DNA – Whether you're making more or just damaging it, double-helices are nature's way of telling us how simple and yet how complex it all can be.

7. RecD – See my lab notebook for my thoughts on you.

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# List of Abbreviations

Amp - ampicillin

bp – base pair

CD – Circular Dichroism

CFU – Colony Forming Unit

DSB – Double Stranded Break in DNA

Dr – *Deinococcus radiodurans*

DAPI - 4'-6-diamidino-2-phenylindole

dsDNA – Double-stranded DNA

DMSO – Dimethyl Sulfoxide

D loop – Displacement loop

E.O.P. – Efficiency of Plating

ESDSA – Extended Synthesis-Dependent Strand Annealing

ExoV – Exonuclease V or RecBCD

HR – Homologous Recombination

HJ – Holliday junction

IPTG - Isopropyl-beta-D-1-thiogalactopyranoside

kD - kiloDalton

MMC – mitomycin C

MCS – Multiple Cloning Site

M.O.I. – Multiplicity of Infection

mGy – milliGray (Gray is a unit of radiation measurement)

O.D. – Optical Density

$O.D._{600}$ – Optical Density at 600 nanometers

PFGE – Pulsed Field Gel Electrophoresis

SSA – (Inter and Intra-chromosomal) Single Strand Annealing

SOS – Save Our Souls, international maritime distress signal

SM – Suspension Medium

*tac* – combination *trp* and *lac* promoter

Tet – tetracycline

TSG – Tris-HCl, Salt, Gelatin solution

UV - Ultraviolet

# Chapter 1: Introduction

## 1.1 DNA Damage

The maintenance of an organism's genome requires an array of interacting activities from a diverse set of enzymes. The DNA from prokaryotes to eukaryotes is engaged in numerous pathways relating to replication, transcription, and repair of damage incurred from both endogenous and exogenous agents. Often, the biological machinery of the cell is required to correct errors in the template when some of these pathways are occurring. The presence of an efficient and error-proof repair system allows for an organism to grow, divide, and differentiate without lethal consequences to its DNA in a typical physiological environment.

The injuries to DNA and their potential consequences are plentiful. From within the cell the products of metabolic processes can cause single-base lesions and single-stranded nicks from activated free radicals. Exogenous agents from the environment can cross the membrane and cause severe damage. These can include toxic chemicals, heavy metals, and many forms of ionizing radiation, among others. The most detrimental harm comes from that which causes double-stranded breaks in the DNA. The template is no longer contiguous, and the end result is often mutation, aberrant protein production, and eventually cell death (62). Each organism has at its disposal a number of biological pathways and safety systems to maintain the integrity of its genome.

## 1.2 Repair of DNA damage in *Escherichia coli*

### 1.2.1 Use of Homologous Recombination

Recombining homologous sections of DNA, known as homologous recombination (HR), is one method by which cells can acquire a flawless template to be used in transcription and replication. The connection between recombination and replication has been extensively studied. *E. coli* has been the model prokaryote for researchers in this respect, and its genomic repair systems and associated enzymes have been thoroughly characterized. Many believe that the repair of faltering replication forks is mainly performed via homologous recombination pathways (40). It is also surmised that HR is a method to begin replication and the central means to repair deleterious double-stranded breaks (DSB) in the genome (38, 40, 41). The *E. coli* recombination pathway has been studied, and some general rules can be applied to bacterial, fungal, and mammalian systems. Use of recombination and the proteins implicated in that respect for repair (and possible initiation of DNA replication) are found across all the kingdoms (38).

### 1.2.2 First Step in Homologous Recombination: Initiation by RecBCD

Homologous recombination has been shown to have at least four distinct steps: initiation, location of complementary DNA and ensuing strand exchange, heteroduplex extension, and finally resolution (38). Figure **1** illustrates these four steps. The first, initiation, directly involves one of two pathways, RecBCD or RecFOR. The former is a heterotrimer that only acts as one unit, whereas the latter exists as separate enzymes with different biochemical activities. The preliminary substrate for RecBCD differs from that of RecFOR as well. The obligatory blunt end indicative of a DSB is the necessary element in the early step of HR by RecBCD (2, 19, 41). The initiation step requires the

removal of any single-stranded DNA binding proteins from the invading single strand, and replacement by RecA on the processed strand. The actual manufacture of the single-stranded DNA (ssDNA) section is performed by the aforementioned repair protein(s), and consists of a simultaneous helicase unwinding of the duplex DNA at the blunt end, and subsequent nuclease action (5' to 3'). The degraded DNA is the duplex which contained the original lesion. A RecA-coated ssDNA is necessary for the second step of HR (19, 22, 38).

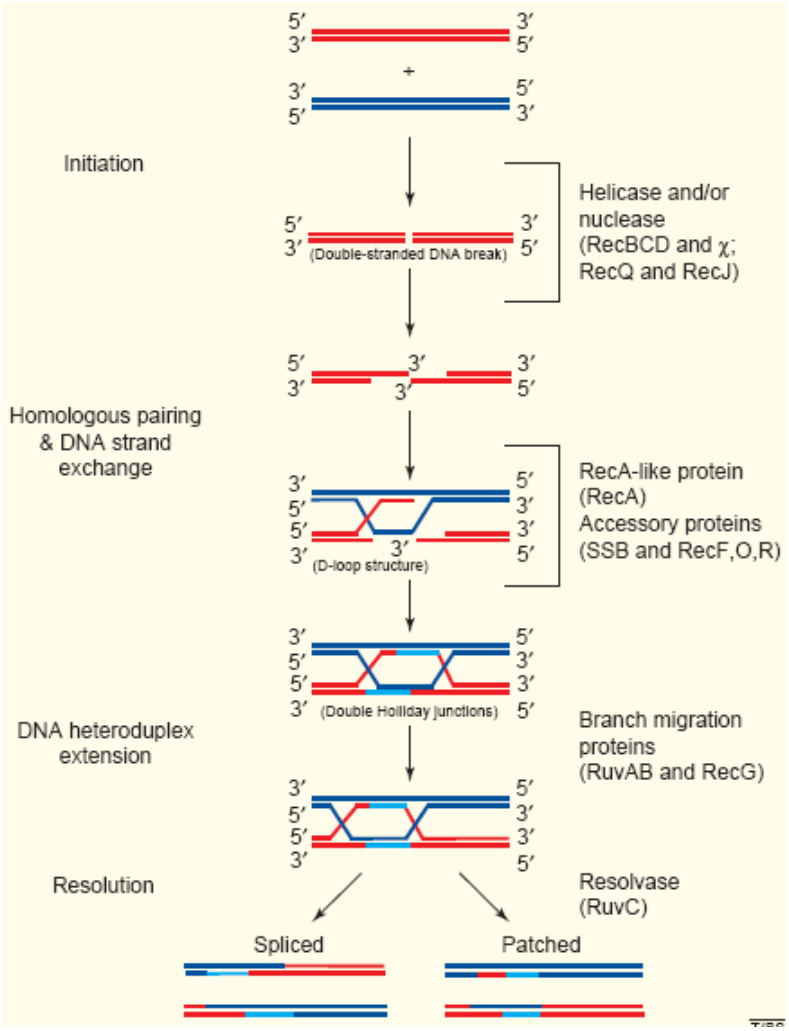


**Figure 1** – A general model for homologous recombination. There are 4 main enzymatic processes to HR, of which RecBCD participates in the first, Initiation. (38)

### 1.2.3 Step Two in Homologous Recombination: RecA-coated Strand Exchange

After the generation of a RecA-coated ssDNA, the next step begins whereby the nucleoprotein filament locates a complementary sequence on a homologous section of DNA. Once found, the RecA-ssDNA invades the undamaged duplex and forms a displacement loop or D-loop, a structure that is defined as a heteroduplex of the old strand and the new strand.

### 1.2.4 Step Three in Homologous Recombination: Heteroduplex Extension

Prior to beginning the next phase of HR, new DNA is synthesized by DNA polymerase I to join the broken ends of the double-stranded region. The newly synthesized DNA can be seen as light blue lines in Figure **1** after the homologous pairing and strand exchange step. At this point the heteroduplex is extended by the motor protein complex RuvAB. RuvAB acts as a helicase to unwind the target DNA, thereby creating new double-stranded DNA between the invading strand and the corresponding duplex DNA as they reform new hydrogen bonds in Watson-Crick base pairs. This is achieved via branch migration at the cross-over point (19, 22, 38, 41).

### 1.2.5 Step Four in Homologous Recombination: Resolution by RuvABC

The final step in HR is termed resolution, and features the Holliday junction (HJ), named by Robin Holliday in 1964. The Holliday junction is an intermolecular series of phosphodiester bonds among the two heteroduplexes which is surrounded by the RuvABC protein complex. The endonuclease RuvC, in coordination with RuvAB,

cleaves this spatially twisted conformation in one of two ways. If the same strands that broke the first time break again, a non-crossover recombination product is yielded, resulting in two DNA double-strands with small sections of heteroduplex. These patches contain one strand from each of the resolved HJ. Figure **2** illustrates this type of resolution. Alternatively, if new nucleic acid backbones are separated and rejoined that were not the first time around, crossover recombination occurs. These new duplexes have DNA from arms flanking the site of the junction. These series of molecular events by the RuvABC resolvasome finish the task of HR, and the integrity of the DNA is retained. Replication, at this point, can be resumed, and often initiated by the action of the recombination enzymes (22, 38, 41, 62).

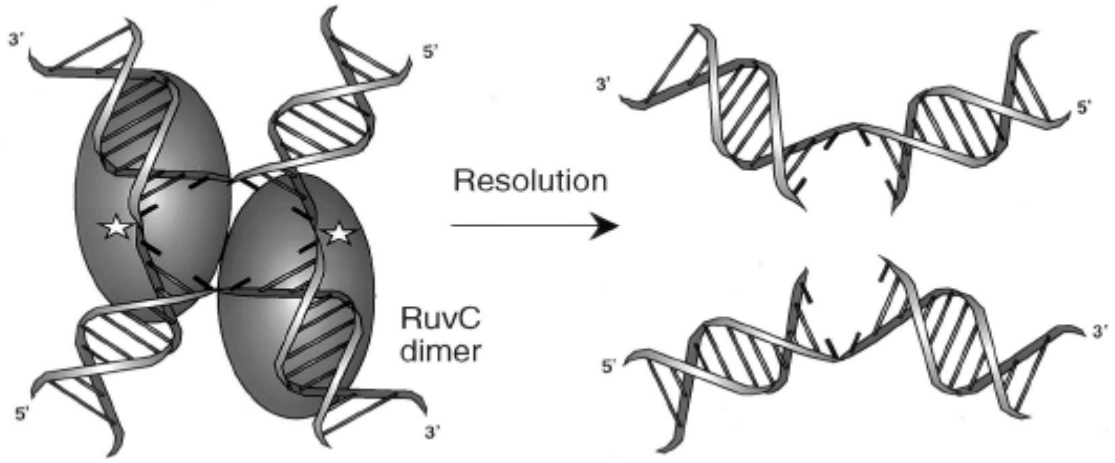


**Figure 2 -** A representation of Holliday junction resolution by the RuvC endonuclease. The stars on the left part of the image are meant to symbolize the nuclease active sites in each half of the dimer. A non-crossover event is shown. (63)

## 1.3 RecBCD

RecBCD (ExoV) is used for over 90 percent of the digestion of foreign DNA and over 99 percent of recombination events in normal *E. coli*. RecBCD is a 330 kD multisubunit enzyme consisting of three polypeptides. Its main substrate is blunt, or nearly blunt DNA ends typically induced by DSBs or stalled replication forks. The

activities of RecBCD include ATP-dependent single and double stranded exonuclease, ssDNA endonuclease, and a DNA unwinding function contributed by its helicase segments (2, 38, 41). These all act in concert to bring about the initiation step of HR, and generate the proper starting material for the second step with RecA. These various activities of RecBCD are shown in Figure **3**.

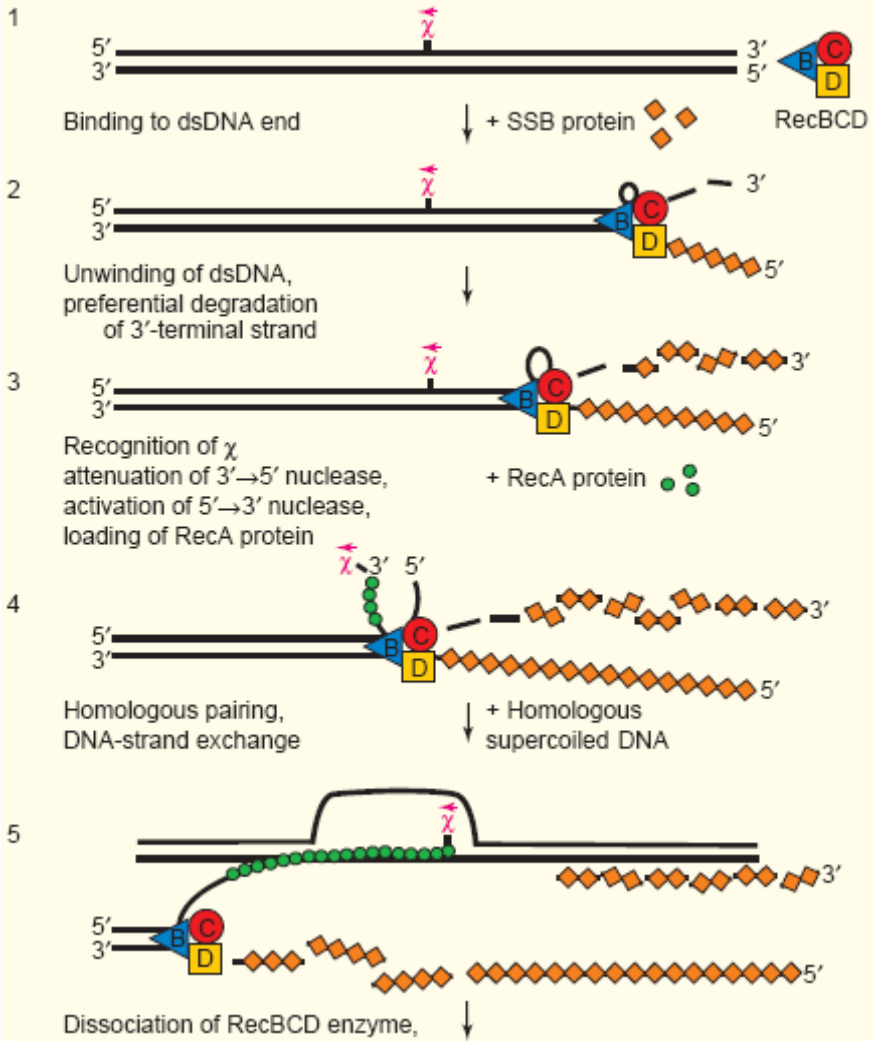


**Figure 3 –** Enzymatic activities of RecBCD in the initiation stage of HR. Steps 1 - 5 detail the involvement of RecBCD, RecA, and the hotspot χ in the early stages of homologous recombination. The production of the RecA-coated, χ-containing ssDNA results from the coordinated nuclease and helicase action of the RecBCD multisubunit complex. The role of the RecD protein is found in steps 2 and 3, where it is necessary for the digestion of the 3' strand and unwinding of the duplex, as well as χ recognition. (38)

### 1.3.1 Chi site interaction with RecBCD

The Chi sequence (χ), designated 5'-GCTGGTGG-3', is recognized by the RecBCD enzyme as it travels along the DNA, and plays a crucial role in HR as well as resistance to phage DNA. RecBCD is normally a highly processive unit, and the χ sequence, which is highly concentrated around the origin of replication in *E. coli*, attenuates the 3' to 5' exonuclease activity (by inactivation of the RecD subunit), and a subsequent (but weaker) 5' to 3' exonuclease proceeds from the point of Chi encounter (2, 38). The nucleic acid is still unwound upon χ detection, demonstrating that the helicase activity is inherent to RecB and/or a combination of RecBC. RecD is required for exonuclease activity (despite not having a nuclease domain), Chi recognition, and increased rate of DNA unwinding (2, 16, 22). Therefore, χ protects both the bacterial genome from continuous degradation and also permits the removal of foreign DNA from infecting phages or other extracellular sources. The truly meaningful portion of the RecBCD-Chi meeting is the assistance in loading RecA onto the ssDNA substrate necessary for the second step of homologous recombination (2, 38, 41).

### 1.3.2 Phenotypes of *E. coli* mutant for RecB, RecC, and/or RecD proteins

The phenotypes of *Escherichia coli* mutants lacking RecB, RecD, or RecBCD have been established through numerous biological and biochemical tests. See Table **1** for a listing of known phenotypes for selected strains. *E. coli* that have had all three *recBCD* genes deleted from the genome, such as V186, have phenotypes of reduced viability (30%), sensitivity to DNA damaging agents, and are vulnerable to some bacteriophages. A strain lacking a functional RecB rendered that strain essentially null for RecBCD, as it was sensitive to DNA damaging agents and had reduced viability when

compared to that same wild-type parent. Any *E. coli* that is missing or has a truncated *recD* gene will produce a RecBC(+)D(-) phenotype that has the following characteristics: wild-type viability, resistant to most forms of DNA damage that results in DSBs, recombination proficient, χ nonactivating, and susceptible to bacteriophage infection (1, 14). There exist only two definite tests for proper RecD function *in vivo*. The first was used to identify *recD* mutants when the gene was first discovered in 1986. The test measures plasmid stability in transformed RecD(-) strains, where those *E. coli* lacking the native subunit tend to form multimers of the transformed plasmid (6). The second test, the bacteriophage T4 gene2- nuclease assay, will be discussed here.

**Table 1 -** List of phenotypes for selected *Escherichia coli* strains[a]

| Strain | Genotype | T4 gene 2$^-$ | mitomycin C | cell viability |
|---|---|---|---|---|
| WA234 | wild-type | resistant | resistant | normal |
| WA632 | WA234, *recB21* (recBCD$^-$) | not resistant | sensitive | reduced 70% |
| BT125 | WA234, *recD1011* | not resistant | resistant | normal |
| AFT228 | wild-type | resistant | resistant | normal |
| V324 | AFT228, *recD1009* | not resistant | resistant | normal |
| V186 | AFT228, Δ*recBCD* | not resistant | sensitive | reduced 70% |
| V1119 | AFT228, *recA*$^-$ | resistant | sensitive | reduced 50% |

The T4 gene2$^-$ assay measures ExoV nuclease activity, mitomycin C crosslinks bases and disrupts the replication fork, which requires RecA and RecBCD to resume, and cell viability is a method to gauge whether a particular strain can form viable colonies.
[a] – See Section 2.1, Table **2** for reference on data found in this table.

The bacteriophage T4 has a genome of approximately 166 kilobase pairs and contains over 200 genes. The genome is linear, and there exists a cap protein encoded by the *gene2* product that protects the DNA ends from exonuclease degradation. Researchers constructed a T4 phage that was mutant for *gene2*, thereby exposing the blunt ends of the DNA for RecBCD digestion (33). A wild-type *E. coli* is not normally lysed by this engineered phage, but it was discovered that strains that were *recB-, recD-*,

or had the *recB recC recD* genes removed could not engage the required nuclease activity that makes the wild-type resistant to the T4 gene2$^{-}$ bacteriophage (1, 14).

## 1.4 *Deinococcus radiodurans*

Mending the harm caused by double-stranded breaks is crucial to survival. The task HR and the RecBCD recombination machinery play in the balance between DNA metabolism and repair has already been stated. The gram positive bacterium *Deinococcus radiodurans* has achieved the status of super-survivalist as it is able to withstand thousands of potentially deadly double-stranded breaks in its genome without incurrence of mutagenesis to the DNA or loss of viability. When the genome sequence was released in 1999 (64), no functional RecBCD or AddAB pathways were seen. AddAB is a recombination repair system consisting of two proteins that acts very much like RecBCD, and is found in organisms such as *Bacillus subtilis* who also lack the genes for *recB, recC*, and *recD* (15). The *recB* or *recC* genes were not found in the DNA sequence, but the genes for *recA* and *recD* were seen. The product of the *recA* gene is a protein that is essential for bacterial viability under conditions of DNA damage repair and has enzymatic activities similar in nature to the *E. coli* RecA, except for one major difference. The Dr RecA preferentially binds to dsDNA rather than ssDNA like its counterpart in *E. coli* (20, 27, 34). This indicated the use of some form of recombination machinery to fix the result of DSBs (46). The *recD* gene encodes for a protein that was found to have typical helicase activities (61).

### 1.4.1 Characteristics of *Deinococcus radiodurans*

There are eleven members in the *Deinococcaceae* genus. *Deinococcus radiodurans* is the most investigated due to its ability to efficiently repair double-strand breaks acquired from exposure to ionizing radiation, UV light, and desiccation as well as being naturally transformable(4, 18, 51). *D. radiodurans* is several thousand fold more tolerant than the model microorganism *E. coli* (4, 5, 50, 51). Investigations into the genome since its release in 1999 have revealed a few novel proteins implicated in resistance to DNA damage (31, 54). *D. radiodurans* does seem to possess a high redundancy in its repair gene content (46, 64). The locales that it has been found include organically rich soil, meat cans, weathered granite in the Dry Antarctica Valleys, room dust, and irradiated medical instruments (46, 52, 64). Gamma radiation levels found on earth are at their highest only 100 to 400 mGy, whereas *Deinococcus* family members can withstand hundreds of Grays or even thousands. Therefore the only putative link between the normal phenotype of *Deinococcus radiodurans* and the agents causing the damage is that surroundings low in water content (desiccation) can cause a high amount of DSBs in the DNA (47). Recent work has indicated its multigenomic nature (up to 8 to 10 copies of its chromosome per cell in exponential phase, 4 in stationary phase) is not solely responsible for *D. radiodurans* hardiness against nucleic acid damage (46), though the replication of said DNA is completely stopped until repair is completed.

A recent paper by a multi-institutional group in Europe (65) suggests a two-stage DNA repair process for *Deinococcus radiodurans* that would follow in the event of severe double-stranded breaks. The authors propose a novel molecular mechanism known as Extended Synthesis-Dependent Strand Annealing, ESDSA. This is coupled to

and completed by a series of crossovers among the multitude of genomic copies the microorganism carries. The ESDSA model is displayed in Figure **4**. The basic premise of ESDSA is that the many fragments generated by the damage are used as primers for single-strand synthesis, up to 30 kilobases, by DNA polymerase I (encoded by the *polA* gene). This extension, as shown in step 2a under ESDSA, involves a moving displacement loop over a region of undamaged homologous DNA. These newly synthesized single-strand portions will then hydrogen bond to like sequences (steps 3a and 4a of Figure **4**). Once the gaps have been closed by DNA synthesis, and nucleases degrade 5' to 3' to produce blunt ends, RecA-dependent homologous recombination can occur to generate the final, unflawed template (step 5a and b from Figure **4**).

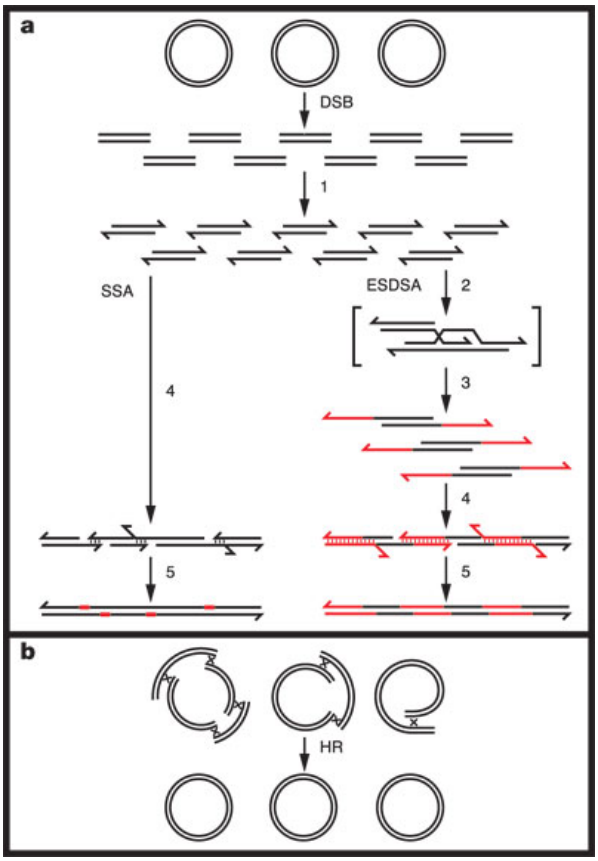

**Figure 4 -** The schematic of the proposed ESDSA model for *D. radiodurans* double-strand break repair. DSB is double-strand break, SSA is inter- and intra-chromosomal single-strand annealing, and HR is homologous recombination with the RecA protein. The red portions of ESDSA and SSA represent newly synthesized DNA by DNA polymerase I (65).

### 1.4.2 The RecD protein from *Deinococcus radiodurans*

Examination of the *D. radiodurans* genome sequence revealed a gene having similarity to the *E. coli* RecD. Sequence comparisons among known helicases showed that the Dr RecD is in the Superfamily I helicase group, of which Ec RecD is a member. The homology between some group members is shown in Figure **5**. There is poor similarity and identity at the amino terminal end of the Dr RecD with other helicases of Superfamily I.

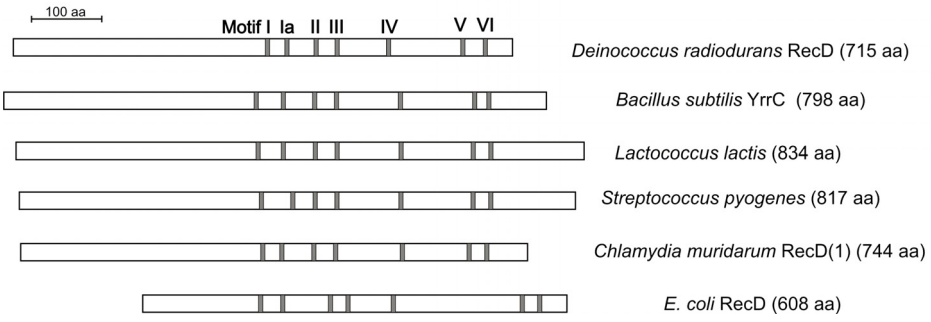


**Figure 5 –** Schematic representation of RecD and RecD-like proteins. Shown above are some members of the Superfamily I helicase group, of which four (*B. subtilis, L. lactis, Strept. pyogenes,* and *C. muridarum*) are closely related to the *D. radiodurans recD* gene. Amino terminus begins on the left side. The vertical bars (labeled I through VI) are seven helicase amino acid motifs conserved among all Superfamily I enzymes (61).

Helicases like the RecD protein from *E. coli* perform a vital operation in DNA metabolism. They are a set of enzymes whose main function is to harness the chemical power of ATP hydrolysis and convert it to mechanical energy to unwind the double helix (12, 21, 22, 29, 45, 58). The RecD gene from the model organism *Escherichia coli* was first cloned in 1986. The activities of the *E.coli* RecD include a single-stranded DNA

dependent ATPase activity, and restoration of nuclease and χ recognition when expressed in RecBC(+)D(-) strains (3, 6, 16). RecD contains both an ATP-binding domain and several helicase motifs, including the classic Walker A and B NTP-binding amino acid sequences. The *recD* gene, as found in the *D. radiodurans* genome, is 2192 basepairs. The translation of this gene yields a protein 715 amino acids in length and an apparent molecular weight of 76,431 Daltons (61).

### 1.4.3 Biochemical and *in vivo* characterization of the Dr RecD helicase

The *Deinococcus radiodurans* RecD protein is able to unwind short duplexes of length 20bp, provided a 5' single-strand overhang exists natively or as found in a stalled replication fork. The *D. radiodurans* RecD is unable to unwind larger duplexes with high processivity *in vitro*. The Dr RecD is an ATPase when placed in a mixture of ATP, $Mg^{2+}$, and short single-stranded oligomers. It binds with high affinity to DNA containing a 5' single-strand overhang, and prefers a length on the overhang of 8 to 12 nucleotides. There was no significant nuclease activity seen in any tests to date. The carboxy terminus has the classic helicase motifs, whereas the amino terminus seems to have no structural or functional domain homologs in any known protein database (61). Recent mutagenesis studies in *Deinococcus radiodurans* show that there is an increase in sensitivity to gamma irradiation and UV, as well as a marked increase in transformability of those strains lacking a RecD (Servinsky and Julin, unpublished results).

## 1.5 Research Justification

As was mentioned in the introduction, much is known about DNA repair in *Escherichia coli*, and more specifically how the organism fixes double-stranded breaks in

its nucleic acid template. The preferred method is homologous recombination through the coordinated actions of RecA and RecBCD. *Deinococcus radiodurans*, the organism most resistant to DNA double-stranded breaks, has a RecA protein and a RecD-like helicase, but does not have genes for *recB* or *recC*. Recent experimental evidence (65) suggests that homologous recombination is used by the organism to generate a flawless template after suffering DNA damage. Therefore our lab decided to pursue the background work on the RecD helicase in order to discern the true nature of this motor protein.

The RecD-like protein first purified from the cloned gene of *Deinococcus radiodurans* displayed typical biochemical activities attributed to helicases. These included the ability to separate short, double-stranded oligonucleotides into the corresponding single-strands in the presence of ATP, in addition to binding to several types of DNA substrates. The next logical step was to determine the *in vivo* function of the Dr RecD. Several experiments in *Escherichia coli* were planned to elucidate the purpose of the *Deinococcus radiodurans* RecD. The *E. coli* strains selected in this research had some type of mutation in RecA or RecBCD. The first goal was to determine if the *D. radiodurans* RecD might complement those strains lacking in native RecD or RecBCD. The secondary goal came about after a Δ*recBCD* strain, V186, showed an unusual negative phenotype when expressing the *Deinococcus radiodurans* RecD. It was then decided to pursue additional cell viability tests, as well as microscope studies to observe the size, shape, and state of the *E. coli* in a *D. radiodurans* RecD (+) background. These observations would then give insights into RecD function.

# Chapter 2: Experimental

## 2.1 Strains and Plasmids

The various experimental *Escherichia coli* strains are shown in Table **2**. The plasmids used in this work are given in Table **3**. The pKK 223-3 expression vector was chosen due to its low to mid copy number, strong *tac* promoter/operator, ampicillin resistance, and that it would generate a non-affinity tagged protein (9). pNM52 is a pACYC184 derived plasmid, and has the gene for the lactose repressor inserted in addition to the gene for tetracycline resistance (25). WA234 is the wild-type parent strain to WA632, V1119, and BT125. AFT228 is the wild-type parent strain to V324 and V186.

**Table 2 -** *Escherichia coli* strains used in this work

| Strain | Alternate designation | Genotype | Reference |
|---|---|---|---|
| WA234 | AB1157 | wild-type | (55) |
| BT125 | | WA234, *recD1011*[a] | (55) |
| WA632 | | WA234, *recB21*[b] | (55) |
| V1119 | | WA234, *recA*$^-$ | Personal Coll.[e] |
| AFT228 | NK5992 | wild-type | (35) |
| V324 | AC321 | AFT228, *recD1009*[c] | (14) |
| V186 | AC113 | AFT228, Δ*recBCD*[d] | (13) |

[a] – The *recD1011* genotype in BT125 is due a nonsense mutation (23)
[b] WA632 is RecBCD (-) due to the *recB* nonsense mutation
[c] – *recD1009* is a nonsense mutation in the *recD* gene (44)
[d] V186 actual genotype is a deletion from *thyA-argA*, which includes the *recB, recC,* and *recD* genes
[e] V1119 was acquired from the personal collection of Dr. Gerald Smith, Fred Hutchinson Cancer Research Center, Seattle, WA

**Table 3 -** Plasmids used in this study

| Plasmid | Type | Reference |
|---|---|---|
| pKK 223-3 | Expression vector, $Amp^r$ [1] | (9) |
| pKK D.r. RecD | *D. radiodurans recD* gene in pKK 223-3, $Amp^r$ | This work |
| pKK K366Q RecD | pKK D.r. RecD with Lys to Gln mutation at aa # 366, $Amp^r$ | This work |
| pNM52[3] | *lacI*$^q$ in pACYC184, $Tet^r$ [2] | (25) |
| pPB100 | *Escherichia coli recD* gene in pKK 223-3, $Amp^r$ | (8) |
| pDr_RecD | Dr RecD in pTZ-19R cloning vector, $Amp^r$ | (61) |

[1] $Amp^r$ denotes resistance to ampicillin
[2] Tetr denotes resistance to tetracycline
[3] pNM52 is a supplemental plasmid expressing the lactose repressor protein where the q denotes quantity. It was used to prevent background expression of the Dr RecD before induction.

## 2.2 Construction of the pKK D.r. RecD wild-type plasmid

The source of the *recD* gene was from a plasmid named pBAD_DrD, containing the Dr RecD in the vector pBAD24 (28). Both the pKK 223-3 vector and the pBAD_DrD plasmid were subjected to a double restriction digest with EcoRI and PstI. The appropriately sized fragments were then gel purified with the Gel Extraction kit from Qiagen, and the purified samples were ligated together with T4 DNA ligase. The ligation mixture was then transformed into XL-1 blue. Several colonies were screened for the presence of the correct insert and vector sizes. This was done via a double restriction digest with the original enzymes EcoRI and PstI. Additional confirmation came from sequencing the promoter region and the full gene on the pKK plasmid. The sequencing was performed by the DNA sequencing facility on the University of Maryland, College Park campus (http://www1.umbi.umd.edu/~cbr/dna.html).

## 2.3 Construction of lysine to glutamine mutation in *recD* gene

Originally, the pKK D.r. RecD plasmid was selected for mutagenesis. This plasmid was abandoned in favor of pDr_RecD due to low yields and lack of a definitive restriction enzyme test for lysine to glutamine codon alteration in pKK D.r. RecD. pDr_RecD is based on the pTZ-19R cloning vector, a 2862 phagemid that is under the control of a *lac* promoter and is derived from the *E. coli* pUC19 plasmid (48). The Dr r*ecD* gene had previously been placed inside this vector (61), and was available in high yields from *E. coli* strains such as HB101 or XL-1 blue.

The primers used in the mutagenesis were ordered from Invitrogen. The forward primer had the sequence 5' – CCGGCACCGGC**CAA**AGCACGACG – 3' and the reverse primer sequence was 5' – CGTCGTGCT**TTG**GCCGGTGCCGG – 3' where the bolded nucleotides are the site of the mutation. The conserved Walker A NTP binding motif was selected as the site of mutagenesis. A lysine residue at amino acid position 366 in the wild-type protein was changed to glutamine. The Quikchange II Site-directed Mutagenesis kit from Stratagene was employed, and the samples were then treated with DpnI to digest away the methylated, parent DNA molecules. A restriction enzyme, EaeI, was used to distinguish between the mutated and unmutated plasmids. EaeI recognizes the sequence 5'-YGGCCR-3' where Y is any pyridimine base and R is any purine base. The expected fragment sizes for the normal pDr_RecD were 1499, 1442, 1136, and 938 basepairs, respectively. The primers would create another restriction site, and the fragment sizes would be 1442, 1136, *1107*, 938, and *392* basepairs. The *392* and *1107* fragments add up to the 1499 basepair fragment of the parent plasmid. Additional

confirmation came by sequencing upstream of the promoter region as well as the entire gene at the University of Maryland, College Park's DNA sequencing center.

The next step was to create a version of the pKK plasmid with the new Lys to Gln mutation in the *recD* gene. This alteration was referred to as K366Q *recD*. The pKK 223-3 vector and the now mutant pDr_RecD plasmids were subject to a double restriction digest with EcoRI and PstI. EcoRI and PstI are located within the Multiple Cloning Site (MCS) of pKK 223-3, and both enzymes cut only once in the mutant pDr_RecD removing the K366Q *recD* gene. The cut vector and the K366Q *recD* fragment were then ligated together with T4 DNA ligase. The ligation mixture was transformed into XL-1 blue. Several colonies were screened for the mutation by re-digesting them with EcoRI and PstI. The pKK plasmid with the K366Q mutation had the promoter region and entire gene verified at the DNA sequencing facility on-campus. The new pKK-derived plasmid was referred to as pKK K366Q RecD.

## 2.4 Bacteriophage T4 gene2$^{-}$ nuclease assay

The T4 bacteriophage has a linear genome whose ends are normally protected by a cap protein encoded by *gene2*. In those phages mutant for this protein, *E. coli* RecBCD is able to digest the foreign DNA, thereby rendering the cell resistant to infection. The native RecD helicase is necessary for this activity, and in RecBC(+)D(-) strains the cell cannot destroy the attacking phage and are lysed. This will appear as plaques, or areas where bacteria did not grow, on typical LB agar plates. Therefore each plaque represents one input bacteriophage. This is one of only two direct tests for RecD function *in vivo* (33).

A T4 gene2$^-$ bacteriophage stock of 3.0 x $10^9$ titer was procured from an existing source in the lab. Phage titer is the amount of viable bacteriophage in one milliliter. The phage stock was diluted into a resuspension buffer known as SM or TSG (0.01 M Tris-HCl pH 7.45, 0.15 M NaCl, 0.03% gelatin). 100 to 500 viable phage particles is the ideal range of bacteriophage per 1.0 x$10^8$ *E. coli* cells (33). The procedure for the T4 test consisted of mixing 100 µl from a mid-log phase culture of experimental *E. coli* and 100 µl of diluted phage stock. The mixed samples were incubated at 37° C for 10 minutes. They were then transferred to a 44° C pre-warmed aliquot of soft top agar (10 g tryptone, 7 g Difco™ agar, 10 g NaCl, 1 g glucose, 1.2 ml of 1 M NaOH for pH 7.1). After gentle mixing, the soft agar and bacteria-phage sample were plated onto 37° C pre-warmed LB agar Petri dishes. Once the soft top agar solidified, the dishes were incubated at 37° C for 16 to 24 hours. Plaques were counted and the overall experimental titer for that strain was determined. The equation for titer was (Number of plaques × plating dilution × phage dilution). The multiplicity of infection or MOI was determined to be between 300-500 bacteriophage particles per culture for most tests.

## 2.5 Mitomycin C test

Mitomycin C (MMC) is a DNA damaging agent that crosslinks bases in complementary strands. The direct result is an interruption in DNA replication. This requires use of the RecA-RecBCD homologous recombination pathway to restart the replication (42). The structure of mitomycin C is shown in Figure **6**.

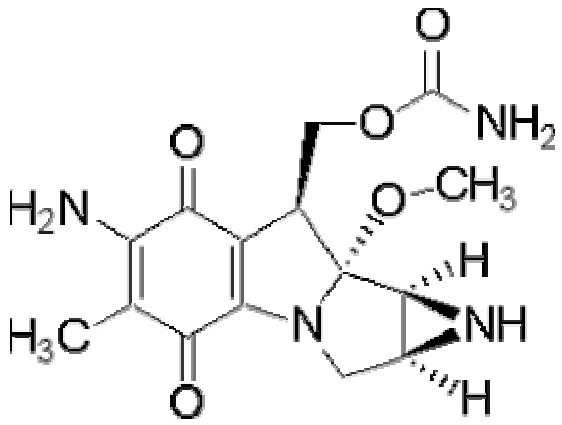


**Figure 6 -** The chemical structure of mitomycin C

Two milligrams of mitomycin C was dissolved in 2.0 ml of 100% ethanol for a stock concentration of 1.0 mg/ml. The procedure for mitomycin C exposure was to grow the experimental *E. coli* strain at 37° C at 250rpm until it reached an optical density of 0.200, and then induce with IPTG to a final concentration of 0.75 mM. The culture was allowed to grow until the mid-log phase ($O.D._{600}$ 0.500 to 0.600). At this point growth was halted and 100 µl of a series of bacterial saline dilutions (0.85% NaCl in $H_2O$) were plated on LB agar Petri dishes with and without mitomycin C. The LB agar dishes with MMC had a final concentration of 1 µg/ml. Both sets of dishes had 100 µg/ml ampicillin added for the experimental plasmids, as well as 50 µg/ml of thymidine for V186 cells. The dishes were incubated at 37° C for 18 to 24 hours and the number of survivor colonies was counted the next day. A CFU / ml / $O.D._{600}$ for each experimental strain was calculated via the equation (number of colonies / plating dilution) × (bacterial dilution / $O.D._{600}$) where CFU stands for colony forming units.

## 2.6 Cell viability

An LB overnight culture with the appropriate media additives for that particular *E. coli* strain and experimental plasmid was diluted 1:50 fold into 7.0 ml of fresh LB with the same additives as the overnight. This diluted sample was then placed inside a 37° C shaker at 250 rpm. The culture was induced with IPTG to a final concentration of 0.75 mM when it reached an $O.D._{600}$ of 0.200. IPTG was added to all bacterial strains regardless of the presence of plasmids. The $O.D._{600}$ was monitored until a final optical density of between 0.500 and 0.600 was reached. The culture was then placed on ice until ready for use. If the *E. coli* samples were to be analyzed on an SDS-PAGE apparatus, approximately 300 µl was removed from the chilled cultures and centrifuged in an Eppendorf 5415D microcentrifuge (Eppendorf, Hamburg, Germany). The pellets were then resuspended in 50 to 100 µl of 2X SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol (v/v), 4% SDS (v/v), 0.02% bromphenol blue), boiled for 3.5 minutes, cooled to room temperature, and run on a 7.5% acrylamide gel at 100 Volts for 1.5 hours. The samples were stained with Coommassie to visualize the protein bands.

A series of saline dilutions (0.85% NaCl in $H_2O$) were made, and three separate aliquots were taken from the chilled culture and carried through the dilutions. One hundred microliters of each saline dilution was plated on LB agar Petri dishes with the same media additives as the day growth. At least two dishes were prepared for each dilution factor. Typical additive concentrations were 100 µg/ml for ampicillin, 15 µg/ml for tetracycline, and 100 µg/ml for thymidine. The LB agar dishes were then incubated at 37° C for 16 to 24 hours and colonies were counted on each dish. Only those with colonies between 30 and 330 were considered significant. The final calculation was for

CFU / ml / $O.D._{600}$ where the equation is (number of colonies / plating dilution) × (bacterial dilution / $O.D._{600}$).

## 2.7 Microscopy analysis

### 2.7.1 Brightfield and DAPI fluorescence studies

The samples for the brightfield and DNA fluorescence assays were prepared as described in Section **2.6** except for the following differences. For the *E. coli* wild-type strain WA234 filamentation study, a 5 mg/ml aqueous solution of nalidixic acid was added to the day growth receiving the chemical to a final concentration of 15 μg/ml. Upon chilling the experimental culture, at least four 1.5 ml aliquots were removed and spun for 1.5 minutes at 13,200 rpm in a microcentrifuge. The pellet was then washed twice with 1.5 ml of a saline solution (0.85% NaCl in $H_2O$), the supernatant discarded, and the pellet resuspended in 300 to 500 μl of the saline solution.

The following method is an adaptation from Hiraga *et al*, (30). Two 5.0 μl samples from each separate aliquot were placed onto fresh, clean glass slides and allowed to dry at 37° C. The sample was fixed with 2 drops of methanol for 5 minutes. The slide was then washed 6 times in tap water, and allowed to dry at 37° C for 5 minutes. Approximately 5.0 to 7.0 μl of a 5.0 μg/ml DAPI solution in 0.85% NaCl was applied to each slide. DAPI is a photosensitive molecule, and therefore all glass slides were kept under aluminum foil to protect the samples from light degradation. A clean glass coverslip washed twice in 100% ethanol was placed over the glass slide. One drop of immersion oil was placed on top of the glass cover slip before viewing the slide.

The fluorescence microscope used in this study was from the laboratory of Dr. Stephen Wolniak (Department of Cell Biology and Molecular Genetics, University of

Maryland, College Park) and was an Axioskop (Carl Zeiss, West Germany) equipped with a Spot Flex Digital Camera (Diagnostic Instruments Inc., Sterling Heights, MI). The program used to acquire the images was Advanced Spot version 4.5.7 (Diagnostic Instruments Inc.). The settings were a 2.0-3.0 s exposure time, 1.0X gain, and 1.1 gamma correction factor for the brightfield microscopy, and 4.0-6.0 s exposure, 8.0X gain, and 1.0 gamma correction factor for the fluorescence microscopy. The filter used to view the DAPI DNA stained pictures was the DAPI fluorescence filter set. At least 30 different fields of view were photographed for each sample, and paired brightfield and fluorescence images were obtained for each field of view.

### 2.7.2 LIVE/DEAD® Baclight™ Bacterial Viability kit

Invitrogen manufactures a kit for viewing living and dead cells in the same field of view called LIVE/DEAD® Baclight™ Bacterial Viability kit. The two components of the kit are two different cellular stains. The first is propidium iodide, a red-fluorescent nucleic-acid stain. The excitation and emission maxima for the dye are 490/635 nm. The second dye is SYTO® 9, a green-fluorescent nucleic acid stain that has excitation and emission maxima of 480/500 nm. The green dye, SYTO 9, will penetrate both damaged and undamaged membranes. Propidium iodide can only travel across damaged membranes. When both dyes are present in a cell, propidium iodide causes a reduction in the fluorescence of SYTO 9 because the emission spectrum for the SYTO 9 dye overlaps with the excitation spectrum for propidium iodide. The end result is that living cells will fluoresce green and dead cells will fluoresce red.

The kit provided two varying mixtures of propidium iodide and SYTO 9 dissolved in DMSO. The two mixtures were combined in equal volumes and mixed

thoroughly. For every 1.0 ml of bacterial culture, 3.0 µl of the above dye mixture was added and mixed thoroughly. This was then incubated for 15 minutes at room temperature and protected from ambient light. 5.0 µl of this mixture was placed on to a clean glass slide, and a clean glass coverslip was placed over the sample. After applying a drop of immersion oil to the slide, the sample was viewed immediately in the dark (to prevent degradation of the dyes). The same camera and microscope as described in Section **2.7.1** were employed. The filter set used to view both the living and dead *E. coli* was a fluorescein longpass filter. The microscope was not equipped with the fluorescein bandpass filter set for observing living cells only, but was equipped with a Texas Red bandpass filter set for the dead bacteria alone. The Advanced Spot software settings were a 3.0-4.0 s exposure time, 4.0X-8.0X gain, and a gamma correction factor of 1.0. Each sample was photographed in different fields of view at least 30 times.

### 2.7.3 Image Analysis

In order to gain information from the images, a program was needed that could count the bacteria based on size and shape. *ImageJ* is an image analysis program distributed by the National Institutes of Health (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006). Protocols used in this study were described in tutorials from the *ImageJ* website (http://rsb.info.nih.gov/ij/), and (http://www.uhnresearch.ca/facilities/wcif/imagej/particle_analysis.htm#particle_auto).

The procedure used to process each batch of images varied according to the contrast and brightness levels of each particular image. Photographs of similar particle and background contrast were processed in sequence. Threshold intensities were set for

image groups as a means to best distinguish actual bacteria from other random artifacts. Next, image sets were run through a particle counter provided in the *ImageJ* program. The typical settings for this included a circularity of between 0.0 and 0.7, and a size$^2$ of 70 to 5000. The circularity refers to the ellipticity of the object, where a value of 1.0 indicates a perfect circle, while any value close to 0.0 is a straight line. The size$^2$ refers to a mean area, in pixels. Any value below 70 was rejected since it was considered too small to be a bacterium. The desired measurement was length of the bacteria (to discern normal from filamentous cells). Once the bacterial samples were collected and measured, a stage micrometer was obtained and a straight line was drawn with the 'Straight line selection' button located on the *ImageJ* toolbar. This was done for a known length of 10 μm. The distance, in pixels, corresponded to 10 μm. This was shown on the *ImageJ* tool bar and recorded.

The results from the image analysis were compiled in a Microsoft Excel™-compatible spreadsheet (Microsoft Corporation, Redmond, WA) that was then compared to the image from which each counted object originated. The objects were numbered by the analysis program to facilitate the discrimination of random objects from bacteria. The output from the *ImageJ* program included an object distance measurement known as Feret's Diameter. Feret's Diameter is the measured distance between parallel lines that are tangential to an object's profile and perpendicular to the ocular scale (http://www.leadtools.com/SDK/Functions/GetFeretsDiameter.htm). This parameter enhanced accuracy in measuring the length of the bacteria in each brightfield image. The Feret's Diameter for each object counted by the program was divided by the pixel length acquired from the stage micrometer to determine the size of that bacterium.

## 2.8 Supplemental Materials List

### 2.8.1 Enzymes

EaeI (Fermentas, Hanover, MD)

EcoRI (New England Biolabs, Ipswich, MA)

XbaI (New England Biolabs)

PstI (New England Biolabs)

DpnI (New England Biolabs)

T4 DNA Ligase (New England Biolabs)

BenchMark™ Pre-stained Protein Ladder (Invitrogen, Carlsbad, CA)

### 2.8.2 Chemicals

IPTG [Isopropyl-beta-D-1-thiogalactopyranoside] (Fisher Chemicals, Pittsburgh, PA)

mitomycin C (Sigma-Aldrich, St. Louis, MO)

ampicillin (Sigma-Aldrich)

tetracycline (Sigma-Aldrich)

nalidixic acid (Sigma-Aldrich)

thymidine (Sigma-Aldrich)

DAPI [4'-6-diamidino-2-phenylindole] (Sigma-Aldrich)

# Chapter 3: Results

## 3.1 The *Deinococcus radiodurans* RecD does not substitute for *E. coli* RecD or RecBCD

### 3.1.1 Bacteriophage T4 gene2$^-$ test

In order to determine whether or not the *D. radiodurans* RecD could complement *E. coli* strains lacking the native RecD subunit, a *recD* mutant, BT125, was used. The bacteriophage T4 normally has the ends of its linear genome protected by a cap protein encoded by *gene2*. When mutant for this cap protein, the phage DNA is susceptible to degradation by ExoV (RecBCD). A strain that has a mutation in the *recD* gene (BT125, V234) or lacks a functional RecBCD complex (V186, WA632) will be sensitive to the T4 gene2$^-$ bacteriophage. This infection results in lysis and formation of a plaque for that colony. BT125 was transformed with the wild-type *D. radiodurans* RecD only. The results are listed in Table **4**. The *Deinococcus radiodurans* protein cannot rescue the BT125 cells as they are still sensitive to the phage. This can be seen in Table **4** under the E.O.P. column where the titer for BT125 with the *D. radiodurans* RecD is more like that of V186, the RecBCD(-) strain (0.19 versus 0.24).

**Table 4 -** Data from the bacteriophage T4 gene2- nuclease assay[a]

| Strain | Genotype | Plasmid | Avg. titer (phage/ml)[b] | E.O.P.[e] |
|---|---|---|---|---|
| WA234 | wild-type | none | $3.3 \times 10^5$ | 0.00057 |
| WA632 | *recB21*[c] | none | $5.8 (\pm 0.2) \times 10^8$ | 1 |
| BT125 | *recD1011* | none | $5.3 (\pm 0.6) \times 10^7$ | 0.091 |
| BT125 | " | pKK D.r. RecD | $1.1 (\pm 0.01) \times 10^8$ | 0.19 |
| V324 | *recD1009* | none | $1.8 \times 10^7$ | 0.031 |
| V186 | Δ*recBCD*[d] | none | $1.4 \times 10^8$ | 0.24 |

[a] – Equal volumes of a diluted phage stock of known titer and a mid-log phase culture of experimental *E. coli* were mixed and placed at 37° C for 10 minutes. The BT125 [pKK D.r. RecD] cultures had IPTG added to a final concentration of 0.75 mM at an $O.D._{600}$ of 0.2. The phage-*E. coli* mixture was gently added to a pre-warmed aliquot of soft top agar. The solution was poured onto pre-warmed LB Petri dishes, incubated for 18 to 24 hours at 37° C and number of plaques counted.
[b] - The average titer for each strain was calculated via the equation (Plating dilution × Dilution factor for phage × Number of plaques).
[c] – The *E. coli* strain WA632 has a nonsense mutation in the *recB* gene that renders it RecBCD(-)
[d] - V186 has the entire gene region from *thyA–argA* deleted from its chromosome. This includes the genes for *recB, recC,* and *recD.*
[e] – The E.O.P. or Efficiency of Plating relates the average titer of an experimental strain that is fully permissive to phage infection to all other strains (Titer experimental strain / WA632 titer). WA632, the *recB* mutant, was chosen as the fully permissive strain. A number closer to zero indicates fully resistant to phage.

### 3.1.2 Mitomycin C exposure

Mitomycin C is a DNA damaging agent that blocks replication fork progression through crosslinking of opposing bases, usually a cytosine and guanine pair (42, 60). The *Deinococcus radiodurans* RecD might function to repair the harm in some way. To this end, two different *Escherichia coli* cell lines were chosen. V186 (Δ*rec*BCD) was transformed with the empty vector and the wild-type *D.radiodurans* RecD. V186 was selected to observe any DNA repair enhancement since the strain cannot resolve the damaged DNA into homologous recombination intermediates necessary to resume replication. Additionally, a RecD mutant strain, V324, was subjected to the same assay with the Dr RecD. This was done to observe any noticeable effect from expressing the foreign protein on the capable DNA repair processes of the RecBC(+)D(-) V324. The results of the MMC assay are shown in Table **5**. The cells lacking RecBCD and containing the *D. radiodurans* RecD remained sensitive to mitomycin C. V324 was still

able to survive on the MMC plates indicating that the presence of the non-native RecD did not hinder the repair ability.

**Table 5 -** Results from the mitomycin C exposure test[a]

| Strain | Genotype | plasmid | CFU / ml[b] |
|---|---|---|---|
| V186 | Δ*recBCD* | none | 0.00 |
| V186 | " | pKK 223-3 | 0.00 |
| V186 | " | pKK D.r. RecD | 0.00 |
| V324 | *recD1009* | none | 3.0 (±0.6) x $10^9$ |
| V324 | " | pKK D.r. RecD | 1.7 (±0.0) x $10^9$ |

[a] - Saline diluted samples of a mid-log phase *E. coli* culture (induced to 0.75 mM IPTG at an $O.D._{600}$ of 0.200) were aliquoted on to LB Petri dishes with and without 2 μg/ml MMC and allowed to grow at 37° C for 18-24 hours. Colonies were then counted.
[b] - CFU (Colony Forming Units) is calculated with the equation (colony count / 0.1 ml) / (dilution factor).

## 3.2 The *Deinococcus radiodurans* RecD negatively affects V186

### 3.2.1 Cell viability tests reveal an unexpected V186 phenotype

V186, lacking RecB, RecC, and RecD will die if the cell encounters any double-stranded breaks or stalled replication forks. The main repair pathway for both of these events is a functional RecA-RecBCD system. In the cell viability test, this will result in a reduction in the number and size of viable colonies seen on LB Petri dishes. WA632 is mutant for RecB and is similar to V186 in that both strains have a nonfunctional RecBCD complex in the cell. Wild-type strains AFT228, parent to V186 and WA234, isogenic to BT125 and WA632, were used in the viability tests. Another strain, V1119, was chosen after the initial V186 results as it is a *recA* mutant, and would allow further examination of the viability results from V186.

The strains in Table **6** were all transformed with the empty expression vector and that same vector having the Dr RecD. A plasmid that encodes for the lactose repressor

protein, pNM52, was included with either the pKK 223-3 or pKK D.r. RecD plasmids in some tests. V186 was also transformed with a RecD mutant, pKK K366Q RecD. Each strain was tested separately at least two times and most were tested three times. The data from the experiments are given in Tables **7** and **8**. To chart the expression level of the plasmids in the cell viability tests, several samples were removed at various time points during the growth and induction. A day-growth culture was sometimes split into two separate samples at an $O.D._{600}$ of 0.200, one of which was induced with IPTG (Isopropyl-beta-D-1-thiogalactopyranoside), while the other was uninduced. SDS-PAGE was performed on these time course samples to estimate the expression level. Figure **7** is an SDS-PAGE gel of V324 (a RecD mutant) with the *Deinococcus radiodurans* RecD, while Figure **8** is an SDS-PAGE gel of V324 with the *Escherichia coli* RecD protein.

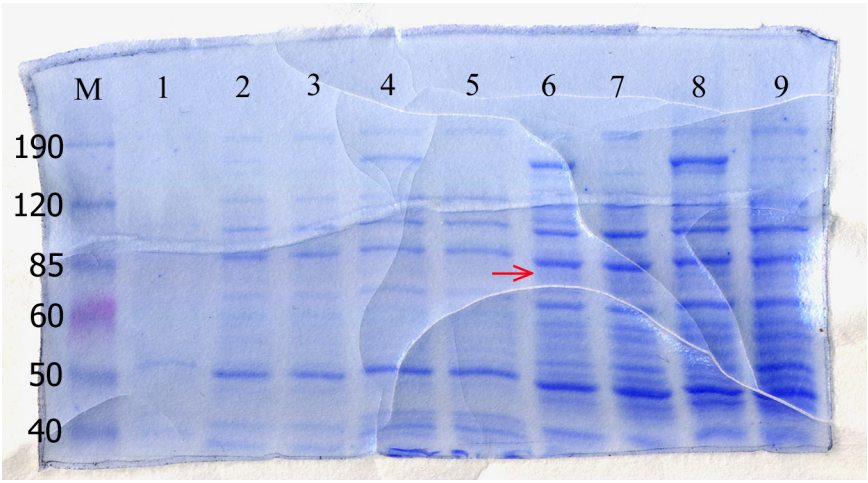


**Figure 7** – 7.5% acrylamide SDS-PAGE gel of the *E. coli* RecD mutant V324 expressing the pKK D.r. RecD plasmid. The day culture was separated into 2 samples at an $O.D._{600}$ of 0.200: One was induced with 1 mM IPTG, the other was untreated. The lane descriptions are as follows: (*lane M*) Pre-stained protein marker, (*lane 1*) preinduction, (*lane 2*) 30 minute induction, (*lane 3*) 30 minute noninduction, (*lane 4*) 1 hour induction, (*lane 5*) 1 hour noninduction, (*lane 6*) 2 hour induction, (*lane 7*) 2 hour noninduction, (*lane 8*) 3 hour induction, (*lane 9*) 3 hour noninduction. The red arrow indicates where the Dr RecD is expected to migrate when Coomassie stained (76 kD). The pre-stained protein marker molecular weights are included on the gel, in kiloDaltons.

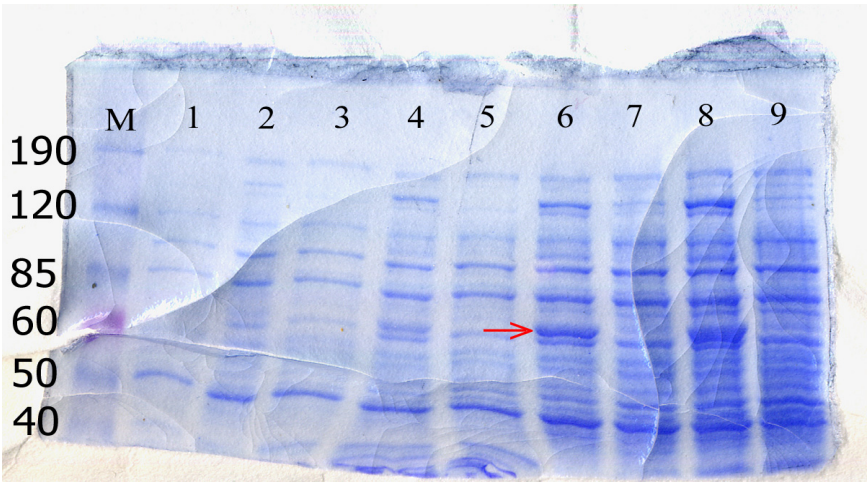


**Figure 8 –** 7.5% acrylamide SDS-PAGE gel of V324 with the pKK 223-3 vector plasmid expressing the *Escherichia coli* RecD protein. The lanes *M* and *1-9* have the same time points and parameters as those in Figure **7**. The red arrow shows the expected migration of the *E. coli* RecD when Coomassie stained (67 kD).

**Table 6 -** List of all *E. coli* strains tested in the cell viability assay

| Strain | Genotype | Relative cell viability[a] | Reference |
|---|---|---|---|
| WA234 | wild-type | 1 | |
| AFT228 | wild-type | 1 | |
| V324 | AFT228, *recD1009* | 0.85 | (14) |
| BT125 | WA234, *recD1011* | 0.95 | (14) |
| WA632 | WA234, *recB21* | 0.31 | (1) |
| V186 | AFT228, Δ*recBCD* | 0.12 | (13) |
| V1119 | WA234, *recA*$^-$ | 0.50 – 0.60 | (11) |

[a] – **Relative cell viability** is the ratio (in CFU/ml/O.D.$_{600}$) of mutant to parental strain as found in previous reports (see reference column in table).

The gels in Figures **7** and **8** show discernable differences in expression levels between the *Deinococcus radiodurans* RecD and the *Escherichia coli* RecD. A thick band corresponding to the molecular weight of the Ec RecD is readily visible after 2 hours of IPTG induction (see Figure **8**, lane 6), whereas there appears to be little to no over-expression in the same untreated samples (see Figure **8**, lanes 3, 5, 7, and 9). In contrast, the V324 expressing the *D. radiodurans* RecD supplemented with the same

stock of IPTG does not show this same inducible level of expression for the protein (compare induced in lanes 2, 4, 6, and 8 with uninduced lanes 3, 5, 7, and 9 in Figure **7**).

There were many trends observed throughout the cell viability tests. First, expression of the Dr RecD in a RecBC(+)D(-) background did not significantly affect the viability of that strain. This can be seen for V324 in Table **7** and BT125 in Table **8**. While the overall viability relative to AFT228 was less than expected, the V324 cultures expressing either the *Deinococcus radiodurans* RecD or the *Escherichia coli* RecD in the same vector led to similar relative cell viability results (0.44±0.05 for V324 [pKK D.r. RecD] versus 0.44±0.02 for V324 [pPB100]), and were comparable to V324 with the empty vector (0.49±0.04). The *recD1011* mutant, BT125, did have a slight decrease in viability when the pNM52 plasmid was omitted (0.76 relative to BT125 [pNM52/pKK D.r. RecD]), thereby permitting any background expression (and presumably any negative effect) from occurring in the presence of the *D. radiodurans* RecD protein. The viability relative to the wild-type was, however, very similar given the standard error (1.31±0.33 for BT125 [pNM52/pKK D.r. RecD] versus 1.00±0.28 for BT125 [pKK D.r. RecD]).

Second, those strains (V186 and WA632) lacking a functional RecBCD complex were negatively affected by the presence of the *D. radiodurans* RecD protein (for V186, see the bottom of Table **7**, and for WA632, the middle of Table **8**). This was unexpected given the results reported by other groups on RecD overexpression in a RecBCD(-) background (53). In those viability tests, a Δ*recBCD* strain was found to have very little difference in viability (<4%) between the vector alone and that same vector producing the *E. coli* RecD. The variation in viability in V186 with the Dr RecD and the empty vector

was evident among the isogenic AFT228 strains (0.04±0.01 versus 0.34±0.06 compared to AFT228 [pKK 223-3]), as well as within V186 where there was over a 9-fold reduction in viability (0.11) between pKK 223-3 and the pKK D.r. RecD strains. WA632, the *recB21* mutant, also displayed a decrease in cellular viability when compared to both the empty vector (0.10±0.04 versus 0.43±0.11) and that of WA632 without any plasmids (0.10±0.04 versus 0.25±0.07). Within the strain itself, there appeared a 60% reduction in the viability tests when the pKK D.r. RecD was present versus in its absence. An even more striking result is where in WA632 the viability for vector alone is contrasted with WA632 expressing the *Deinococcus radiodurans* RecD (1.7 for WA632 [pKK 223-3] versus 0.4 for WA632 [pKK D.r. RecD], a decrease of 425%).

Third, the harmful effect of the Dr RecD in V186 and WA632 was mitigated when the lactose repressor protein was included in the tests (observe V186 with pNM52, bottom of Table **7** and WA632 with pNM52, middle of Table **8**). Because the expression system relies on an inducible hybrid *lac* and *trp* promoter/operator, the lactose repressor protein should prevent any leaky expression of the *D. radiodurans* RecD in the absence of IPTG. The intended effect of the pNM52 plasmid was apparent as the V186 [pNM52/pKK D.r. RecD] showed a doubling in viability (0.08±0.01 versus 0.04±0.01 in V186 [pKK D.r. RecD]), and there was little difference between V186 with either the *D. radiodurans* RecD (0.08±0.01) or the vector alone (0.09±0.01). In WA632 strains harboring the pNM52 plasmid, little to no variation was observed among the pKK 223-3 (0.28±0.09), pKK D.r. RecD (0.27±0.07), and no additional plasmid (0.28±0.07) samples.

Fourth, expression of a mutant form of the *D. radiodurans* RecD (pKK K366Q RecD) in V186 lessened the harmful phenotype noticed in V186 [pKK D.r. RecD] cultures (see bottom of Table **7**). An increase of 18% relative to AFT228 (0.04±0.01 to 0.22±0.07), and >40% within the strain (0.11 to 0.65) was seen for V186. This mutation had been shown (16) to eliminate the ATP hydrolysis reaction required for the *Escherichia coli* RecD helicase (36, 37).

Previous reports (53) had indicated that it was possible to generate a ~20% decrease in viability in the same wild-type strain (AB1157) used in this study from overexpression of the native RecD polypeptide. Wild-type *E. coli* strains such as WA234(=AB1157) and AFT228 had approximately the same viability in the experiment when expressing the *D. radiodurans* RecD (for AFT228, see top of Table **7** and for WA234 see top of Table **8**). The same Kuzminov article demonstrated that in *recA*$^{-}$ *E. coli* strains, over production of the Ec RecD subunit had no discernable consequence on viability when compared to the empty vector. The same phenomenon was seen in the RecA mutant V1119 where there was even a modest 20% increase between V1119 [pKK 223-3] and V1119 [pKK D.r. RecD] (see V1119, bottom of Table **8**), but no decrease as had been observed in V186 and WA632.

**Table 7 -** Cell viability data for the wild-type strain AFT228 and isogenic V186 and V324[a]

| Strain | Genotype | Plasmid | CFU/ml /OD (± std. dev.)[b] | Relative Viability[c] | Viability Relative to AFT228[d] |
|---|---|---|---|---|---|
| AFT228 | wild-type | pKK 223-3 | 1.8 (±0.09) x $10^9$ | 1 | 1 |
| AFT228 | " | pKK D.r. RecD | 1.5 (±0.07) x $10^9$ | 0.83 | 0.83 (±0.06) |
| | | | | | |
| V324 | *recD1009* | pKK 223-3 | 8.8 (±0.5) x $10^8$ | 1 | 0.49 (±0.04) |
| V324 | " | pKK D.r. RecD | 8.0 (±0.8) x $10^8$ | 0.91 | 0.44 (±0.05) |
| V324 | " | pPB100[e] | 8.0 (±0.2) x $10^8$ | 0.91 | 0.44 (±0.02) |
| | | | | | |
| V186 | " | pKK 223-3 | 6.2 (±1.1) x $10^8$ | 1 | 0.34 (±0.06) |
| V186 | " | pKK D.r. RecD | 0.7 (±0.1) x $10^8$ | 0.11 | 0.04 (±0.01) |
| V186 | " | pKK K366Q RecD[f] | 4.0 (±1.3) x $10^8$ | 0.65 | 0.22 (±0.07) |
| V186 [pNM52][g] | " , lacI$^q$ | pKK 223-3 | 1.4 (±0.2) x $10^8$ | 0.23 | 0.08 (±0.01) |
| V186 [pNM52] | " , lacI$^q$ | pKK D.r. RecD | 1.6 (±0.1) x $10^8$ | 0.26 | 0.09 (±0.01) |

[a] – A saturated overnight culture of experimental *E. coli* was diluted 1:50 into fresh LB media. The $O.D._{600}$ was monitored until the culture reached 0.200, when it was induced with IPTG to 0.75 mM. The growth was halted at an $O.D._{600}$ of 0.500-0.600. The culture was saline diluted and each trial was plated in triplicate and incubated for 18-24 hours at 37° C. Only those samples having between 30 and 330 colonies were counted.

[b] - The calculation for CFU / ml / $O.D._{600}$ is (colony count / 0.1 ml) / (dilution factor / $O.D._{600}$).

[c] – Relative viability is the ratio of CFU/ml/O.D. of a strain with vector to any other plasmid in that same strain.

[d] - The viability relative to AFT228 [pKK 223-3] is represented as F(±Z) where F is the ratio of CFU/ml/OD for any strain divided by the CFU/ml/OD for AFT228 [pKK223-3], and Z is calculated via the equation $(F)\times((x^2/a^2)+(y^2/b^2))^{½}$ where *x* is the standard deviation and *a* is the CFU/ml/OD for any strain and *y* is the standard deviation and *b* is the CFU/ml/OD for AFT228 [pKK 223-3].

[e] – The pPB100 plasmid is the pKK 223-3 vector with the *E. coli* RecD protein.

[f] – pKK K366Q RecD expresses a mutant form of *Deinococcus radiodurans* RecD that should be unable to bind ATP.

[g] – pNM52 is an additional plasmid that encodes the lactose repressor protein.

**Table 8 -** Results from the cell viability assay with wild-type WA234 and isogenic strains BT125, WA632, and V1119[a]

| Strain | Genotype | Plasmid | CFU / ml / OD (± std. dev.) | Relative Viability | Viability Relative to WA234 |
|---|---|---|---|---|---|
| WA234 | wild-type | none | 1.6 (±0.4) x $10^9$ | 1 | 1 |
| WA234 | | pKK 223-3 | 2.0 (±0.4) x $10^9$ | 1.25 | 1.25 (±0.40) |
| WA234 | | pKK D.r. RecD | 1.7 (±0.3) x $10^9$ | 1.06 | 1.06 (±0.33) |
| | | | | | |
| BT125 | *recD1011* | none | 2.1 (±0.08) x $10^9$ | 1 | 1.31 (±0.33) |
| BT125 [pNM52] | " , $lacI^q$ | pKK D.r. RecD | 1.6 (±0.2) x $10^9$ | 0.76 | 1.00 (±0.28) |
| | | | | | |
| WA632 [pNM52] | *recB21*, $lacI^q$ | none | 4.5 (±0.4) x $10^8$ | 1 | 0.28 (±0.07) |
| WA632 [pNM52] | " | pKK 223-3 | 4.5 (±0.9) x $10^8$ | 1 | 0.28 (±0.09) |
| WA632 [pNM52] | " | pKK D.r. RecD | 4.3 (±0.2) x $10^8$ | 0.96 | 0.27 (±0.07) |
| | | | | | |
| WA632 | *recB21* | none | 4.0 (±0.5) x $10^8$ | 1 | 0.25 (±0.07) |
| WA632 | " | pKK 223-3 | 6.8 (±0.5) x $10^8$ | 1.7 | 0.43 (±0.11) |
| WA632 | " | pKK D.r. RecD | 1.6 (±0.4) x $10^8$ | 0.4 | 0.10 (±0.04) |
| | | | | | |
| V1119 | *recA*$^-$ | pKK 223-3 | 6.8 (±6.1) x $10^8$ | 1 | 0.43 (±0.40) |
| V1119 | " | pKK D.r. RecD | 8.2 (±0.3) x $10^8$ | 1.2 | 0.51 (±0.13) |

[a] - The experiment was performed three times except in the case of BT125 and V1119 where it was only done twice. See Table (**7**) footnotes (a)-(d) for experimental details.

### 3.2.2 Microscopy complements V186 [pKK D.r. RecD] cell viability data

The large negative effect seen in V186 was further pursued with microscopy studies to resolve the issue of low colony count on V186 [pKK D.r. RecD] plates. Cells, when under stress, can trigger the SOS response to DNA damage. Several cellular events can take place, and among these is filamentation. Filaments are many cell equivalents which have failed to resolve into individual units (32). Typical wild-type *Escherichia coli* sizes are on average 2.0 μm and can reach up to 10 μm (24). The measurement of optical density in a UV/Visible spectrophotometer does not distinguish between viable and non-viable cells, only those objects able to scatter light. Therefore the rise in the O.D. might come from long, filamentous structures scattering the input light that form in V186 expressing the *D. radiodurans* RecD.

Both brightfield (for size and shape of *E. coli*) and fluorescence (for DAPI DNA staining) were used, as well as a kit from Invitrogen titled LIVE/DEAD® Baclight™ Bacterial Viability. This kit permitted the viewing of living and dead cells in the same field of view (see experimental section for kit contents and explanation of technique). Most microscopy experiments were coupled to a cell viability test on the same day. All microscope work was performed in the lab of Dr. Stephen Wolniak from the Department of Cell Biology and Molecular Genetics of the University of Maryland, College Park. The strains used were V186 and WA632, as well as WA234 for size and shape reference of *E. coli*. V186 and WA632 were each transformed with the empty vector and the *D. radiodurans* RecD, and the two cultures were compared on the same day.

Several representative pictures of V186 with empty vector (pKK223-3) and with the *Deinococcus radiodurans* RecD are shown in Figures **11** and **12**. Any cell with a

length greater than 10 μm was considered to be filamentous. A distribution of the various cell sizes in V186 observed can be seen in Figure **9**. Overall, the V186 [pKK D.r. RecD] cells had a higher number of and larger size filament(s) than the corresponding strain with empty vector. Only 0.51% of the 6651 counted cells were above 10 μm in the V186 [pKK 223-3] cultures, whereas 6.1% of the 6111 cells in V186 [pKK D.r. RecD] were filaments. The largest filamentous cell observed in V186 [pKK 223-3] was 16 μm, and the average filament size was 13.1 (±1.7) μm. The V186 [pKK D.r. RecD] samples had 37 filaments in excess of 40 μm, and the average filament size was 22.8 (±12.1) μm. Some may have been longer given the limited field of view of the digital camera.

The initial conclusion that can be reached from Figure **9** was that there were a low number of filaments per general population of V186 cells expressing the Dr RecD. The majority of the growing V186 [pKK D.r. RecD] appeared to be of the typical size range expected (2 to 3 μm for nondividing, 4 to 6 μm for actively dividing). In order to gain a better perspective, it was reasoned that because the filaments themselves were actually comprised of several smaller units, that is to say, the individual *E. coli* cells that had failed to separate upon division, the filamentous units could not be considered one object, as the *ImageJ* program interpreted. Thus further data analysis was done whereby the concept of 1 cell equivalent equaling the known *Escherichia coli* cell length of 2 μm was applied to the existing measurement data seen in Figure **9**. Figure **10** details the cell equivalents found in the V186 [pKK D.r. RecD] cultures compared to that of V186 [pKK 223-3]. The original percent filaments of the total cell population was 0.51% for V186 with empty vector, and modestly increases to 1.5% cell equivalents in those filaments.

The intriguing result was with the V186 [pKK D.r. RecD] strain, where the cell equivalents in the filaments was found to be 29.0% of the total cell equivalents.

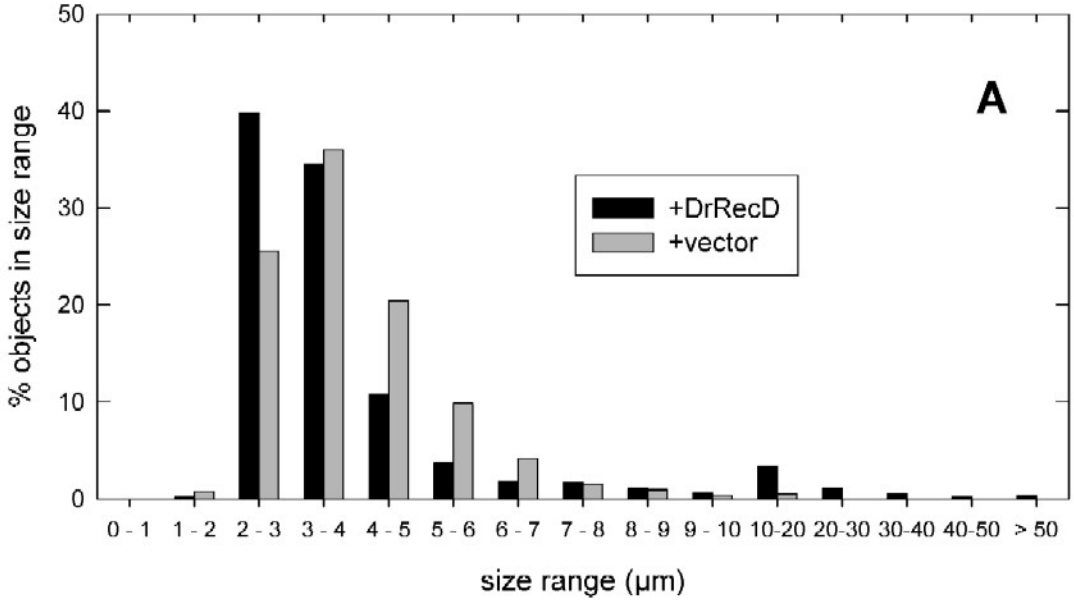


**Figure 9 –** A histogram detailing the ranges of V186 cell sizes in the brightfield microscopy experiments. The empty vector is the pKK 223-3 plasmid. The total number of counted *E. coli* cells for the V186 [pKK 223-3] strain was 6650, while that of the V186 [pKK D.r. RecD] was 6109.

The WA632 [pKK D.r. RecD] had a slightly different result than V186 [pKK D.r. RecD]. Pictures of WA632 with Dr RecD or pKK223-3 are shown in Figure **14**. The WA632, while being essentially a RecBCD(-) cell as V186, displayed filamentation in 6.6% of the total cell population. The longest detectable filament was 48 µm, and the average filament size was 17.1 (±8.6) µm. WA632 [pKK223-3], in contrast, showed no filaments or any cell lengths above 5 µm. The reaction of the wild-type WA234 strain to the SOS-inducing nalidixic acid can be seen in Figure **13**. There was an increase in cell length from 3.2 (±0.7) µm to 5.0 (±1.6) µm, with no significant filamentation over 11 µm.

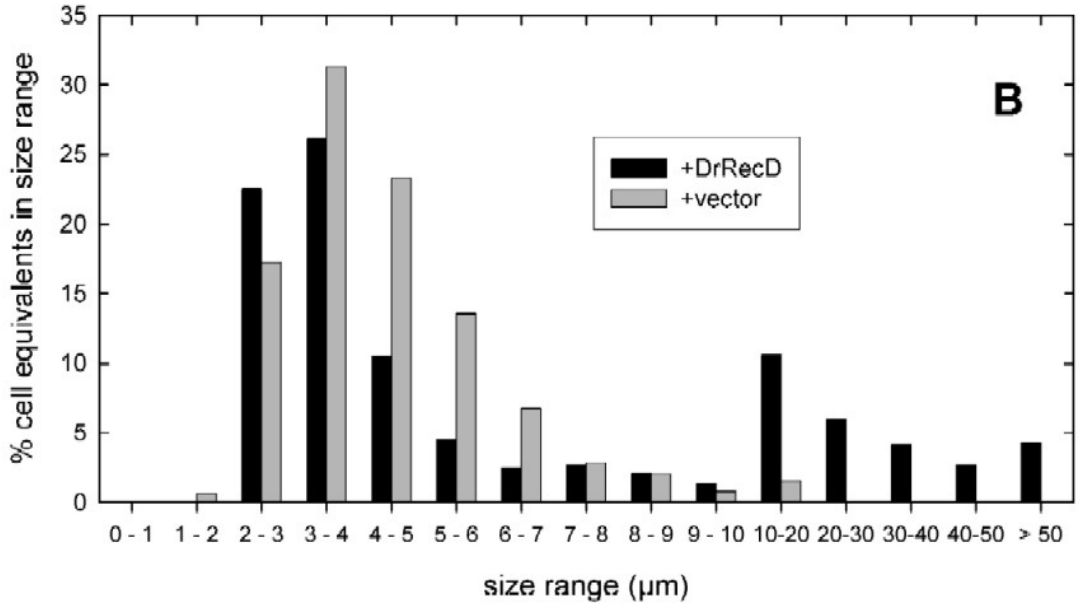


**Figure 10** – Histogram of the number of cell equivalents seen in the V186 [pKK D.r. RecD] and V186 [pKK 223-3] samples in the brightfield microscopy experiments. Each cell equivalent is equal to 2 µm.

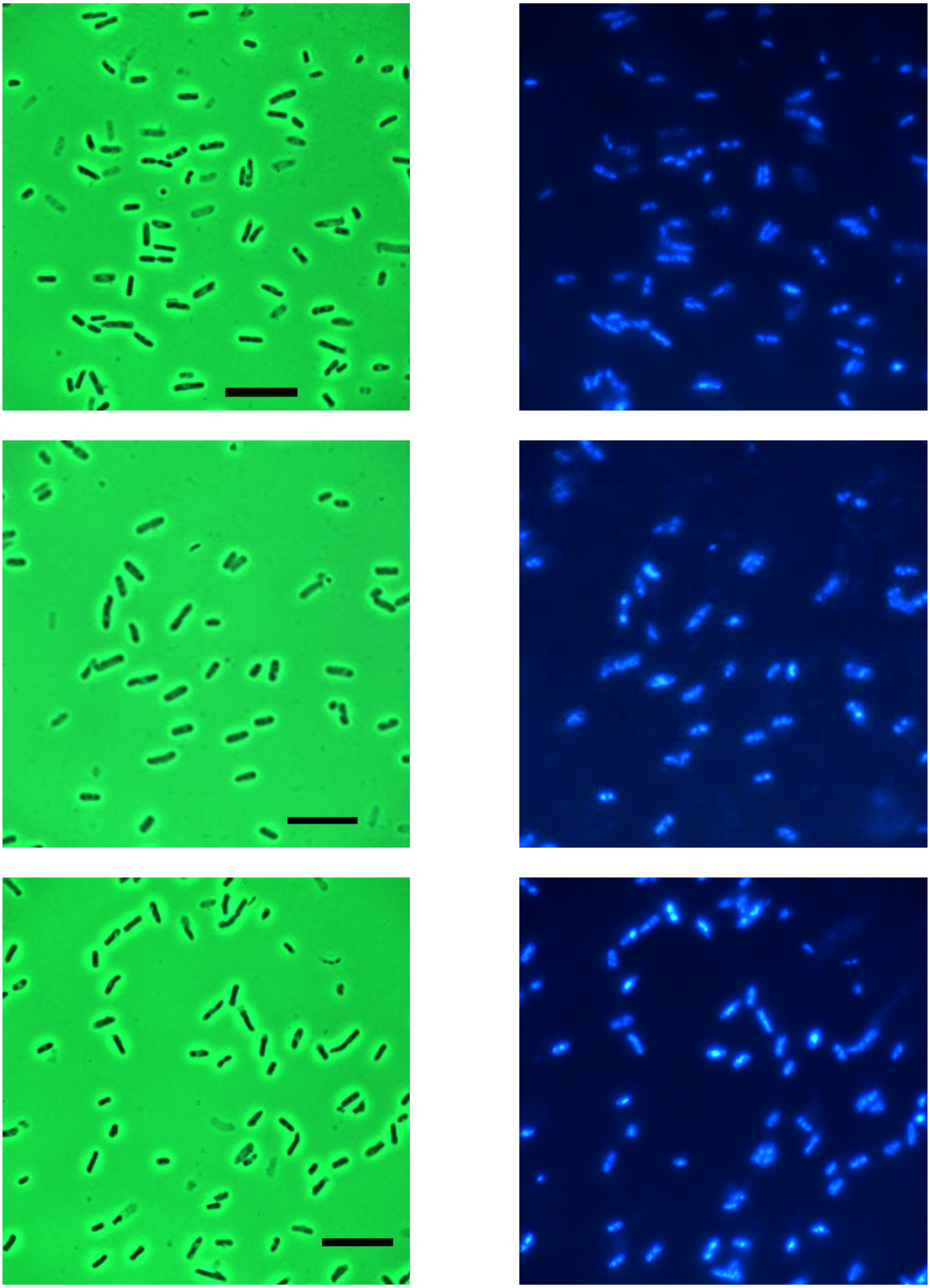

**Figure 11 -** The V186 cells with the empty vector pKK 223-3. The left panels are brightfield, while the right panels are the corresponding images stained with DAPI to visualize the nucleoid. All pictures were taken with 630X magnification. The black bar represents 10 μm.

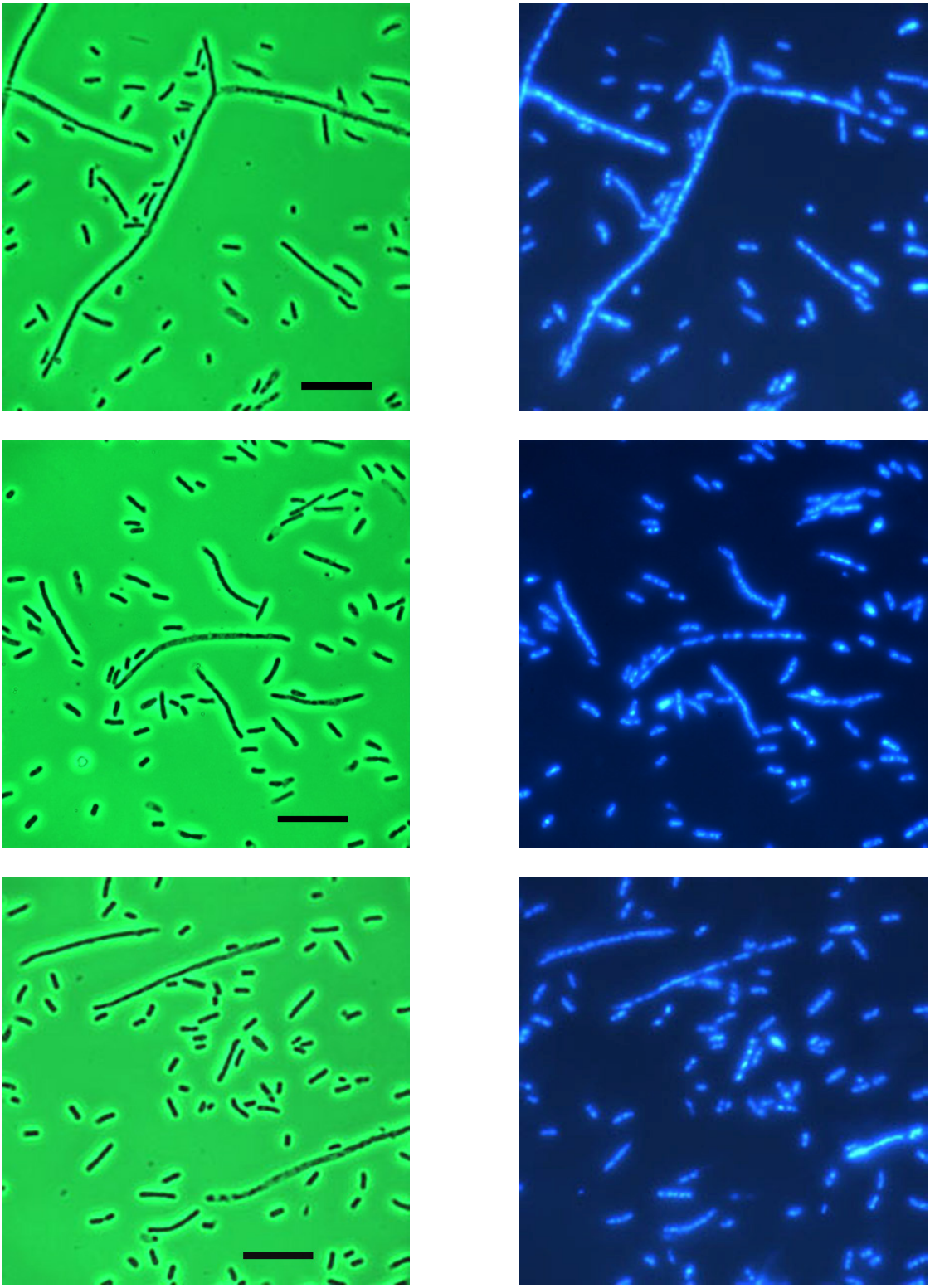

**Figure 12 -** The V186 cells expressing the wild-type Dr RecD. Brightfield images are the left panels and the corresponding DAPI stained are the right panels. The black bar represents 10 μm.

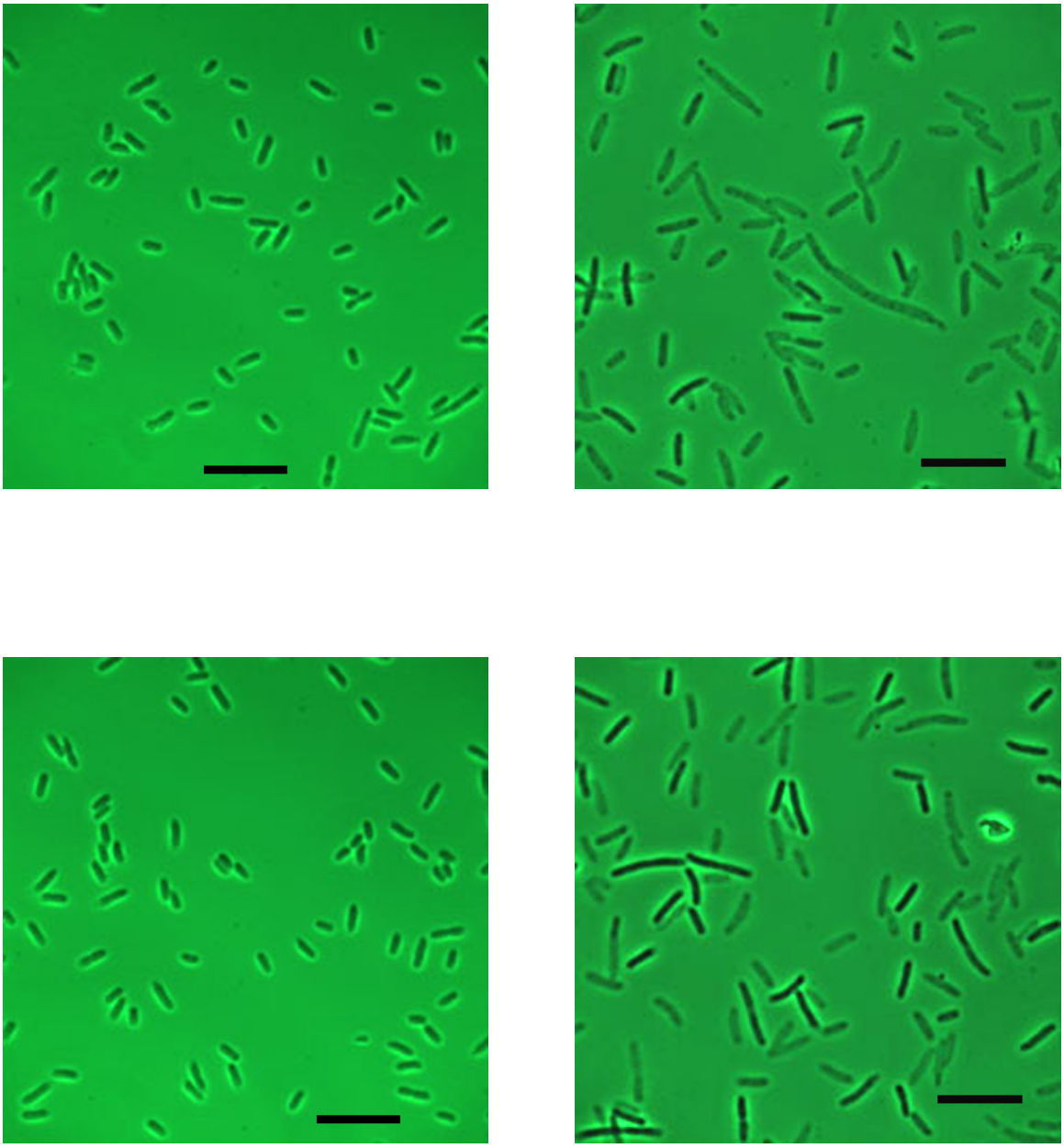

**Figure 13 -** Brightfield images of WA234 wild-type *E. coli*. The left panels are WA234 under normal growth conditions with no additives. The images on the right panel were taken from a WA234 culture with nalidixic acid at a final concentration of 15 μg/ml. Nalidixic acid inhibits replication and triggers the SOS response and filamentation. The black bar represents 10 μm. Each photograph is from a new field of view for both WA234 samples.

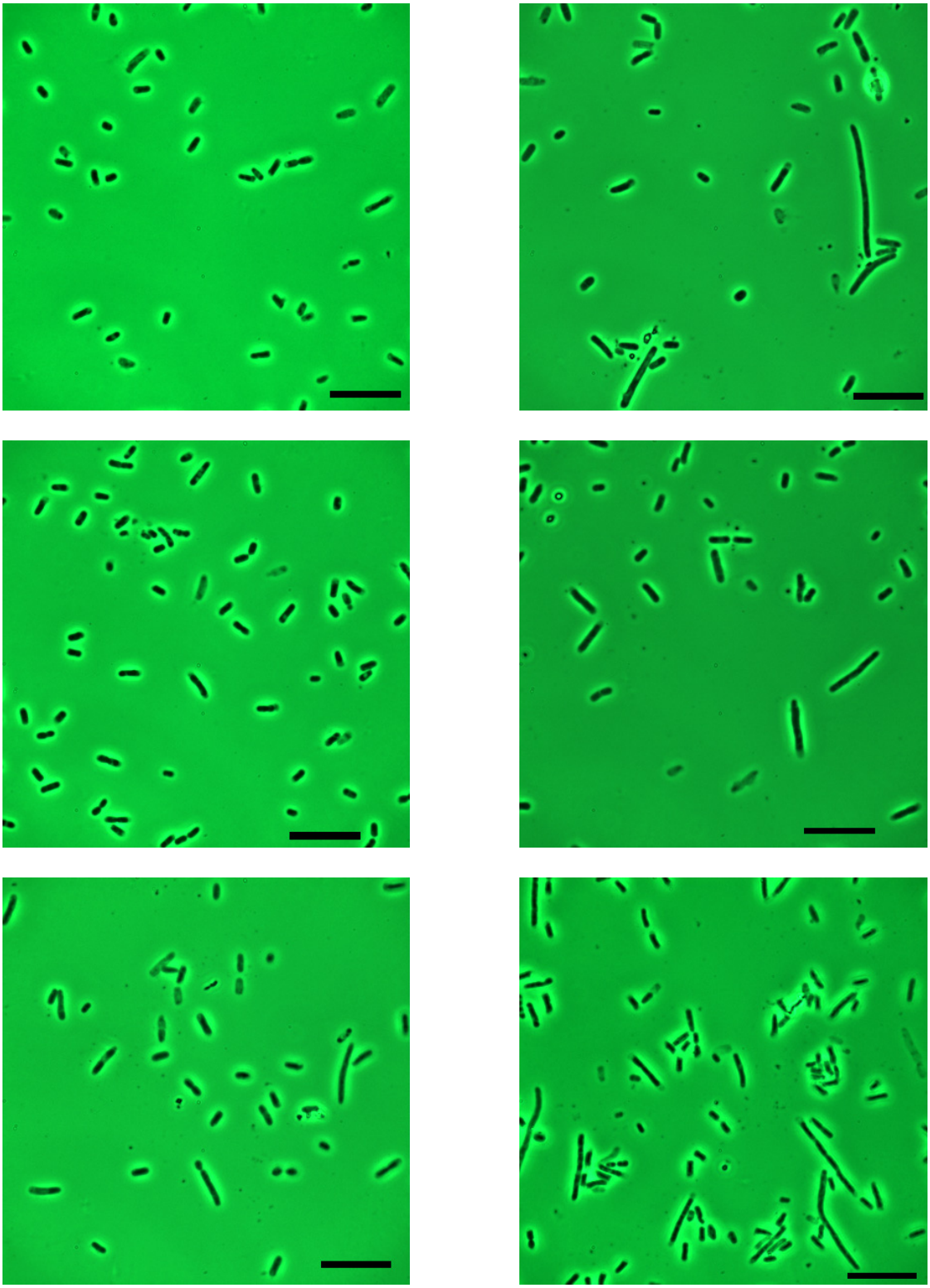

**Figure 14 -** Brightfield images of the *recB* mutant WA632. The left panels are WA632 with the empty vector pKK 223-3. The right panels are WA632 with the wild-type Dr RecD. The black bar represents 10 μm. All images seen are from different fields of view in both samples.

The LIVE/DEAD® kit from Invitrogen permits the researcher to view living and dead bacterial cells in the same field of view, provided the microscope has the proper optical filter sets. V186 (Δ*recBCD*) was transformed with the empty vector pKK223-3, pKK D.r. RecD, as well as the pKK K366Q RecD. The number of filamentous cells, or those that were longer than 10 μm, were counted and sorted according to whether they appeared green (alive) versus red (dead). This part of the microscopy work was performed in order to determine whether the long cellular structures observed previously were truly dead, as was initially believed. The average of two separate experiments with the kit was taken. Several representative images from each sample can be seen in Figures **15**, **16**, and **17**.

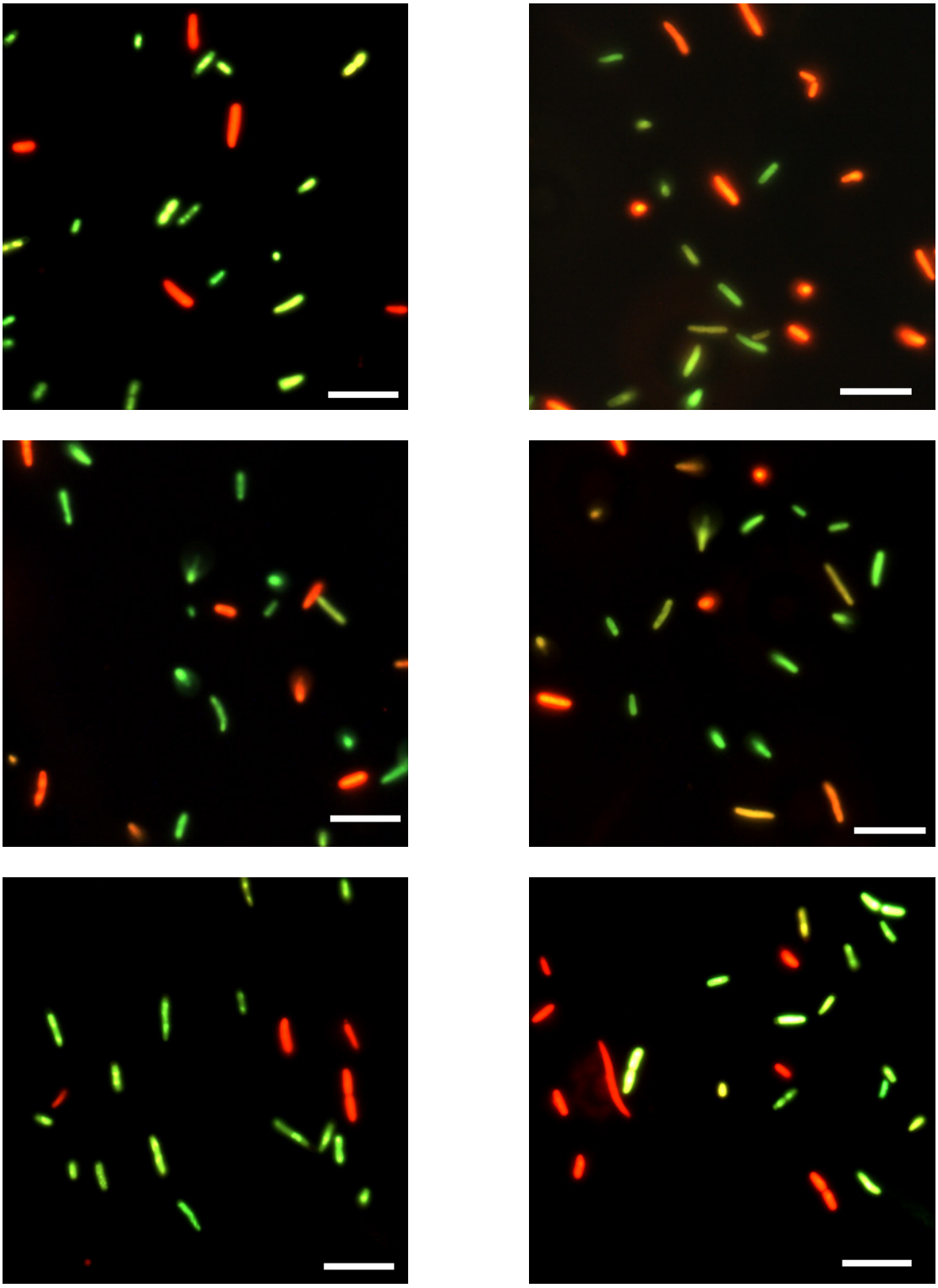

**Figure 15 -** V186 with the empty vector pKK 223-3 as seen with the LIVE/DEAD® Baclight™ bacterial viability kit from Invitrogen. All images taken at 630X magnification. Dead bacteria are red, while live bacteria are green. The white bar represents 10 μm. All images were photographed in a different field of view.

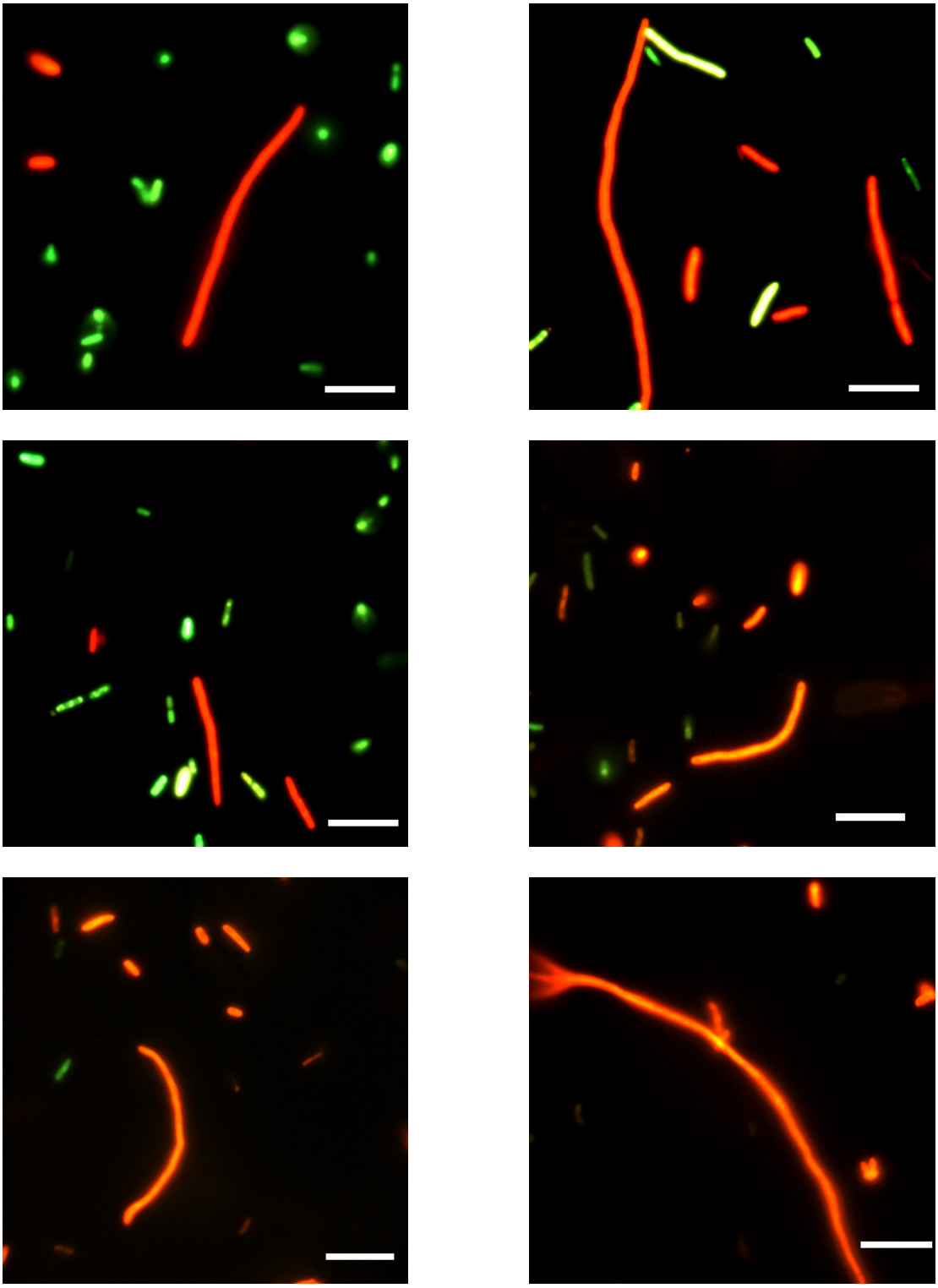

**Figure 16 -** V186 with the pKK 223-3 plasmid expressing the *D. radiodurans* wild-type RecD protein, as seen with the Invitrogen LIVE/DEAD® Baclight™ bacterial viability kit. All images taken at 630X magnification. The white bar represents 10 μm. Each image was taken in a new field of view.

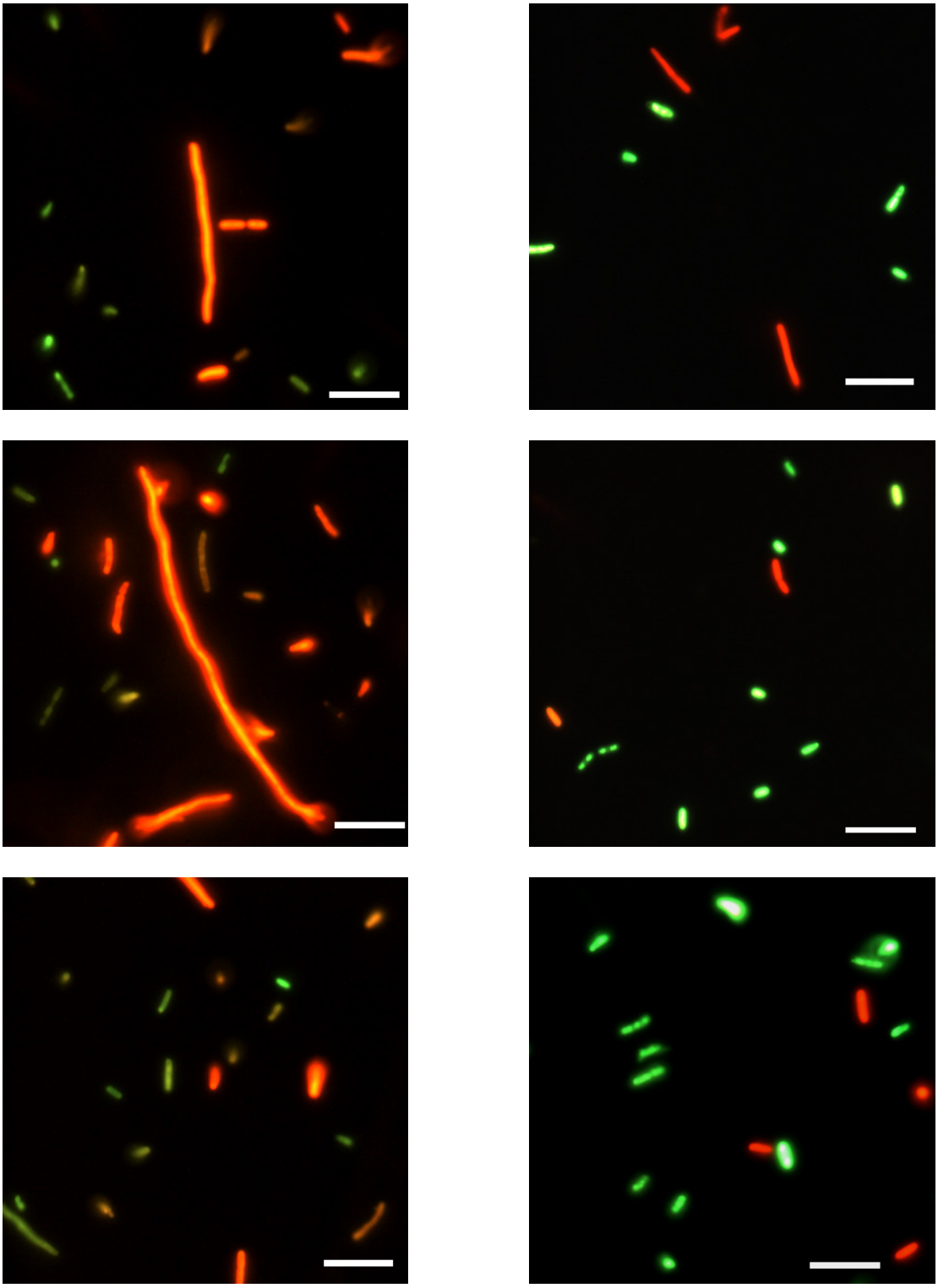

**Figure 17 -** V186 with the pKK 223-3 plasmid expressing the K366Q RecD mutant protein, as seen with the LIVE/DEAD® Baclight™ bacterial viability kit from Invitrogen. All images taken at 630X magnification. The white bar represents 10 μm. Each photo is from a different field of view.

Preliminary results with the LIVE/DEAD® Baclight™ bacterial viability kit indicate that the V186 [pKK D.r. RecD] strains produce more dead filaments than that of V186 [pKK 223-3]. Out of the 78 filaments observed in the *D. radiodurans* RecD-containing samples, 62 appeared dead under the fluorescent microscope, or 79.5%. This contrasts with the V186 [pKK 223-3] samples that had 7 dead filaments out of a total population of 14 filamentous cells. The total number of counted *E. coli* cells in both strains in the test were 1265 cells for V186 [pKK 223-3] and 1059 for V186 [pKK D.r. RecD]. The microscope pictures were not analyzed for the amount of dead, normally shaped cells. There was insufficient data to make a conclusion on the V186 [pKK K366Q RecD] sample as not enough pictures were taken to be studied.

# Chapter 4: Discussion

## 4.1 *Deinococcus radiodurans* RecD protein does not complement *E.coli* RecD(-) or RecBCD(-) strains

The RecD protein from *D. radiodurans* had been biochemically characterized *in vitro* prior to this work, and was shown to have helicase activity (61). The amino acid sequence and subsequent homology search revealed the seven helicase motifs typical of Superfamily I helicases, including the conserved Walker A and Walker B purine NTP binding sequences. There is a region beginning at the amino terminus and extending to roughly 300 residues that did not show any homology with other helicase domains or other potentially functional domains (See Figure **5**). This deficit in knowledge about the *in vivo* function of the *Deinococcus radiodurans* RecD protein led to explorations in several RecBCD (-) and RecD (-) *Escherichia coli* strains. The bacteriophage T4 gene2$^{-}$ nuclease assay, exposure to mitomycin C, and cell viability assay were three tests selected to discern the biological action of Dr RecD in *E. coli*.

Results from the T4 and mitomycin C tests for RecBC(+)D(-) strains such as BT125 and V324 indicated that the *D. radiodurans* RecD protein cannot replace the missing native subunit in the RecBC complex of those cells. The cell viability of V324 and BT125 showed little deviance in the presence of the Dr RecD compared to the empty vector. Additionally, the same viability was seen when the same vector containing the *Escherichia coli recD* gene was transformed into the V324, and was expressed at much higher levels (see Figures **7** and **8** under Section **3.2.1**) than the *Deinococcus radiodurans* RecD protein. The *E. coli* strains that do not have a functional native RecD are still capable of repairing DSB, or stalled or collapsed replication forks. Therefore whether or

not the *Deinococcus radiodurans* RecD can assemble into a competent trimer with RecB and RecC would have no impact on the DNA damage the foreign product would continue to exert upon expression (either naturally through a leaky promoter or IPTG induced). This interruption in replication caused by the Dr RecD would still be repaired, and would be masked in the cell viability or microscope studies. Possible tests to discern whether or not the *D. radiodurans* RecD was still causing the same disruption of the replication fork include fluorescently tagging the RecD and observing whether or not the protein localized to the replication fork. Also, any proteins (such as PriA, RecA, or other replication fork proteins) could be fluorescently tagged, and their locations and lifetimes in the cell analyzed for any variations associated with recruitment to sites of DNA damage and/or replication fork collapse. Pulsed Field Gel Electrophoresis (PFGE) could also be applied to measure the level of double-stranded breaks in V324 with and without the Dr RecD over a long enough time course with short sample intervals to detect the chromosomal breakage presuming to occur in those cultures.

The crystal structure of *Escherichia coli* RecBCD bound to a short double-stranded DNA oligomer was released in 2004 (57). The RecD subunit of this heterotrimer was observed to have 3 ordered domains, of which the 1$^{st}$ domain makes extensive contacts with subdomain 2B of RecC. Figure **18** is representation of the RecD and RecC interaction regions using the PyMol molecular modeling program and PDB file 1W36. We speculate that the *D. radiodurans* RecD helicase is unable to form a competent complex with the RecB and RecC proteins of *E. coli*. The large amino terminus of the helicase (see Figure **5** of Introduction) may form some kind of domain or domains that precludes formation of the heterotrimer with RecB and RecC. Figure **18** is

labeled for the amino terminal region of the *E. coli* RecD subunit where it forms interactions necessary for binding to and creating a functional complex. The 3 domains of the *E. coli* RecD subunit can be viewed in Figure **19**.

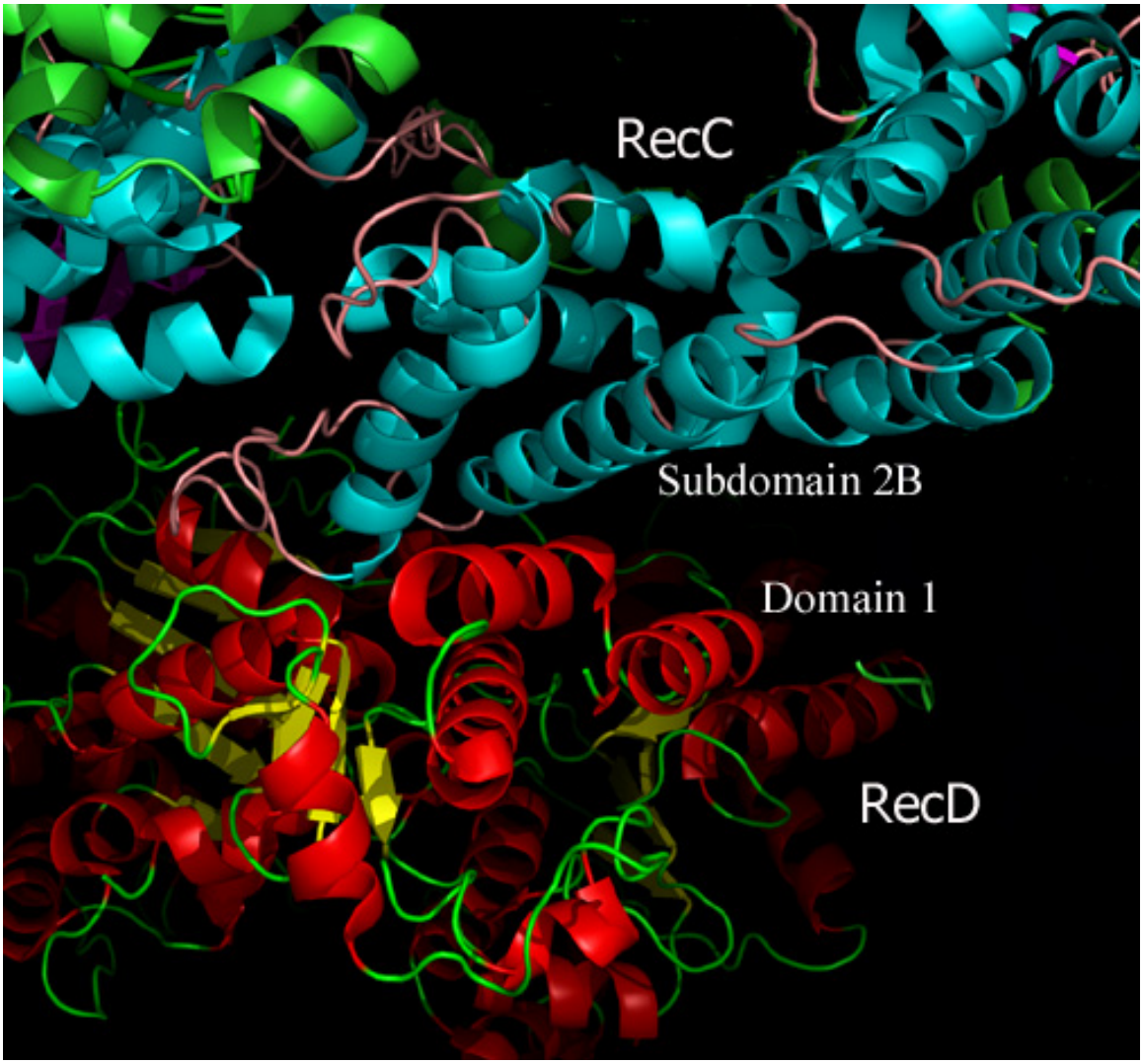


**Figure 18 –** A view of the interface of the RecC and RecD subunits, part of the RecBCD heterotrimer. The structure of RecD is represented as red helices and green unordered residues, while RecC is shown in blue helices and pink unordered residues. The amino terminal domain, known as Domain 1 of RecD makes many contacts with Subdomain 2B of RecC. RecB and the bound oligomer have been eliminated from the field of view for convenience (Figure made with PyMol using coordinates in PDB file 1W36)

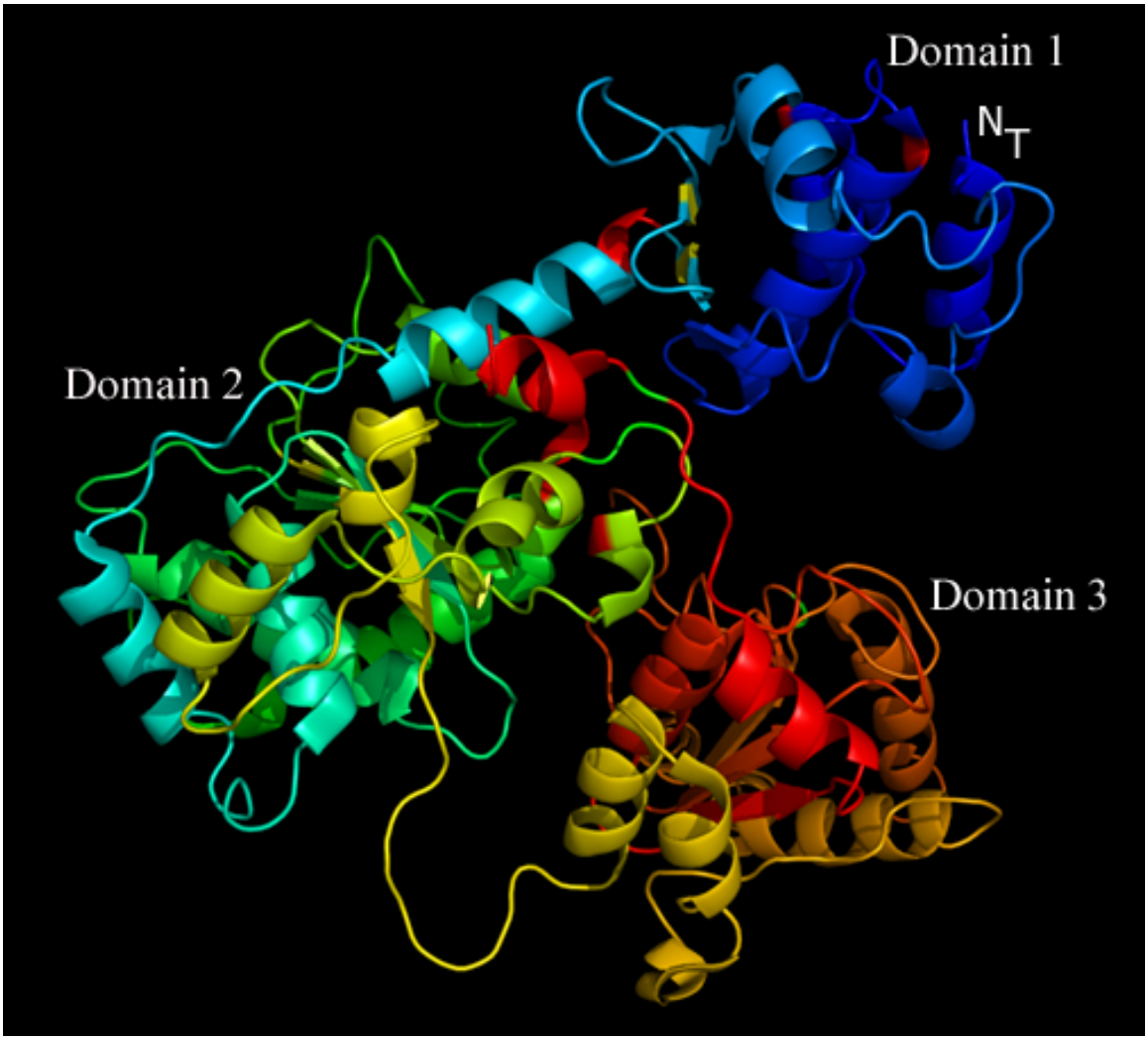


**Figure 19 –** The RecD subunit of *E. coli* RecBCD, as depicted with the PyMol molecular modeling program. The 3 main domains seen in the crystal structure of the helicase are labeled Domain 1, Domain 2, and Domain 3. The amino terminal end of the protein is indicated by the $N_T$ symbol. It is Domain 1 that forms the interface with Subdomain 2B of RecC in the heterotrimer.

In the MMC and cell viability assays with RecBCD(-) strains like V186 and WA632, a negative phenotype emerged. The chemical DNA damaging agent had the same lethal effect on the V186 with and without the plasmid expressing the *D. radiodurans* RecD protein. This showed that the helicase did not contain some other intrinsic enzymatic activity that could assist in regenerating the replication fork through homologous recombination intermediates. These intermediates are normally formed by

RecA and RecBCD in wild-type *E. coli* after mitomycin C interrupts the replication process.

## 4.2 Cell viability results with the *D. radiodurans* RecD

The RecD mutant strains BT125 and V324 did not display negative effects from expression of the *Deinococcus radiodurans* RecD protein. This observation can be deduced from the proposal presented in Section **4.1** and Figures **18** and **19** where it is proposed that the Dr RecD cannot fit into the RecBC complex owing to its large, possibly ordered amino terminal region. The harmful phenotype derived from the expression of the *D. radiodurans* RecD would not come from its capacity to form nonfunctional heterotrimers with the RecB and RecC subunits (a form of competitive inhibition), but from its localization to moving replication forks, thereby causing stalls or collapse of the cellular machinery. These errors can be fixed in a RecA-RecBC strain, and the temporary damage incurred from Dr RecD overexpression would not be apparent.

V186 and WA632 exhibited large decreases in cell viability when expressing the *D. radiodurans* RecD. Section **4.3** discusses the reasons for this phenomenon. The reduction in the ability to form viable colonies was only relieved when the pNM52 plasmid encoding the lactose repressor protein was co-transformed along with the pKK D.r. RecD plasmid. It is believed that the negative phenotype was not observed because the presence of the lactose repressor on the *tac* promoter prevented background expression of RecD during the period before induction. Had V186 [pNM52/pKK D.r. RecD] or WA632 [pNM52/pKK D.r. RecD] been permitted to grow to a higher optical density, the same drop in viability may have been seen.

Wild-type strains WA234 and AFT228 did not show a negative or significantly altered growth phenotype in the cell viability tests. This is consistent with the speculation in Section **4.1** about the Dr RecD's inability to bind to the RecBCD complex, which, if possible, might have lowered the viability to levels seen in previous research on expression of Ec RecD in a RecBCD(+) background. The authors of that report conclude that in typical physiological conditions, wild-type cells have RecBCD macromolecules that are inactivated for the exonuclease action on the *E. coli* genome due to the presence of Chi sites. This arrives from inactivation of the RecD subunit upon χ encounter. That was circumnavigated in strains carrying expression plasmids harboring the Ec RecD. Through simple mass action principles, enough functional RecD exists in those cells to regenerate the exonuclease activity via replacement of bound, inactivated RecD subunits and therefore lower the viability. The current system suggested in WA234 [pKK D.r. RecD] or AFT228 [pKK D.r. RecD] precludes this scenario observed in the AB1157 strain with the *E. coli* RecD helicase. Were it able to substitute for the native subunit, overexpression of the *Deinococcus radiodurans* RecD would have resulted in the same phenotypes seen by the authors in their research.

A mutation was made in the Walker A NTP binding motif in the *Deinococcus radiodurans* RecD. The mutagenesis of the *recD* gene from *D. radiodurans* was done to see if the negative effects emanating from expression of the wild-type RecD in V186 were from a functional enzyme. Previous studies had demonstrated that this mutation made the protein unable to bind and hydrolyze ATP, a required co-factor in the DNA unwinding activity. When a plasmid encoding this mutant Dr RecD was transformed inside the V186 strain, an improvement in viability was seen over the wild-type protein.

There are two possible explanations for the increase in the cell viability assay. The first is that the binding to, and blockage of, the replication fork in *Escherichia coli* by the Dr RecD is derived from an enzymatic activity and not simply a physical impairment of progression. The second is that the mutation somehow altered the secondary and tertiary conformation of the protein, preventing the cell from producing the functional, stable monomers that would normally interfere with the fork. The effect of the lysine to glutamine mutation needs to be verified through enzyme assays such as ATPase and DNA unwinding, as well as taking near and far UV CD spectra of the K366Q mutant and comparing it to the wild-type protein to detect structural changes in the helicase.

The *recA* mutant V1119 gave relatively normal cell viability results when the *D. radiodurans* RecD was present. As was shown in Section **3.2.1**, the absence of any detrimental phenotype in V1119 [pKK D.r. RecD] correlated with the observations found in the Kuzminov paper. They found similar results with a *recA* mutant strain transformed with an expression vector carrying the *Escherichia coli* RecD.

These data suggested that the lack of RecBCD conferred a genotypic background favorable towards the detrimental action of the *D. radiodurans* RecD that was not similarly apparent in strains having RecBCD, RecBC, or missing the RecA protein. It is possible that in recombination-proficient bacteria, whatever harm comes from the *Deinococcus radiodurans* helicase is mitigated by and hidden by the ability of the cell to repair the damage without significant loss of viability. This would be done through homologous recombination or other pathways necessitating use of RecBCD.

## 4.3 V186 and WA632 trigger the SOS response when expressing the Dr RecD protein

V186 is an *E. coli* strain that has had its genes for *recB*, *recC*, and *recD* deleted from the genome. WA632 has a nonsense mutation in its *recB* allele, designated *recB21*. Therefore both strains are completely deficient in any form of double-strand break repair, and are hindered in their ability to perform any homologous recombination reactions required by the cell. The LexA-RecA SOS response pathway is a collection of over 40 genes, all regulated in the same manner by the LexA repressor protein (32, 56). This pathway is engaged when substantial amounts of DNA damage persist, or when the replication fork has stalled and collapsed at some blockage or lesion. The extreme end of this pathway is the appearance, under the microscope, of abnormally long cells termed filaments. Filamentation is a failure of the cell to septate properly, and is caused by the action of the SulA (=*sfiA*) protein which inhibits normal cell division (32, 43, 59). Restart of replication after the SOS pathway has been down-regulated often involves priming through homologous recombination or DNA intermediates made by RecA and RecBCD activities (26, 39, 49). There are two main causes for filamentation: problems with DNA replication, or disruption of the pathway for proper septation of two dividing cells. The *Deinococcus radiodurans* RecD is believed to be involved with the former as we surmise it can bind to, and cause collapse of the replication fork in the *E. coli* chromosome.

The cell viability test with V186 expressing the *D. radiodurans* RecD displayed reduced viability when compared with the empty vector alone. The same was seen for WA632 [pKK D.r. RecD] when compared to WA632 [pKK 223-3]. Further exploration

of this phenomenon was conducted with a microscopy study of the cell size and shape from both V186 [pKK D.r. RecD] and WA632 [pKK D.r. RecD] cultures. More images were captured of the V186 samples than WA632; therefore the V186 cultures received a more rigorous data analysis. Filamentation, while not extensive (never more than 7% of total population, see Figure **9**), was more prominent in V186 with the *D. radiodurans* RecD than with the pKK 223-3 vector. When the number of cell equivalents for the V186 [pKK D.r. RecD] cultures are considered, the percentage of cells in filaments rises to 29%, whereas it is only 1.5% of total cells in V186 [pKK 223-3]. The overall length of the filaments observed was also significant, with some measuring 60 μm or more in the cultures with *D. radiodurans* RecD.

Since filamentation is at the latter end of the SOS pathway, it was evident that the amount of stress being placed on the biological machinery of the V186 and WA632 strains was large. These strains, without any exogenous plasmids and in a typical physiological environment, still encounter DNA damage that cannot be fixed, such as interruptions in replication from single-strand gaps, or double-strand breaks in the template from metabolic by-products (10). The *D. radiodurans* RecD was able to further disrupt the cell by some action on the DNA itself. The only evidence that it might be from a functional interaction (in addition to the possibility of its physical presence blocking fork progression) with the *E. coli* chromosome is from the introduction of the K366Q RecD mutant. This mutant enzyme should be unable to bind and hydrolyze ATP, eliminating the unwinding activity. While the ATPase and DNA binding properties of this mutant were never measured, cell viability as well as microscopy studies show improvement in both viability and reduced length and amount of filaments. Whether this

increase in the V186 viability is directly related to the enzyme properties being disrupted has yet to be determined.

Filamentation is part of a broader response to severe DNA damage. As was mentioned, a perturbation in replication can often trigger the cell to use the SOS pathway. The *in vitro* substrates of the *D. radiodurans* RecD include a replication fork-like structure (61). The non-native RecD might be binding to, and acting on, replication fork proteins such as other *E. coli* helicases or the actual polymerase holoenzyme. It may displace a native enzyme required for proper functioning of the moving replication fork. Replication fork stalling can occur due to impediments along the track as the fork progresses, such as DNA-binding proteins (7, 17, 39). The binding of the *D. radiodurans* RecD protein to DNA may precipitate a collision between it and the replication proteins. This would explain why filaments still persisted in the K366Q RecD mutant, and the viability did not return to values like with the empty vector alone in V186. The observations from the cell viability and microscopy studies suggest that the Dr RecD can bind to the *E. coli* replication fork, and interrupt replication of the cell's genome.

The *Deinococcus radiodurans* RecD prefers DNA substrates with fork-like structures and 5' tails. It is unknown at this time if the protein can bind to other DNA conformations. Replication of the cell's DNA involves many other nucleic acid structural intermediates produced by the replication process itself, or from repair of gaps, lesions, or other errors found on the template. The Dr RecD could be localizing to any one of these and causing the negative phenotype to emerge. Future studies should include the procurement of an *E. coli* strain constitutive in the SOS response and examination of the amount and length of its filaments. Also, the *D. radiodurans* RecD

and proteins implicated in the moving replication fork should be fluorescently tagged. These could then be measured with fluorescence microscopy for changes in the lifetimes at the replication fork when compared to those same strains without the Dr RecD.

## 4.4 Concluding Remarks

The radioresistant microbe *Deinococcus radiodurans* can repair thousands of double-stranded breaks in its genome without severe mutagenesis or considerable loss of viability. A pathway termed ESDSA has been proposed whereby DNA polymerase I extends the 3' ends of the fragmented DNA, homologous chromosomes pair and anneal, DNA contigs are formed, and finally homologous recombination is performed amongst the many linear pieces to re-generate the DNA template. Most organisms use some form of a RecA-RecBCD mechanism to repair DSB. *D. radiodurans* possesses a RecA protein, but not RecB or RecC; only a RecD-like helicase has been characterized with typical helicase properties. The current biological function of this helicase in *Deinococcus radiodurans* is speculative at the current time.

Expression of the Dr RecD in *Escherichia coli* lacking the native RecD did not produce any difference in enzymatic activities missing in the RecBC complex. The viability of these RecD(-) strains were also relatively unaffected in the presence of the *D. radiodurans* RecD protein. The same result was observed in viability tests with wild-type strains and the Dr RecD. A negative phenotype was only observed in cells lacking a fully functional RecBCD heterotrimer. (RecD has no other functions in these *E. coli* cells). The RecBCD(-) strains expressing the Dr RecD appeared to filament in the microscope; filamentation is a part of the SOS response engaged during periods of heavy damage to the chromosome or a perturbation in the septation process in dividing cells.

We believe that the Dr RecD is binding to the moving replication fork *in vivo*, and causing stalling and eventual collapse of this fork. The helicase may achieve this outcome in two related ways. The first is that of a physical presence on the DNA, whereby the effect is a blockage of fork progression. The second could be derived from an enzymatic activity on either the replication fork itself, or one of the many replication proteins. It is also possible that it is a combination of these activities that results in the cell triggering the SOS pathway and filamentation.

Future research on the *Deinococcus radiodurans* RecD should include characterization of the K366Q mutant in ATPase and DNA-binding studies. *In vitro* assays on the wild-type proteins ability to localize to replication fork-like structures (Holliday junctions, the 'chicken-foot' intermediate in a reversed replication fork, etc.) should also be considered. To determine whether the harmful phenotype from Dr RecD expression is from some enzyme property, the carboxyl terminal portion of RecD that contains the helicase motifs should be made and expressed in those same cells. This would then be compared to the amino terminal region in the same assays to gauge if that large, unknown region has some as yet undiscovered function. Finally, Pulsed Field Gel Electrophoresis should be done on the wild-type, RecD(-), and RecBCD(-) strains producing the *D. radiodurans* RecD to see if in the presence of the foreign protein there are a greater number of double-strand breaks in the DNA compared to the empty vector. More microscopy studies are needed on V186 [pKK D.r. RecD] and WA632 [pKK D.r. RecD], as well as V186 [pKK K366Q RecD] with the LIVE/DEAD® kit to determine if more normally shaped cells are actually dead in those samples than that of the pKK 223-3 vector. It may also be advantageous to use fluorescence microscopy to measure lifetimes

and localization of tagged Dr RecD and other replication proteins at the replication fork during *Deinococcus radiodurans* RecD overexpression.

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