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THE SOS RESPONSE IN ESCHERICHIA COLI K12: AN EXPLORATION OF MUTATIONS IN LEXA AND RECA USING FLUORESCENCE MICROSCOPY

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A Dissertation Presented

By

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ABSTRACT

THE SOS RESPONSE IN ESCHERICHIA COLI K12: AN EXPLORATION OF MUTATIONS IN LEXA AND RECA USING FLUORESCENCE MICROSCOPY

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Faithful replication of the genome is paramount for maintaining the fitness of an organism. Therefore, life has evolved inducible mechanisms to be able to repair damaged DNA and maintain evolutionary fitness. The SOS response is a highly conserved DNA damage inducible response that is tightly regulated. Multiple factors contribute to the ability of the cell to perform proper DNA repair and induction of the SOS response including the amount of RecA, mutations in RecA that affect competition for DNA, and other proteins that interact with the RecA filament. The complex relationship between RecA and LexA is the subject of this work.

This dissertation is comprised of two projects examining the genetics of the SOS response. In the first chapter, we overexpress a noncleavable mutant of *lexA*, *lexA3*, and demonstrate an association between high expression of *lexA3*, severe ultraviolet light sensitivity, and an increased number of punctate RecA-GFP structures. While the explanation for this phenotype is not completely clear, we were able to show that a four-

fold increase in *lexA3* expression led to a nearly ten-fold decrease in *recA* expression. The second chapter examines the role of charge at position 38 and position 184 in RecA, that are the sites of the *recA730* and *recA1202* mutations, respectively. These mutations confer a phenotype whose hallmark is constitutive expression of the SOS response. Upon substituting different residues at positions 38 and 184, we were able to show that a positive charge at these positions is associated with constitutive SOS expression. Together, these studies show the complexity that surrounds the SOS response in *E. coli*.

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LIST OF ABBREVIATIONS

Amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
Cam	chloramphenicol
cat	chloramphenicol acetyl transferase
DNA	deoxyribonucleic acid
DSB	double-strand break
FRT	Flp recombinase recognition target
GFP	green fluorescent protein
Kan	kanamycin
OD	optical density
op	overproducer
P1	bacteriophage strain P1
PCR	polymerase chain reaction
rec+	recombination proficient
rec–	recombination deficient
Tet	tetracycline
UV	ultraviolet
WT	wild type

CHAPTER 1

THE SOS RESPONSE IN ESCHERICHIA COLI: LITERATURE REVIEW AND OVERVIEW

Recombination and DNA repair are essential processes conserved throughout all lifeforms (Brendel et al., 1997; Karlin & Brocchieri, 1996). Proficiency in performing these functions is paramount to survival and withstanding stress (Baharoglu & Mazel, 2014; Mo et al., 2016). DNA repair touches many fields from cancer biology to antibiotic resistance to aspects of evolution (Brendel et al., 1997; Culyba et al., 2015; Prakash et al., 2015). The best understood DNA repair system is the SOS response in *Escherichia coli*. The genetics and biochemistry for this system have been the most widely characterized providing an ample resource of mutants, molecular structures, and mechanistic understanding (Michel, 2005). Recombination regulation and DNA repair have been reviewed in (Bell & Kowalczykowski, 2016; McGrew & Knight, 2008; Persky & Lovett, 2009; Roca et al., 2008).

The RecA protein is central to DNA repair (Howard-Flanders & Theriot, 1966; Willetts & Clark, 1969). After DNA damage, RecA forms a filament on single stranded DNA (Figure 1.1B) (Cox & Lehman, 1982; Egelman & Yu, 1989; West et al., 1980; Yancey & Porter, 1984). RecA filament formation initiates the process of recombination (Cox & Lehman, 1982) and induction of the SOS response(Miura & Tomizawa, 1968; Witkin, 1976; Yu & Egelman, 1993). The repressor of the SOS response, LexA, interacts with the RecA filament, which increases the rate of autoproteolysis, and is then rapidly degraded by Lon and ClpXP (Figure 1.1CD) (Luo et al., 2001; Neher et al., 2003; Yu & Egelman, 1993). LexA depletion leads to derepression of the SOS regulon which includes, but is not limited to *recA*, nucleotide excision repair genes, the mutagenic

polymerase *umuD*, and the cell division inhibitor *sulA* (Brent & Ptashne, 1981; Courcelle et al., 2001; Huisman et al., 1984; Little & Mount, 1982). When appropriately induced, the SOS response contributes to the cell's survival under genotoxic conditions. However, unrepaired lesions or excessive mutagenesis can lead to deleterious mutations and a decrease in fitness. Therefore, the cell needs to be able to determine when to perform recombination and when to induce the SOS response.

Different recombination scenarios need to be handled according the DNA substrate. More specifically, the DNA motif will determine the factors used to load RecA (Figure 1.2). The first of these loading factors to be discovered was the RecBCD complex. It was discovered as a mutant deficient in conjugation (Barbour et al., 1970; Howard-Flanders & Theriot, 1966). RecBCD is a complex with ATP dependent helicase and nuclease activity that recognizes double stranded ends of DNA in the cell (Arnold & Kowalczykowski, 2000; Dillingham & Kowalczykowski, 2008; Goldmark & Linn, 1972). Double stranded ends of DNA are manifest in multiple situations to the cell. RecBCD cannot differentiate between exogenous DNA and the bacterial nucleoid solely on the double stranded end. On one hand, it might be phage DNA and should be degraded as quickly as possible to prevent infection. On the other hand, it might be a double stranded break in the genome and should not be degraded. Or in yet another circumstance, the phage particle that injected the DNA was a transducing phage and the DNA may not lead to lytic infection, rather horizontal gene transfer.

How does RecBCD determine between friend and foe? Once the enzyme binds to a double stranded end of DNA it starts degrading both strands of DNA until it reaches a χ (or crossover hotspot instigator) site (Bianco & Kowalczykowski, 1997; Lam et al.,

1974). Upon reaching this, the nuclease activity favors the 5' strand which leads to the production of a single stranded 3' overhang (Masterson et al., 1992). Single stranded DNA is bound by single stranded DNA binding protein (SSB) which competes with RecA for single stranded DNA substrates. RecBCD displaces SSB and allows RecA to load on single stranded DNA (Arnold & Kowalczykowski, 2000). The RecA nucleation process is slow but extension of the filament occurs at an accelerated rate (Bell et al., 2012; Shivashankar et al., 1999). The RecA filament then searches the cell for homology and upon finding it starts to undergo strand exchange, forming what is called a D-loop (Cox & Lehman, 1982). Upon successful strand exchange, DNA polymerase I is recruited to synthesize the DNA between the two ends (Bonura & Smith, 1975). DNA ligase then seals the strands together. The Holliday Junctions that are formed by the crossover are then resolved by the RuvABC complex (Stacy & Lloyd, 1976). The end result can be either two intact chromosomes or the site for replication restart (See Figure 1.2).

Not all DNA damage comes in the form of double stranded ends. In some cases, recombinational repair needs to help fix gaps in the DNA where the double stranded DNA becomes single stranded DNA (Figure 1.2). One instance of this is repairing damage after ultraviolet radiation which forms pyrimidine dimers. The pyrimidine dimers are usually fixed by nucleotide excision repair. However, if the DNA replication machinery tries to replicate the genome before the lesion can be repaired, replication forks stall at the lesion. This can lead to a section of the DNA where there is only one strand of DNA. RecFOR enzymes are recruited to a junction of double stranded DNA turning to single stranded DNA. RecFOR allows for the displacement of SSB so that

RecA can be loaded onto the DNA (Umezu et al., 1993). Upon loading RecA, the rest of the process is very similar to repair when RecA was loaded by RecBCD. Aside from single stranded gaps, the RecFOR pathway can also use double stranded ends with small overhangs with the help of RecQ helicase and the RecJ exonuclease. RecQ separates the DNA strands in a 3' to 5' direction and allows for RecJ to perform its 5' to 3' exonuclease activity which leaves a 3' overhang (Lovett & Clark, 1984). This substrate is recognized by RecFOR and recombination continues as described before with the RecBCD pathway.

The SOS response is transcriptionally regulated by the LexA protein. Mutations in *lexA* can make the protein noncleavable and therefore the cell cannot induce the SOS response (Luo et al., 2001; Mount et al., 1972). One of these mutations is *lexA3*. This mutation encodes for a G85D amino acid change which mutates the cleavage site of LexA. A cell can experience DNA damage and form a RecA filament as usual; however, since LexA is noncleavable, the SOS response is not induced and the cell maintains the roughly 10,000 molecules of RecA in rich media and nearly 3,000 molecules in minimal media (Li et al., 2014; Slilaty & Little, 1987). This differs from wildtype strains where the amount of RecA would increase roughly ten-fold after DNA damage.

When the *lexA3* mutation was first discovered in 1972, David Mount and colleagues performed a few recombination assays on the mutant strain (Mount et al., 1972). They discovered that the *lexA3* mutation did not affect conjugation to an appreciable amount, but the ability to repair damage after exposure to ultraviolet light was somewhat inhibited. The authors concluded that since LexA was the repressor for the SOS response, that induction of the SOS response was important for survival after

ultraviolet light exposure, but was not as important for conjugation. To further highlight the importance of RecA, this ultraviolet light sensitivity can be partially suppressed by increasing *recA* expression using a *recAo281* operator mutation (Ginsburg et al., 1982). This operator mutation decreases the affinity LexA has for the *recA* operator so that *recA* is expressed at levels similar to full SOS response induction (Volkert et al., 1976, 1979, 1981). Importantly, high levels of *recA* expression does not induce the SOS response. From this data, the ultraviolet light sensitivity suppression would most likely be due to increased RecA levels.

Aside from transcriptional regulation, recombination is regulated at the protein level. DNA substrates, RecA loading factors, and RecA protein amount play a role in regulation; specifically, in how RecA is loaded onto the DNA. However, RecA loading can be affected by mutations in *recA*. Most mutations that disrupt normal RecA activity cause a loss of function. Interestingly, there are a few mutations that lead to a gain of function. These mutant proteins have gained an increased affinity for single stranded DNA and are referred to as *srf* mutations or suppressors of *recF* (Thoms & Wackernagel, 1988; T. C. Wang & Smith, 1986; T.-C. V. Wang et al., 1993). Since RecF is an important protein in loading RecA, strains harboring a *recF* mutation are not able to load RecA as effectively and thus are sensitive to DNA damaging agents. To bypass the need for RecF and restore DNA repair function, mutations in *recA* increase the protein's ability to compete for SSB-coated single stranded DNA better than wildtype RecA (Handa & Kowalczykowski, 2007; Long et al., 2010; W. B. Wang & Tessman, 1986). Furthermore, RecA unloads at a slower rate from the DNA. The result is a more stable RecA filament. Stable RecA filaments are able to cleave more LexA and induce the SOS response. If the

RecA filament is consistently stabilized, LexA is consistently being cleaved, leading to constant SOS induction (i.e. SOS constitutive phenotype) (Lavery & Kowalczykowski, 1992). One might expect with this line of reasoning that all *srf* mutants would be SOS constitutive. Interestingly, not all *srf* mutants express the SOS response constitutively when expressed by itself as a single mutant (M. V. Madiraju et al., 1988; Thoms & Wackernagel, 1988; T. C. Wang & Smith, 1986; T. C. V. Wang et al., 1991; T.-C. V. Wang et al., 1993). One SOS constitutive *srf* mutation, *recA730*, and two SOS constitutive *recA* mutants which were measured as *srf* in this work, *recA1202* and *recA4161*, will be explored further in the following paragraph.

Strains harboring *recA730* can partially suppress the ultraviolet light sensitivity of *recF* mutants and are expressing the SOS response constitutively (T.-C. V. Wang et al., 1993). Strains harboring either *recA1202* or *recAo1403 recA4161* double mutant are also SOS constitutive although before this current study there was no published data on whether they suppress *recF* mutations or not (W. B. Wang & Tessman, 1986) (Long J.E Doctoral Thesis). What makes these three mutations so interesting is their proximity in three-dimensional space and their similarities phenotypically. The *recA730* mutation at position 38 (E38K), and *recA1202* (Q184K) are relatively close to each other in physical space (Figure 3.1) (Britt et al., 2010; Eggler et al., 2003; Handa & Kowalczykowski, 2007; Lavery & Kowalczykowski, 1992; Lusetti, Shaw, et al., 2003; Lusetti, Wood, et al., 2003; W. B. Wang & Tessman, 1986). Phenotypically, *recA730* and *recA1202* strains constitutively express the SOS response. By increasing the basal transcription of *recA* by two to three-fold, *recA4161* strains are also SOS constitutive (Long, J.E. 2009, Doctoral Thesis). Could these three mutations potentially be connected? Work from Michael Cox's

lab has demonstrated that *in vitro* both RecA730 and RecA4161 have an increased ability to compete single stranded DNA compared to wildtype RecA. Work from Ethel Tessman's lab demonstrated that *in vitro* RecA1202 has an increased ability to compete for single stranded DNA compared to wildtype RecA (W. B. Wang, Sassanfar, et al., 1988).

Two examples of how RecA's ability to compete for single stranded DNA are RecA's affinity for DNA or polymerizing into a filament. Could the region around position 38 and 184 be important for binding DNA or polymerizing into a filament? Mutating residues in the DNA binding domain or residues that affect the cooperativity of binding could lead to RecA forming a more stable filament. Saturation mutagenesis of the primary DNA binding domain, the L2 loop, yielded no mutations with increased affinity for DNA (Hörtnagel et al., 1999). A mutation in the oligomerization domain, *recA4142* (F217Y), has an increased cooperativity when forming a filament on DNA (Eldin et al., 2000). When combined with an operator mutation, *recAo1403*, that increases the basal level of transcription 2 to 3-fold, strains with this mutation have an SOS constitutive phenotype (Long et al., 2008). However, instead of lying in the obvious candidate domains, *recA730*, *recA1202*, and *recA4161* are nowhere near the DNA binding domains or oligomeric interface domains. In fact, when RecA is in a filament, the C-terminal domain and position 38 and 184 are on the outside of the filament (Figure 3.1).

The C-terminal domain contains residues 270-352. It has been proposed to serve as a "gateway" to regulate double-stranded DNA binding (Kurumizaka et al., 1996). The C-terminal domain has also been shown to affect single-stranded DNA binding *in vitro* (Eggler et al., 2003). Deletions of the last seventeen amino acids in RecA lead to an

increased ability to compete for SSB-bound single-stranded DNA (Benedict & Kowalczykowski, 1988; Eggler et al., 2003; Lusetti, Wood, et al., 2003). When expressed *in vivo* as a single mutant, the cell expresses SOS at a normal level. However, with an operator mutation, *recAo1403,* that increases basal expression by two-fold, this is enough to cause an SOS constitutive phenotype (Long, J.E. 2009, Doctoral Thesis).

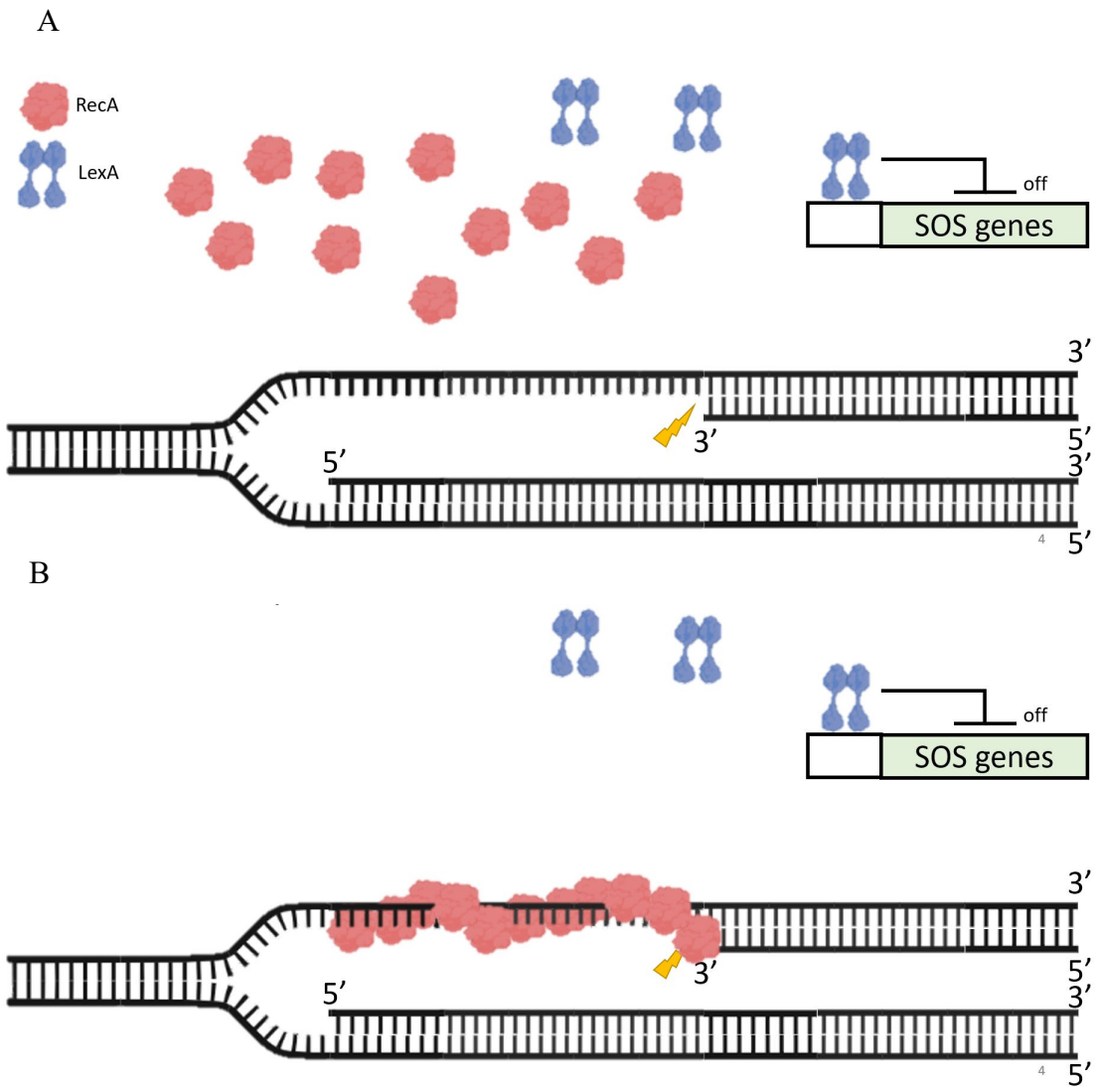
1.1 Overview of Studies

The SOS response in *Escherichia coli* serves as the archetype of inducible DNA repair systems. Careful regulation of the SOS response allows for the cell to be able to maintain genome fidelity. The two main regulators in the SOS response are RecA and LexA. Previous work on the SOS response is based upon the observations that about 15% of cells in an undamaged population of log cells have a RecA-GFP structure, but only 1% of the population is induced for the SOS response at any one time in the absence of DNA damaging agents. This contrast indicates there are additional layers of SOS response regulation that have yet to be revealed. Mutations in either *lexA* or *recA* can disrupt proper regulation of the SOS response. On one hand, the SOS response can be defective for induction. This can be achieved by noncleavable mutants of *lexA* or deletion of *recA*. On the other hand, the SOS response can be constitutively active. This can be achieved by deletion of *lexA* or point mutations in *recA* that allow RecA to form a filament in the cell in the absence of DNA damage. The aim of this research was to study the complex relationship between LexA and RecA. The SOS response, which includes *recA*, is transcriptionally regulated by LexA. Overexpression of a noncleavable mutant of LexA drives down expression of *recA* and presumably the SOS response genes. Chapter two will study the affect overexpression of *lexA3* has on RecA-GFP. Strains harboring the *recA730* or *recA1202* allele constitutively express the SOS response in the absence of

DNA damage. Both of these mutations are spatially proximal to each other and replace a residue with a lysine. Amino acid substitutions at the positions of *recA730* (E38K) and *recA1202* (Q184K) reveal the importance of a positive charge at that location for the SOS constitutive phenotype. Chapter three is studying the role of charge at position 38 and 184. This thesis will focus on the complex relationship between LexA and RecA and how this complexity contributes to the function and dynamism of the SOS response.

1.2 Figures

Figure 1.2.1 Overview of the induction of the SOS response



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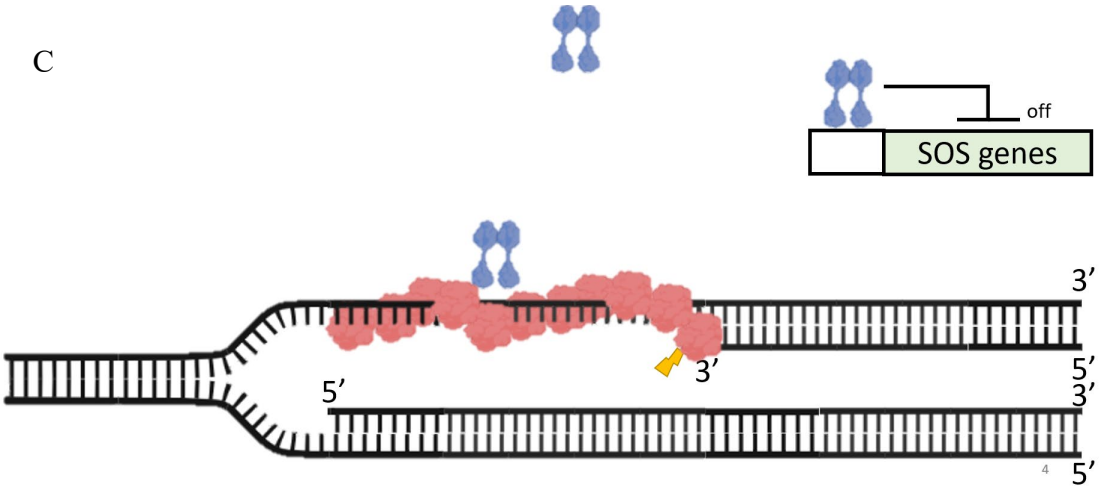
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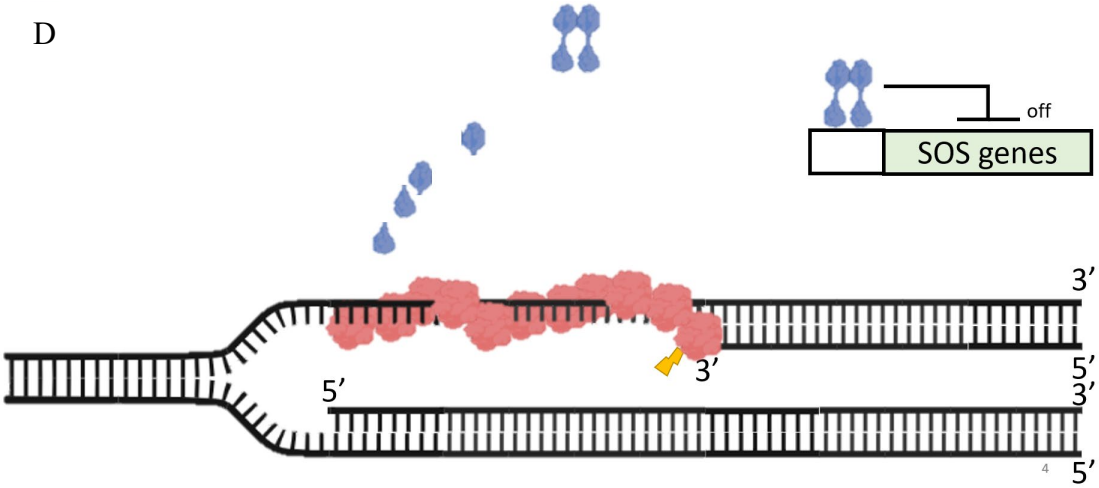
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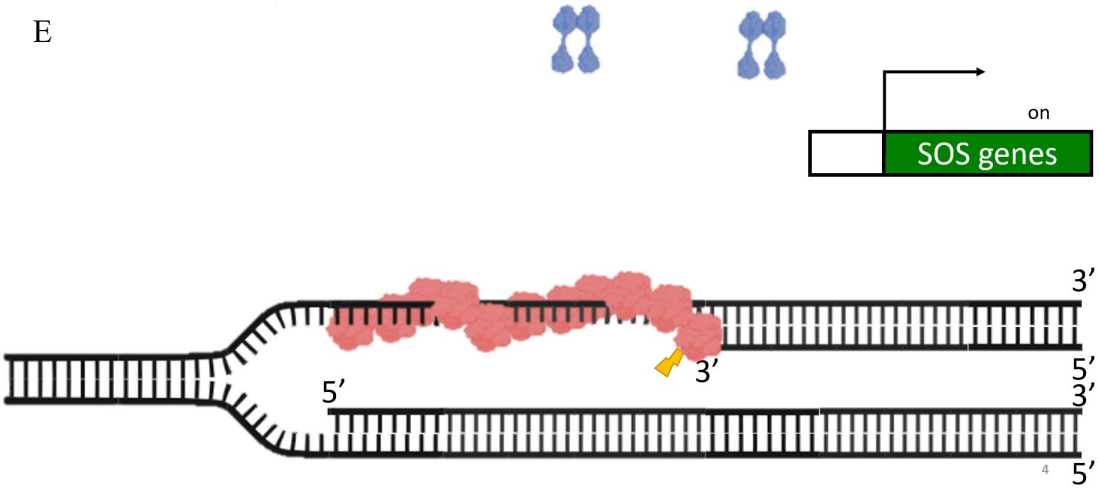
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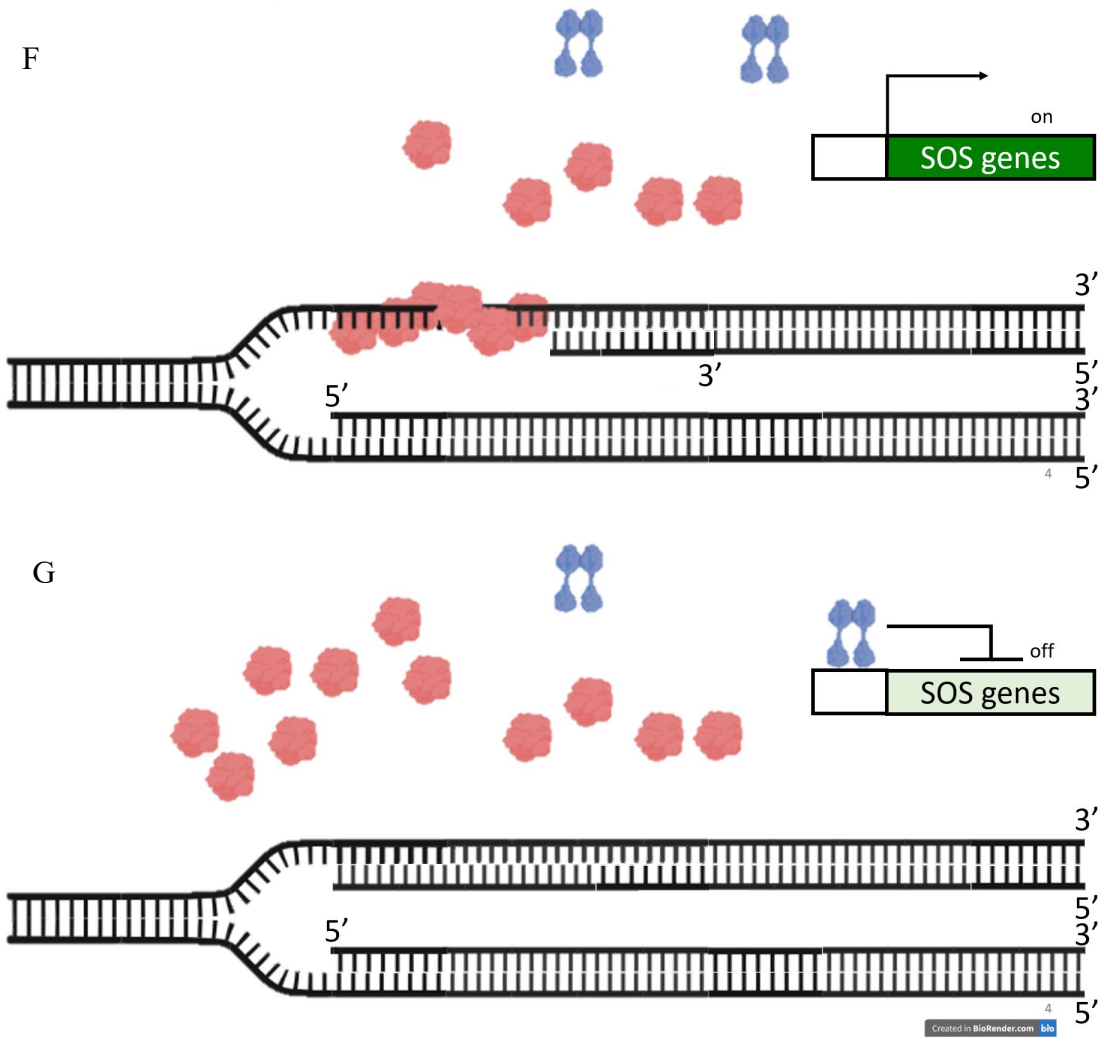


Figure 1.1.1: Overview of induction of the SOS response. (A) The two main components of the SOS response are the DNA damage sensor, RecA, and the repressor for the DNA damage response, LexA. In conditions without DNA damage, the SOS response is kept at a basal level of transcription. If a replication fork encounters a lesion in the DNA template and generate a single stranded DNA gap. (B) This substrate will be recognized by RecA loading factors that will load RecA onto the DNA and form a nucleoprotein helical filament. (C) The RecA filament is recognized by LexA, which upon interacting with the RecA filament, (D) will increase its rate of autoproteolysis. (E) Approximately five minutes after damage, almost all of the LexA in the cell has been cleaved and degraded and the genes in the SOS response regulon increase transcription. (F) Once the DNA damage has been repaired, the RecA filament will disassemble. (G) With no RecA filament to cleave LexA, the amount of LexA will increase to sufficiently repress the SOS response again.

Figure 1.2

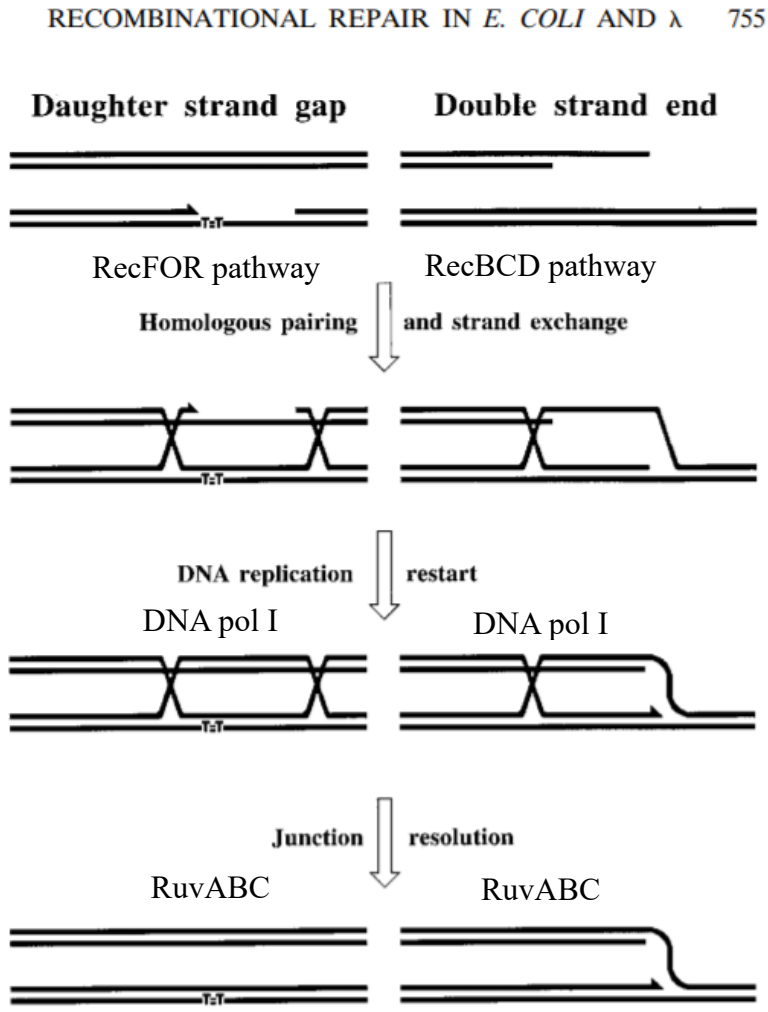


Figure 1.2: RecA loading pathways are determined by DNA substrate. RecA is loaded onto different DNA substrates by different RecA loading pathways. Daughter strand gap repair is facilitated by the RecFOR pathway. Double strand end repair is facilitated by the RecBCD pathway. Once RecA has been loaded, recombination resolves in a similar manner regardless of how the RecA filament was formed. (Adapted from Kuzminov 1999)

CHAPTER 2

DECREASED RECA EXPRESSION FROM LEXA3 OVEREXPRESSION CONTRIBUTES TO ULTRAVIOLET SENSITIVITY IN ESCHERICHIA COLI K12

Summary:

Recombination and regulation of the SOS response in *Escherichia coli* centers mainly around two genes, *recA* and *lexA*. RecA is central to the process of homologous recombination, DNA repair and induction the SOS response via cleavage of LexA. LexA is the global repressor for the over forty genes in the SOS response, including *recA*, which aid in the process of DNA repair. *In vivo*, RecA-GFP has been used to identify structures associated with RecA's function in recombination, DNA repair, and induction of the SOS response. RecA-GFP structures are observed as circular or linear in shape (or undecided, a category for RecA-GFP structures that are between circular and linear) with the linear shaped structures being associated with double strand break repair. Certain alleles of *lexA*, such as *lexA3*, are noncleavable and make the cell more sensitive to DNA damage due to an inability to induce the SOS response. Previous work overexpressing *lexA3* from a plasmid has shown to make the cells even more sensitive to UV. It was hypothesized that this was due to decreased RecA expression. In this work, we overexpress *lexA3* to test the effect of suboptimal concentrations of RecA and RecA-GFP in the cell. Western blot analysis showed that a three to four-fold increase in LexA3 decreases RecA and RecA-GFP expression nearly ten-fold. As was observed previously, overexpression of *lexA3* made $recA^+$ cells more UV sensitive. Surprisingly, the number of RecA-GFP structures in a log phase population increased instead of decreased as was expected. Additionally, after UV irradiation, the RecA-GFP structures form mostly circular structures instead of linear structures which is observed in $lexA^+$ strains. The

amount of RecA and RecA-GFP can be increased five to ten-fold with a *recAo281* operator mutation. A strain with *recAo281* and *lexA3* overproducer has similar or more RecA than a strain with *recAo*$^{+}$ and *lexA*$^{+}$, but does not fully restore UV resistance or normal distribution of RecA-GFP to the cells. The other SOS genes are likely expressed at a lower level due to *lexA3* overexpression which may contribute to why full UV resistance is not restored. We hypothesize that *lexA3* overproduction has two effects: decreasing the level of RecA in the cell and binding to RecA-GFP structures, stabilizing them in the circular form.

2.1 Introduction

Escherichia coli responds to DNA damage by inducing the SOS response which is comprised of over forty genes involved in DNA repair, homologous recombination, and mutagenesis (reviewed in Little & Mount, 1982; Michel, 2005; Radman, 1975; Walker, 1984). The two main regulatory elements used to induce the SOS response and facilitate DNA repair are RecA, the DNA damage sensor, and LexA, the repressor to the SOS response (Brent & Ptashne, 1981; Thliveris et al., 1991). Depending on the sequence of the operator and promoter of the gene or operon, LexA will bind with different affinities and lead to a wide range of regulation (Arthur & Eastlake, 1983; Casaregola et al., 1982; Courcelle et al., 2001; Friedman et al., 2005; Picksley et al., 1984; Salles & Paoletti, 1983; Sandler, 1994; Woodgate & Ennis, 1991). For example, *recA* has a high basal level of expression of 3,000 molecules in minimal media as measured by ribosomal profiling (Brar & Weissman, 2015; Li et al., 2014). In contrast, *umuD*, a subunit of the error-prone DNA polymerase V, has a low basal level of expression of 27 molecules in the same conditions (Brar & Weissman, 2015; Li et al., 2014). The rate of induction after

DNA damage can be different depending on the gene. While *recA* is heavily induced within five minutes of UV irradiation (Casaregola et al., 1982; Courcelle et al., 2001), *umuD* is not fully induced until twenty minutes after UV irradiation (Woodgate & Ennis, 1991). This is important since the cell may want to perform housekeeping functions such as fixing a broken replication fork, but not want to initiate mutagenesis or inhibit cell division which can have a fitness cost to the cell and would be beneficial to the cell only if the DNA damage is severe. There is a limited understanding on how the cell makes the decision to induce the SOS response; however, the amount of RecA, RecX, and RadA (both of which affect RecA filament stability) are known to play a role (Beam et al., 2002; Drees et al., 2004; Massoni et al., 2012).

RecA is a cytosolic protein that in its monomeric form is inactive for recombinational repair and SOS response induction (Yancey & Porter, 1984). In order to become active, RecA must bind a molecule of ATP and then a single-stranded DNA substrate (Craig & Roberts, 1980; Flory et al., 1984) and, along with multiple other ATP-bound RecA monomers, form a helical protein-single-stranded DNA filament (Bell et al., 2012; Egelman & Yu, 1989; Shibata et al., 1979; Yancey & Porter, 1984). Once in this polymerized form, RecA is active and can perform homologous recombination (Yancey & Porter, 1984) and induction of the SOS response (Rehrauer et al., 1996). Homologous recombination involves finding a homologous sequence in the genome and performing strand exchange to repair the damaged DNA (Cox & Lehman, 1982; Cunningham et al., 1980). SOS response induction involves LexA binding in the helical groove of the RecA filament (Yu & Egelman, 1993) which increases the rate of auto-proteolysis (Little, 1984, 1991; Little et al., 1980). This decreases the level of LexA repressor in the cell which

allows for increased expression of the SOS genes (Sassanfar & Roberts, 1990). Since *lexA* is autoregulated (Brent & Ptashne, 1980; Little & Harper, 1979), *lexA* transcription is upregulated during the SOS response. After the DNA damage has been repaired and RecA is no longer in a filament, the amount of LexA increases and the SOS response gene expression returns to normal (Sassanfar & Roberts, 1990).

Recombination is thought to occur in three stages (reviewed in (Lusetti & Cox, 2002). The first, Pre-Synapsis, is when RecA binds to the ssDNA to create a protein/DNA helical filament (Cox & Lehman, 1982; Flory et al., 1984). This structure can extend for hundreds to thousands of RecA monomers *in vitro* but the minimal critical length *in vivo* is not known. In the second stage, Synapsis, the RecA filament interacts with a duplex of DNA to create a three stranded structure (Cunningham et al., 1979; Hsieh et al., 1992). This is often called a D-loop and can lead to the formation of a Holiday structure (DasGupta et al., 1981). This is the stage at which the RecA filament searches for homology in the duplex. If homology is found, then RecA exchanges the strands of DNA. The final stage, Post-Synapsis, the DNA structures are separated by resolving the Holliday structures (Connolly & West, 1990).

A functional RecA-GFP fusion protein was constructed to study the structures formed *in vivo* (Renzette et al., 2005). It was found that RecA-GFP formed a range of structures who eccentricity ranges from fully circular to linear. These structures ranged in size and intensities. It was also seen that some of the structures were not on the DNA and these are thought to be storage structures (Renzette et al., 2005). These had been previously hypothesized based on structural and biochemical studies (Logan et al., 1997; Story et al., 1992). In biochemical studies, several residues were identified that were

critical for storage structure formation that when mutated had no negative on RecA's functions in recombination, DNA repair or SOS induction (Eldin et al., 2000). When one of these mutations, *recA4155* (R28A) was incorporated into the RecA-GFP (Eldin et al., 2000), the average number of structures per area of cell decreased by 50% (Renzette et al., 2005). Using other tests, it was ascertained that the remaining structures are associated with the DNA. These structures also form a distribution of circular to linear, varying in size. Studies have shown that after DNA damage (UV and DSB), linear structures tend to predominate and are associated with second stage of recombination, Synapsis (Amarh et al., 2018; Ghodke et al., 2019; Lesterlin et al., 2014; Renzette et al., 2005).

LexA is the archetypal transcriptional repressor. It has two domains: dimerization (Giese et al., 2008; Mohana-Borges et al., 2000) and DNA binding (Hurstel et al., 1988; Oertel-Buchheit et al., 1990). The DNA binding is due to the Helix-Turn-Helix motif (Zhang et al., 2010). LexA binds as a dimer (Thliveris et al., 1991) to an operator site with dyad axis of symmetry (Brent & Ptashne, 1981; Little et al., 1981; Little & Harper, 1979; Zhang et al., 2010). These sites regulate a number of genes and their binding affinities have been measured and vary. This, coupled with promoters of different strengths, allows for a range of expression under both repressed and induced conditions (Culyba et al., 2018; Ronen et al., 2002). LexA has a tendency to auto-proteolyze into two fragments (Little, 1984, 1993). This reaction is accelerated when LexA interacts with the RecA filament (Little, 1991). These fragments are then quickly degraded by ClpXP and Lon proteases (Neher et al., 2003). The increase in this reaction and the subsequent removal of the LexA fragments from the cell is the molecular basis for induction of the

SOS response (Sassanfar & Roberts, 1990). Several mutants of *lexA* were found that rendered the cell sensitive to UV light (Mount et al., 1972, 1980). Some of these have been characterized and found to lead to a LexA repressor that does not undergo the auto-proteolytic reaction and cannot induce the SOS response in vivo (Little, 1991, 1993; Slilaty & Little, 1987; Volkert et al., 1976). It has been shown that overproduction of one highly studied LexA mutant, LexA3, from a plasmid could increase the UV sensitivity of the strain more than have a single copy of *lexA3* on the chromosome (Ginsburg et al., 1982; Mount et al., 1980). It was hypothesized that this LexA3 protein could decrease the basal levels of RecA in the cell by occupying the operator site in the *recA* promoter more often due the its higher concentration and chemical equilibria (Ginsburg et al., 1982; Mount et al., 1980).

The *recA* operator has been studied in detail (Ginsburg et al., 1982; Volkert et al., 1981; Wertman & Mount, 1985). Several mutations have been identified that increase the level of recA transcription. Some of these are in the highly conserved regions of the *lexA* box (*lexA* binding site) (Little et al., 1981; Wertman & Mount, 1985; Zhang et al., 2010). One mutation is called *recAo281*. This was found as a suppressor the UV sensitivity of a *lexA102 uvrA155* mutant and increases the level of RecA expression approximately 10-fold or to completely unrepressed levels (Volkert et al., 1976, 1979, 1981). A second mutant, not in the highly conserved region but in the LexA box, called *recAo1403*, increases the level of *recA* transcription about 2-3 (Wertman & Mount, 1985) and has been shown to be needed to get optimal expression and complementation of *recA-gfp* (Renzette et al., 2005).

In this chapter we tested whether overproducing *lexA3* from a strong constitutive promoter (Figure 2.1) would decrease the levels of RecA and RecA-GFP expression so that we could study the phenotypes associated with less than wildtype levels of *recA* in the cell. We show that increasing the level of *lexA3* expression 4-fold decreases the level of RecA or RecA-GFP about 5 to 7-fold (Figure 2.2 & Figure 2.4). We found the expected UV sensitivity (Figure 2.3) that others have seen in the RecA strain when overproducing LexA3 from a plasmid (Ginsburg et al., 1982; Mount et al., 1980). Surprisingly, we found that the number of RecA-GFP foci increased and the distribution of the shape of the RecA-GFP structures shifted to more circular forms (Figure 2.5, Table 2.1). This latter phenotype was also seen after UV irradiation when more linear forms typically predominate (Figure 2.6, Table 2.2). If we increase the level of RecA and RecA-GFP production with a *recAo281* mutation, we find that levels of *recA* and *recA*-GFP increase 5 to10-fold (Figure 2.7 & Figure 2.9), greater than wildtype levels, but yet the level of UV resistance (Figure 2.8) and the distribution of shapes of RecA structures does not return to wildtype (Figure 2.10 & Figure 2.11). Of several possible models, we favor the model that our results can be explained by a combination of decreased RecA protein expression and the ability of LexA3 to either interact with the RecA-ssDNA helical filaments and cause the circular form to predominate or cause the repression of other SOS genes that somehow lead to the same. We hypothesize this form is not productive for DNA repair.

2.2 Results

2.2.1 *lexA2005* increases the level of LexA3 protein 3 to 4-fold in the cell

To construct a *lexA3* overproducer, we used a technique that we have used previously to overproduce the RadA protein (Massoni et al., 2012). This is to place a

strong constitutive promoter upstream of the *lexA3* gene on the chromosome. In this case, the promoter is the *recA* promoter with two mutations in its *lexA* binding box to negate any influence of LexA binding (Volkert et al., 1981; Wertman & Mount, 1985). Figure 2.1 shows that it is inserted just upstream of the *lexA3* gene and leaves the *lexA* promoter operator region intact, but displaced. To measure the amount of LexA3 produced in the *lexA2005* strain, we measured the level of LexA production by Western blot with a commercially available antibody. $LexA^+$ and Δ(*lexA*) are used as controls. Figure 2.2 shows the level of LexA in the deletion strain is undetectable, the level of $LexA^+$ and LexA3 are essentially identical and that the level of LexA3 in the *lexA2005* strain is about 3 to 4-fold higher than the level of LexA in the $lexA^+$ or *lexA3* strain. We conclude that increasing the level of transcription by inserting the constitutive promoter upstream of *lexA* increases the level of LexA protein production by about 3 to 4-fold.

2.2.2 *lexA2005* decreases the level of RecA protein production about 10-fold.

To measure the effect of *lexA2005* on the amount of RecA and its effect on the cell, we conducted several tests. First, we measured the level of RecA in the cell. This was done in a $lexA^+$ Δ*(recA)*, $lexA^+$ $recA^+$, *lexA3* $recA^+$ and *lexA2005* $recA^+$ strains. Figure 2.2 shows that *lexA3* decreased levels by about 40% relative to $lexA^+$ and that expression of *lexA2005* decreased RecA protein levels by about 85% or about 7-fold. To check that our system was similar to experiments previously reported, we measure the survival to UV irradiation in our $lexA^+$, *lexA3* and *lexA2005* strains. We found that like previous reports, LexA3 was much more UV sensitive than wildtype and that *LexA2005* was even more sensitive to ultraviolet light (Figure 2.3). We conclude that *lexA2005* is able to

decrease the level of RecA production by 10-fold and that it causes increased UV sensitivity in the strain.

2.2.3 *lexA2005* increases the number of RecA-GFP structures and changes the distribution of the shapes of the structures.

We then wanted to test the effect of *lexA2005* on the ability of the cell to produce RecA structures using RecA-GFP to visualize the RecA structures in the cell. The construct we used to make these measurements is *recAo1403 recA41455,4136-gfp-901*. As stated above this construct has been optimized for RecA function and all structures we see are on the DNA (they are not storage structures) (Eldin et al., 2000; Renzette et al., 2005). We have previously reported that about 20% of the cell have a RecA-GFP structure at any one time and that the about 75% of the structures are circular, about 10% are linear and about 15% are in-between the circular and linear definitions.

To begin this part of the study, we first measured the level of LexA and RecA-GFP proteins in cells as a function of *lexA* alleles using Western Blots as have been done above. Figure 2.4 shows that like above, LexA3 is overproduced approximately fourfold and that the amount of RecA-GFP decreases about 15% in the *lexA3* strain as compared to the $lexA^+$ and that its level decreases about 90% or 10-fold in the *lexA2005* strains. This is very similar to the results above with RecA. We then grew the cells for 3 hours on agarose pads containing minimal media and visualized the RecA-GFP structures by fluorescence microscopy. We see that the number of total fluorescent structures increases from $lexA^+$ to *lexA3* to *lexA2005* from 0.23 structures/cell area to 0.34 to 0.99. This is almost a 3 to 4-fold increase in the number of structures. We also see that the number of linear structures decreased from 10% to 3% and the number of circular

structures increased from about 72% to 89% (Figure 2.5, Table 2.1). We then tested when one irradiated the cells on the pad with 10J of UV. In Figure 2.6 and Table 2.2, we see that in wild type cells, the number of structures increases dramatically from about 15% of cells having structures to more than 95% of cells after 90 minutes. We also see the percentage of linear structures increase from about 8% to 20% with a corresponding decrease in the number of circular structures 68% to 39%. This is seen to a lesser degree with the *lexA3* cells. However, *lexA2005* cells show a remarkably different behavior. While they start out with many more circular foci than wildtype (about 95% of cells have structures), after UV treatment, more appear (about 2-fold) so that the same percentage of *lexA*$^+$ and *lexA2005* cells have foci. Unlike the *lexA*$^+$ case, *lexA2005* distribution of shapes does not change much with the circular ones predominating (Table 2.2).

From this we conclude that *lexA2005* decreases the amount of RecA-GFP in the cell but at the same time causes more RecA-GFP structures to form in log phase cell and the distribution of the shape of the foci become even more circular. The situation seems to be exacerbated when one irradiated the cells with UV light, while one sees an increase in the number structures and that they remain in the circular form throughout the time course.

2.2.4 *recAo281* increases the amount of RecA 3 to 4-fold but only partially suppresses the UVS phenotype of *lexA2005*.

The *lexA2005* effect in cells could be due to its ability to decrease the level of any (or combination of) of nearly 40 SOS genes. It had been shown previously that *recAo281* (then called *rnmB281*) would partially suppress the UV sensitivity of a *lexA102 uvrA155* strain (Volkert et al., 1976). This was in fact how *recAo281* was originally isolated. It has

been shown that a different *recA* constitutive mutation, *recAo98*, found as a suppressor of *lexA3*, would increase the level of recA mRNA about 7-fold in the presence of *lexA3* on the chromosome and the level of *recA* protein by a little less than 2-fold (when a *lexA3* was on the chromosome and on a plasmid in the cell) (Ginsburg et al., 1982).

To test if increasing only the level of RecA in the cell could suppress the phenotypes associated with the low RecA protein levels of a *lexA2005* strain in our system, we added *recAo281* to the strain. Figure 2.7 showed that in strain SS12198, the level of LexA was still about three-fold above $LexA^+$ or LexA3 levels and that the amount of RecA protein increased about four-fold from $recAo^+$ $lexA^+$. There was about a sixteen-fold increase in RecA from the $recAo^+$ to *recAo281* in the *lexA3* strain and about a twenty eight-fold increase in the *lexA2005* strain. It should be noted that even though the fold increase in the *lexA2005* strain is greater, the total amount of RecA is lower than the *lexA3* strain because it was starting at a lower level. It is clear, however, in both the *lexA3* and *lexA2005* strains that there is at least 3-fold more RecA protein than in wild type cells. We then tested the amount of suppression offered by *recAo281* on the UV sensitivity caused by *lexA3* and *lexA2005* (Figure 2.8). We see that the level of suppression offered by *recAo281* is about equal in the two strains and the level of suppression is only partial relative to wild type. We conclude that the amount of RecA protein is only one factor that is limiting the amount of UV survival in strains that have either *lexA3* or *lexA2005*.

2.2.5 *recAo281* increases the number of RecA-GFP foci and increases the percentage of linear foci regardless of the *lexA* allele in log phase cells.

Above we found that while *recAo281* suppressed the negative effects of *lexA3* and *lexA2005* on RecA protein expression, all strains produced more than two-fold more RecA protein than *recAo*$^+$ strains. However, the level of UV survival was only partially suppressed to the same level for both *lexA3* and *lexA2005* cells. To determine the effect of increased amounts of RecA-GFP in the cell, we replaced *recAo1403* with *recAo281* in our RecA-GFP constructs. As above, we first measured the amount of LexA and RecA-GFP protein in our strains. We saw a 3-fold increase in the amount of LexA3 in the *lexA2005* strain relative to either the *lexA*$^+$ vs *lexA3* strains and an increase in the amount of RecA-GFP produced in all three backgrounds relative to their *recAo*$^+$ controls (Figure 2.9). The total amount of RecA-GFP in the *recAo281 lexA2005* strain was about two to three-fold higher compared to the *recAo1403 lexA*$^+$ strain. From this we conclude that *recAo281* increases the amount of RecA-GFP in strains regardless of their *lexA* allele. The increases however are more modest than with RecA.

We then examined the strains using fluorescence microscopy as above. We found that that number of foci per area of cell for the *recAo281 lexA*$^+$ strain increase from 0.23 to 1.1 or nearly 5-fold (Figure 2.10 & Table 2.3). We also saw that the distribution of shapes foci also shifted with an increase (4% to 23%) in the percentage of linear structures in the population. This is opposite trend that we saw before with the *recAo1403* strains. It should be noted that the *recAo1403 lexA*+ and *recAo281 lexA2005* strains have almost identical levels of RecA-GFP protein.

2.2.6 In *recAo281* cells, UV light still increases the total number of cells with RecA-GFP structures but *lexA3* and *lexA2005* strains still tend away from linear structures.

We then tested the effect of UV light on the production of RecA-GFP structures. This time we saw that all three *recAo281* strains with *lexA*$^{+}$, *lexA3* and *lexA2005* increased their number of cells with foci about 2 to 3-fold to a level that is about equal to what was seen with the *recAo1403* strains (Figure 2.11 & Table 2.4). The *lexA*$^{+}$ increased its percentage of linear structures with time. The *lexA3* and *lexA2005* strains saw a small shift away from the linear structures to the undecided and circular structures.

2.3 Discussion

The regulation and execution of DNA repair in the cell is highly interrelated. In *E. coli*, this is concretely seen as the LexA repressor of the SOS Repressor is regulated by the activity/stability of the main DNA repair/recombination protein, RecA (Little, 1991; Little et al., 1980), initial response to the production of ssDNA, a typical by-product of DNA damage by interacting with the RecA-DNA protein filament in its major groove (Yu & Egelman, 1993) where the second duplex of DNA also needs to interact to initiate the process of synapsis (Harmon et al., 1996). It is further complicated by the facts that LexA regulates RecA at the level of transcription (Little et al., 1981) as well as several other proteins that affects the stability and activity of RecA (RecX, DinI (Lusetti, Drees, et al., 2004; Lusetti, Voloshin, et al., 2004) and DinD (Uranga et al., 2011)). All genes of the SOS response have different basal levels of expression as well as different induced rates transcription as well as different temporal induction depending on the strength of the LexA binding/promoter site (Culyba et al., 2018). Thus, any experiments that modulate the levels of expression of *recA* and/or *lexA* in the cell are difficult to interpret.

In this chapter, we have tried to recreate previous experiments where researchers have increased the levels of *recA* and *lexA* in the cell on plasmids. This was often complicated because the plasmids used contained the operator site for *recA* or *lexA* and while *lexA* is auto-regulated this would further complicate the interpretation of the results. We sought to simplify the interpretations by placing mutations at the locations of these genes' endogenous location on the chromosome. In the case of *lexA*, we added a strong constitutive promoter just upstream and for *recA* we used known operator mutations. We also used *lexA3* so that as more RecA structures were made, the level of LexA would remain constant. This had the consequence of also not allowing increased expression of the other genes in the SOS response during DNA damage. Lastly, we measure the amount of LexA and RecA by Western blot in each strain studied. In this, we were fairly successful in that we were able to overproduce LexA3 and that it decreased the amount of RecA (and RecA-GFP) in the cell and this in turned increased the UV sensitivity of an otherwise wild type strain. As others have also seen, we saw its partial rescue by introducing a *recAo281* mutation.

The above work, enabled us to set the stage for us to see how changing the levels of *lexA3* in *recA-gfp* strains would change the structures we would see. Our expectation was that the absolute numbers of RecA-GFP structures should have been less because there was less RecA in the *lexA2005* strains. Instead, the number of RecA-GFP structures increased three-fold. It is widely held that recombination is substrate limited so cells that have more RecA-GFP structures should have more substrates. Where do these come from in the *lexA2005* cells? There are at least two possibilities. The first is that decreased levels of RecA and other SOS proteins that are normally available in higher amounts

were less and therefore, repair that should have occurred did not. Support is given to the idea that other SOS genes are likely rate-limiting by the data that increasing the level of RecA by a *recAo281* mutation did not completely rescue the UV sensitivity of the *lexA2005* strain nor did it decrease the level of the RecA-GFP structures. It is also possible that the LexA3 blocked the second DNA from interacting with the RecA-ssDNA filament (Harmon et al., 1996) and this inhibition led to reduced levels of repair and therefore led to more repair structures or more stable structures (that were not competent for repair). The second is that it is known that replication fork collapse due to protein obstruction on the DNA can lead to DSBs. If more LexA3 is available to saturate the LexA-binding sites on the chromosome then this might lead to more obstacles. This, however, seem unlikely since it has been shown with LacI or TetR that multiple occupied sites in a row (around 240) are needed to inhibit the replication machinery (Lau et al., 2003; Possoz et al., 2006). However, other proteins such as Tus binding to *ter* sites can pause forks when only one protein bound site is present (Gottlieb et al., 1992; Pandey et al., 2015). The fact that there are more structures with less RecA-GFP protein is contradictory. It is possible that that the structures formed could have less proteins per structures. This would predict that they would be significantly less intense, but preliminary results show this is not the case.

An interesting finding from this work is that both LexA3 and the amount of RecA-GFP influence the shape of the RecA-GFP structures. In a wild type cell, there is both circular and linear structures with the circular structures predominating. While this is still true in the *lexA2005* strain, the circular structures dominate the population even more. It is curious that when *recAo281* is added to this strain, the amount of RecA-GFP

increases about 3 to 4-fold and then percentage of linear increases greatly from 3% to 23% of the total structures. It is interesting that while the absolute level of RecA-GFP in the *recAo281 lexA2005* strain is about equal to the wild type strain but the percentage of linear structures are much more. It is possible that either LexA3 binding directly to the RecA-ssDNA filament changes the structures or the lowering of one or more SOS proteins is responsible. It is also possible that it is a combination of the two.

What is the relance of the shape of the RecA-GFP structure to its function? It is known that RecA is able to nucleate and then polymerize onto ssDNA (Flory et al., 1984) and then expand into regions of dsDNA (Pugh & Cox, 1988) and that this structure is linear *in vitro* and that the appearance of linear structures during Double Strand Break Repair is found *in vivo* (Lesterlin et al., 2014). What, however, is the function of the circular structures? Are they early structures where RecA has just nucleated onto the DNA and has not yet polymerized onto all the ssDNA and into the dsDNA region or are they inactive structures? It is known that circular structures vary in size and intensity. If they were only structures associated with nucleation, then they would be expected to be only small and not intense but this is not the case. We know that they are not storage structures because all constructs in this work have the *recA4155* (R28A) mutation that inhibits storage structure formation (Eldin et al., 2000; Renzette et al., 2005). More work is need to define what these circular structures are and how they relate to RecA function in the cell.

2.4 Experimental Procedures

2.4.1 Bacterial strains and growth conditions

All bacterial strains are derivatives of E. coli K-12 and are characterized in Supplemental Material, Table S1. The strains were generated using either linear transformation or P1 transduction, according to previously described protocols (Datsenko & Wanner, 2000; Willetts & Clark, 1969). Transformants and transductants were selected on 2% agar plates containing either Luria broth (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) or 56/2 minimal medium (Willetts & Clark, 1969) supplemented with 0.2% glucose, 0.001% thiamine, 0.02% arginine, 0.005% histidine, 0.02% proline, 0.01% leucine, 0.01% threonine, and appropriate antibiotics (Willetts & Clark, 1969). Ampicillin was used at 50 mg/ml, chloramphenicol at 25 mg/ml, kanamycin at 50 mg/ml, and tetracycline at 10 mg/ml. The cells were purified on the same type of media on which they were selected and grown at 30° or 37°. L-arabinose was used for induction of the λ Red expression plasmid pKD46 in a final concentration of 0.5% (w/v).

2.4.2 Generation of *lexA2005*

To generate *lexA2005*, (*lexA3* overproducer) PCR was performed using prSJS1541 and prSJS1553 using SS8253 as template. SS8253 was generated in Massoni et al. 2012 and is the placement of a flippable *cat* gene in front of a *recA* promoter with two operator mutations, *recAo1401* and *recA281*. Both of these operator mutations lead to a decreased affinity for LexA, which in combination with the activity of the *recA* promoter, leads to high constitutive expression of the gene it regulates. A difference between this construct and the one used in Massoni et al., is the *lexA* RBS was used instead of the optimized RBS. The PCR product of prSJS1541 and prSJS1553 was used as template for PCR with prSJS1542 and prSJS1554. The final PCR product was

transformed into SS12162 selecting on LB-Cam, successful genomic integration was PCR screened for with prSJS925 and prSJS926 to make strain SS12163. The mutations were verified by sequencing. P1 was grown on the resulting strain and *lexA2005* was P1 transduced into SS996 and SS6294 for characterization.

2.4.3 Generation of *recAo281 recA4155,4136-gfp901*

To generate *recAo281 recA4155,4136-gfp901*, PCR was performed using prSJS1680 and prSJS508 using SS12727 as template. SS12727 was generated by another lab member combining *ygaD::kan recAo281* and *recA4155*. The PCR product contained upstream of *ygaD::kan* to the PmeI site in *recA*. The PCR product included the *recA4155* mutation. The PCR product was transformed into SS13214 selecting for LB-Kan. Successful genomic integration was screened for with prSJS1680 and prSJS508 followed by screening for *recAo281* with addition of an SphI site and screening for *recA4155* with removal of a BstNI site. The mutations were verified by sequencing. The result was strain SS13336. P1 was grown on this strain to transduce into SS996 and SS6294.

2.4.4 Preparation cells for microscopy

Cells were grown in 56/2 minimal media overnight at 37°C with shaking. 200 µl of the overnight culture was diluted into 3 ml of 56/2 minimal media and grown for 3 hours at 37°C with shaking into early log phase. A total of 3–5ml of the log phase culture was loaded onto a 2% agarose pad prepared from 56/2 minimal medium and low-melting agarose. Placing a coverslip on top the inoculated agarose pad, the slides were incubated for 3–4 hours at 37°C before imaging.

2.4.5 Microscopy and image processing

Cells were visualized using a Nikon E600 microscope equipped with a Z-axis focus drive, automated filter wheels, shutters, Cool LED light source, and an ORCA-ER

camera, as previously described (McCool et al., 2004; Renzette et al., 2005). Phase contrast and fluorescence images were taken for at least 9 different fields of view (three fields on three different days) under total magnification of 1000x. A Z-stack of x-y planes was taken for RecA-GFP fluorescent images that consisted of 13-14 ordered images 2.5-3 μm below to above the focal plane of the phase-contrast image in 0.3 μm steps. Images were deconvolved using Velocity 4.0 software (Improvision, Inc). Single x-y planes were selected and merged with each other to produce analyzable images. Number of cells for each strain was about 1,000 cells.

For UV timelapse, phase and fluorescent pictures were taken immediately after incubation for an initial zero-minute time point. Immediately after the initial timepoint, the slides were flipped and irradiated with 10 Jm^{-2} of UV light from two General Electric 15W germicidal lamps at a rate of 1 Joule/m2/sec. A second set of pictures was taken after ten minutes and a third set of pictures was taken after ninety minutes. Shutters were closed between time points to prevent photobleaching effects.

2.4.6 Analysis of microscopic images

Micrographs were analyzed with the following software: I-Vision (BioVision Technologies), SuperSegger (Stylianidou et al., 2016), and MATLAB R2016a and MATLAB R2019a (MathWorks). Individual cells were outlined using SuperSegger. Strains were analyzed for number of cells, number of foci per cell area, and distribution of foci in cells using specially written MATLAB programs. The minimal focus is determined as four adjacent pixels fourfold about that cell's background fluorescence. The number of foci per cell area was determined by dividing the total number of foci (determined by the GFP fluorescent image) by the total square area of cells (determined

from the phase contrast image) multiplied by one hundred. Difference in distribution of foci in cells between strains was statistically analyzed by a chi-squared test for homogeneity. A P-value of <0.001 was used to determine significance.

2.4.7 UV survival assay

Assay for UV sensitivity has been described elsewhere (Sandler et al., 1996). Briefly, strains were grown in LB broth overnight at 37°C. 200 µl of the cultures was diluted in 10 ml LB broth and grown for 3 hours into the log phase at 37°C with shaking. At the end of 3 hours, the cells were harvested by centrifugation and resuspended in 10ml 56/2 buffer. Cells were transferred to a sterile 10 mm petri dishes and irradiated by UV light from two General Electric 15W germicidal lamps at a rate of 1 J/m^2/sec. Samples were collected as 1 ml aliquots at time intervals and serially diluted to 10^7 in 56/2 buffer. 5µl of each dilution were spotted in duplicate on LB agar and incubated overnight at 37°C. Survival at a certain dose was measured as a ratio between number of colony forming units in the radiated sample and nonirradiated control. Each experiment was repeated three times. Confidence intervals are standard error.

2.4.8 Western Blot analysis

Cells were grown in 56/2 minimal media to a OD600=0.2. Three milliliters of cells normalized to OD600=0.2 were spun down and resuspended in 74 microliters 1X TE buffer (10mMTris-HCl 1mM EDTA). To this 1 microliter of 100x PMSF protease inhibitor was added. 25 microliters of 4x Laemmli buffer from BIO-RAD prepared with 355mM Beta-mercaptoethanol for a final volume of 100ul. Samples were boiled for 10 minutes immediately before loading onto gel. 20 microliters were run on a 4%-20% gradient polyacrylamide gel at 120V for 60 minutes. Protein was transferred to PVDF membranes using BIORAD Trans-Blot Turbo Transfer system at 1.3Amps and 25V for

10 minutes. Membranes were blocked for 1 hour with 5% milk in TBST (50mMTris-HCL 150mMNaCl with 0.2% Tween 20). Primary antibodies were incubated overnight in 5% milk in TBST diluted 1:10,000 for RecA and 1:5,000 for LexA. LexA primary antibodies were purchased from Novus Biologicals. RecA primary antibodies were purchased from Abcam. Secondary antibodies were incubated for 1 hour in 5% milk in TBST diluted 1:10,000. Secondary antibodies were purchased from LiCor. Membranes were imaged using Li-Cor Odyssey CLx Imaging System. Images were quantitated using ImageJ. Band intensities of RecA and LexA were normalized to a loading control. Given values for RecA and LexA protein amounts are in terms of fold change in relation to wildtype RecA and wildtype LexA.

2.5 Tables

Table 2.1: Effect of *lexA3* and *lexA2005* on RecA-GFP structures

				% Cells with[d]				% Structures[c,d]		
Strain[a]	*lexA*	*recAo*	Structures/ area[b]	0 structures	1 structure	2 structures	≥3 structures	Circular	Undecided	Linear
SS6294	+	*1403*	0.2	90	9	1	0	73	17	10
SS13348	*3*	*1403*	0.3	87	10	2	1	66	25	9
SS13345	*2005*	*1403*	1.0	62	26	9	3	89	8	3

[a] All strains have *ygaD1*::*kan recAo1403 recA4155, 4136*::*gfp901*(A206T) (Cormack *et al.*, 1996; Renzette *et al.*, 2005) in JC13509 (Sandler *et al.*, 1996) background. Cells were grown as described in Experimental procedures.
[b] The total number of foci divided by the total area of cells times 1000.
[c] A focus was defined as circular if its length is between one- to two-fold of its width. A focus was defined as linear if its length is at least three-fold longer than its width. Otherwise, a focus was defined as undecided.
[d] The structure distribution by number for *lexA3* was not found to be significantly different from the *lexA*$^+$ strain (p=0.67) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. The structure distribution by number for *lexA2005* was found to be significantly different from *lexA*$^+$ and *lexA3* strains (p<0.001). The foci distribution by shape for *lexA3* is not significantly different from the *lexA*$^+$ strain (p=0.20) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. The foci distribution by shape for *lexA2005* was found to be significantly different from the *lexA*$^+$ and *lexA3* strains (p<0.001).

Table 2.2: Effect of *lexA3* and *lexA2005* on RecA-GFP structures after ultraviolet irradiation

		Version of:			% Cells with:[e]				% Structures:[d,e]		
Strain[a]	Time after UV[b]	*lexA*	*recAo*	Structures/ area[c]	0 structures	1 structure	2 structures	≥3 structures	Circular	Undecided	Linear
SS6294	0	+	*1403*	0.3	88	10	2	0	69	23	8
”	10	“	“	2.6	4	53	35	8	66	27	7
”	90	“	“	1.9	9	62	25	4	39	41	20
SS13348	0	*3*	*1403*	0.5	79	18	2	1	71	26	3
”	10	“	“	2.9	2	44	41	13	69	25	6
”	90	“	“	2.0	7	56	29	8	60	31	9
SS13345	0	*2005*	*1403*	1.1	55	30	11	4	89	8	3
”	10	“	“	2.4	9	46	36	9	87	12	1
”	90	“	“	1.8	18	48	27	7	84	14	2

[a] All strains have *ygaD1*::*kan recAo1403 recA4155, 4136*::*gfp901*(A206T) (Cormack *et al.*, 1996; Renzette *et al.*, 2005) in JC13509 (Sandler *et al.*, 1996) background. Cells were grown as described in experimental procedures.
[b] Log phase cells were irradiated with $10Jm^{-2}$ of ultraviolet light as described in experimental procedures
[c] The total number of foci divided by the total area of cells times 1000.
[d] A focus was defined as circular if its length is between one- to two-fold of its width. A focus was defined as linear if its length is at least three-fold longer than its width. Otherwise, a focus was defined as undecided.
[e] At the zero minute time point, the structure distribution by number for *lexA3* was not found to be significantly different from the $lexA^+$ strain (p=0.13) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. At the zero time point, the structure distribution by number for *lexA2005* was found to be significantly different from $lexA^+$ and *lexA3* strains (p<0.001). At the ten and ninety minute time points, the structure distribution by number for any of the *lexA* alleles was not found to be significantly different any of the other *lexA* strains 10 min:($lexA^+$ vs *lexA3* p=0.08, $lexA^+$ vs *lexA2005* p=0.25, *lexA3* vs *lexA2005* p=0.05) 90 min:($lexA^+$ vs *lexA3* p=0.29, $lexA^+$ vs *lexA2005* p=0.02, *lexA3* vs *lexA2005* p=0.04). At the zero and ten minute time points, the foci distribution by shape for *lexA3* is not significantly different from the $lexA^+$ strain 0 min:(p=0.01) 10 min:(p=0.80) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. At the zero and ten minute time points, the foci distribution by shape for *lexA2005* was found to be significantly different from the $lexA^+$ and *lexA3* strains (p<0.001) At the ninety minute time point, the structure distribution by shape for any of the *lexA* alleles was found to be significantly different any of the other *lexA* strains (p<0.001).

Table 2.3: Combined effect of *lexA3* and *lexA2005* with *recAo281* on RecA-GFP structures

				% Cells with[d]				% Structures[c,d]		
Strain[a]	*lexA*	*recAo*	Structures/ area[b]	0 structures	1 structure	2 structures	≥3 structures	Circular	Undecided	Linear
SS13338	+	*281*	1.1	49	46	5	0	89	7	4
SS13339	*3*	*281*	1.2	45	45	8	2	73	10	17
SS13341	*2005*	*281*	0.9	60	31	7	2	44	33	23

[a] All strains have *ygaD1*::*kan recAo281 recA4155, 4136*::*gfp901*(A206T) (Cormack *et al.*, 1996; Renzette *et al.*, 2005) in JC13509 (Sandler *et al.*, 1996) background. Cells were grown as described in experimental procedures.

[b] The total number of foci divided by the total area of cells times 1000.

[c] A focus was defined as circular if its length is between one- to two-fold of its width. A focus was defined as linear if its length is at least three-fold longer than its width. Otherwise, a focus was defined as undecided.

[d] The structure distribution by number for any of the *lexA* alleles was not found to be significantly different from any of the other *lexA* strains (*lexA*$^+$ vs *lexA3* p=0.32, *lexA*$^+$ vs *lexA2005* p=0.008, *lexA*3 vs *lexA2005* p=0.02) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. The foci distribution by shape for any of the lexA alleles was significantly different from any of the other *lexA* strain (p<0.001) by the $\chi 2$ test of homogeneity for (R x C) contingency tables.

Table 2.4: Combined effect of *lexA3* and *lexA2005* with *recAo281* on RecA-GFP structures after ultraviolet irradiation

		Version of:			% Cells with:[e]				% Structures:[d,e]		
Strain[a]	Time after UV[b]	*lexA*	*recAo*	Structures/ area[c]	0 structures	1 structure	2 structures	≥3 structures	Circular	Undecided	Linear
SS13338	0	+	*281*	1.0	45	46	7	2	80	9	11
”	10	“	“	2.6	8	38	38	16	65	24	11
”	90	“	“	2.0	8	47	37	8	37	38	25
SS13339	0	*3*	*281*	1.2	39	52	8	1	66	15	19
”	10	“	“	2.4	10	40	37	13	65	20	15
”	90	“	“	1.8	12	52	30	6	54	32	14
SS13341	0	*2005*	*281*	1.0	54	33	9	4	45	36	19
”	10	“	“	2.4	9	40	38	13	50	33	17
”	90	“	“	1.9	7	52	34	7	56	33	11

[a] All strains have *ygaD1*::*kan recAo281 recA4155, 4136*::*gfp901*(A206T) (Cormack *et al.*, 1996; Renzette *et al.*, 2005) in JC13509 (Sandler *et al.*, 1996) background. Cells were grown as described in experimental procedures.
[b] Log phase cells were irradiated with $10Jm^{-2}$ of ultraviolet light as described in experimental procedures
[c] The total number of foci divided by the total area of cells times 1000.
[d] A focus was defined as circular if its length is between one- to two-fold of its width. A focus was defined as linear if its length is at least three-fold longer than its width. Otherwise, a focus was defined as undecided.
[e] At the zero minute time point, the structure distribution by number for *lexA3* and *lexA2005* was not found to be significantly different from the $lexA^+$ strain ($lexA^+$ vs *lexA3* p=0.43, $lexA^+$ vs *lexA2005* p=0.04) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. At the zero time point, the structure distribution by number for *lexA2005* was found to be significantly different from *lexA3* strain (p<0.001). At the ten and ninety minute time points, the structure distribution by number for any of the *lexA* alleles was not found to be significantly different any of the other *lexA* strains 10 min:($lexA^+$ vs *lexA3* p=0.75, $lexA^+$ vs *lexA2005* p=0.82, *lexA3* vs *lexA2005* p=0.98) 90 min:($lexA^+$ vs *lexA3* p=0.25, $lexA^+$ vs *lexA2005* p=0.79, *lexA3* vs *lexA2005* p=0.25). At the zero minute time point, the foci distribution by shape for *lexA3* is not significantly different from the $lexA^+$ strain (p=0.01) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. At the zero minute time points, the structure distribution by shape for *lexA2005* was found to be significantly different from the $lexA^+$ and *lexA3* strains (p<0.001) At the ten minute time point, the structure distribution by shape for any of the *lexA* alleles is not significantly different from any of the other *lexA* strains ($lexA^+$ vs *lexA3* p=0.39, $lexA^+$ vs *lexA2005* p=0.01, *lexA3* vs *lexA2005* p=0.007) by the $\chi 2$ test of homogeneity for (R x C) contingency tables. At the ninety minute time point, the structure distribution by shape for the *lexA3* allele was found to not be significantly different from the *lexA2005* strain (p=0.63). At the ninety minute time point, the structure distribution by shape for the *lexA3* and *lexA2005* alleles was found to be significantly different from the $lexA^+$ strain (p<0.001).

Table 2.5: Strains used in this study

Strain	*attλ*	*lexAp*	*lexA*	*recAo*	*recA*	Other relevant genotype	Reference
JC19098	+	+	*3*	+	+	*malE::Tn10*	Laboratory stock
SS775	+	+	*3*	+	+	*malE::Tn10-9 (cam)*	Laboratory stock
SS996	*Ωgfp*	+	+	+	+		McCool et al. 2004
SS2385	*Ωgfp*	+	*3*	+	+	*malE::Tn10*	McCool et al. 2004
SS4454	+	+	+	+	*recA4136::gfp901*	*zfj-3131::Tn10*	Laboratory stock
SS5958	*Ωgfp*	+	+	+	*Δ(recA)100::kan*		Laboratory stock
SS6020	*Ωgfp*	+	+	*281*	+	*srlC300::Tn10*	Laboratory stock
SS6294	*ΩmCherry*	+	+	*1403*	*4155::gfp901*	*Δ(galK)200:frt*	Renzette et al.
SS7117	*ΩmCherry*	+	+	+	+	*Δ(galK)200:frt*	Warr et al. 2019
SS11776	*ΩmCherry*	+	*3*	+	+	*Δ(galK)200:frt malE::Tn10*	JC19098→SS7117[c]
SS12161	*ΩmCherry*	+	*3*	+	*Δ(recA)100::kan*	*malE::Tn10 Δ(galK)200:frt*	SS5958→SS11776[a]
SS12162	*ΩmCherry*	+	*3*	+	*Δ(recA)100::kan*	*malE::Tn10 Δ(galK)200:frt* (w/pKD46)	SS12161 transformed with pKD46[d]
SS12163	*ΩmCherry*	*2005*	*3*	+	*Δ(recA)100::kan*	*malE::Tn10 Δ(galK)200:frt*	This study
SS12198	*Ωgfp*	*2005*	*3*	*281*	+	*srlC300::Tn10*	SS12163→SS6020[b]
SS12727	*ΩmCherry*	+	+	*281*	*4155*	*Δ(galK)200:frt*	Laboratory stock
SS12852	*Ωgfp*	+	Δ	+	+		SS12118→SS996[b]
SS13214	+	+	+	+	*recA4136::gfp901*	*zfj-3131::Tn10* (w/pKD46)	SS4454 Transformed with pKD46[d]
SS13336	*ΩmCherry*	+	3?	*281*	*4155::gfp901*	*Δ(galK)200:frt*	This study
SS13337	*Ωgfp*	*2005*	*3*	+	+		SS12179→SS996[b]
SS13338	*ΩmCherry*	+	+	*281*	*4155::gfp901*	*Δ(galK)200:frt*	SS13336→SS7117[a]
SS13339	*ΩmCherry*	+	*3*	*281*	*4155::gfp901*	*Δ(galK)200:frt malE::Tn10-9 (cam)*	SS13336→SS10973[a]
SS13341	*ΩmCherry*	*2005*	*3*	*281*	*4155::gfp901*	*Δ(galK)200:frt*	SS12179→SS13338[b]
SS13345	*ΩmCherry*	*2005*	*3*	*1403*	*4155::gfp901*	*Δ(galK)200:frt*	SS12163→SS6294[b]

SS13346	*Ωgfp*	+	*3*	*281*	+	*malE::Tn10-9 (cam) srlC300::Tn10*	SS775→SS6020 [b]
SS13348	*ΩmCherry*	+	*3*	*1403*	*4155::gfp901*	*malE::Tn10 Δ(galK)200:frt*	JC19098→SS6294[c]

All strains have a JC13509 background. JC13509 is derived from SK362 strain and has the following genotype: F^- *lacMS286 Φ80dIIlacBK1 sulB103 argE4 his-4 thi-1 xyl-5 mtl-1* Sm^R $T6^R$. The *lacMS286 Φ80dIIlacBK1* codes for two partial non-overlapping deletions of the lac operon (Konrad, 1977; Zieg & Kushner, 1977). Unless notated otherwise, all *recA* mutants generated in this study have *ygaD1::kan* in addition to noted mutations (Renzette et al., 2005).

[a] Select for kanamycin resistance and then screen by phenotypic marker

[b] Select for chloramphenicol resistance and then screen by either UV sensitivity or red or green cells

[c] Select for tetracycline resistance and then screen UV sensitivity

[d] Select for ampicillin resistance at 30ºC

Table 2.6: Oligonucleotides used in this study

Name	Sequence (5' to 3')	Specificity
prSJS508	AGAGGATCTGGAATTCAGCC	Reverse primer to make *recAo281 recA4155,4136-gfp901*
prSJS1541	TGTATATACACCCAGGGGGCGGAC GTACGGAAGTTCCTATTCTCTAGA AAGTATAGGAACTTCGGCGAAAAT GAGACGTTG	First forward primer to make *lexA2005*
prSJS1553	GATCACGGATGAGATCAAACACCT CTTGTTGCCTGGCCGTTAACGCTTT CATTTTCTTCCTCCTTCATGCCGGG TAATACC	First reverse primer to make *lexA2005*
prSJS1542	TATTGTGCAGTTTATGGTTCCAAA ATCGCCTTTTGCTGTATATACTCAC AGCATAACTGTATATACACCCAGG GGGCGGACG	Second forward primer to make *lexA2005*
prSJS1554	GCTGCGCGATTTCCGCACGCGTCG GCGGCATACCTGTCTGGCTGATGT GATCACGGATGAGATCAAACACCT CTTGTTGCC	Second reverse primer to make *lexA2005*
prSJS1680	GCCGGATCCTGACGAAAGTGCTAT CTTGTCCGG	Forward primer to make *recAo281 recA4155,4136-gfp901*

2.6 Figures

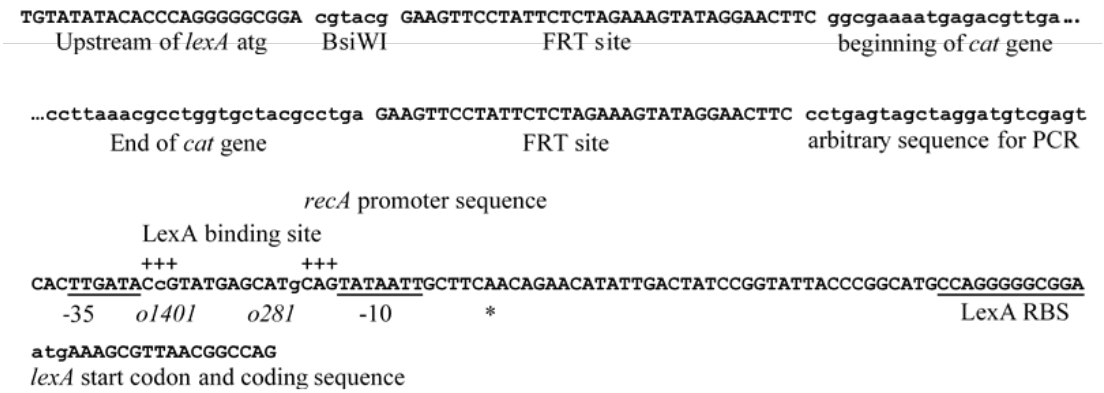
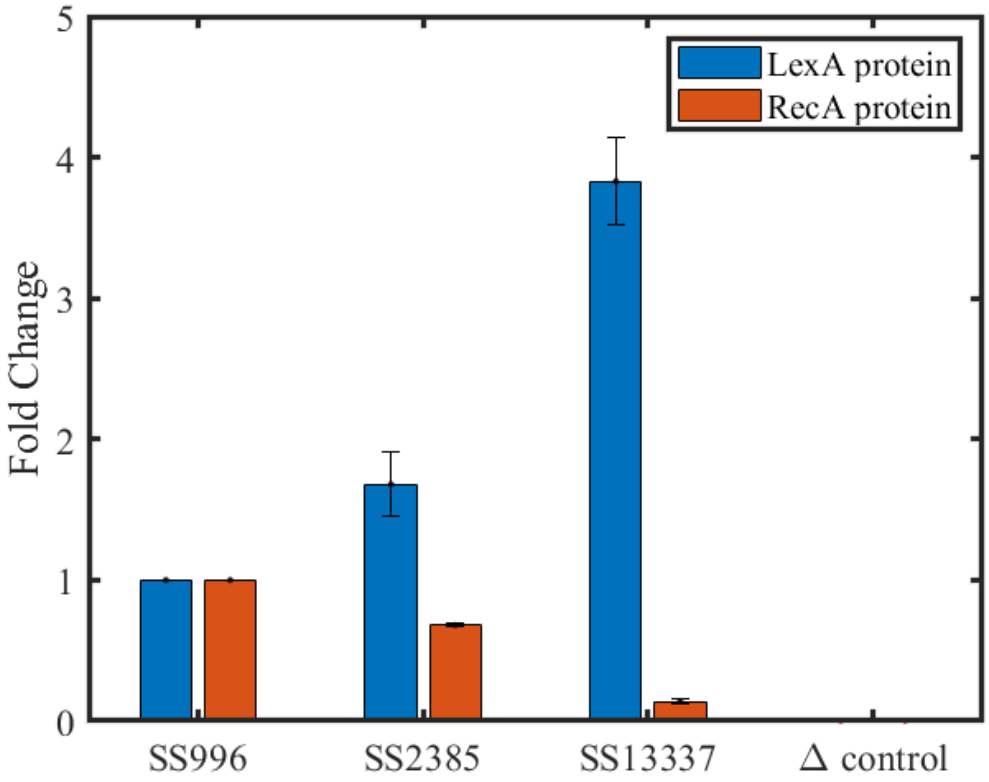


Figure 2.1: Construction of *lexA3* overproducer (*lexA2005*)

Sequence of DNA that has been added in front of the *lexA3* gene to increase its level of transcription. Spaces in the sequence are placed there to separate functional sequences of DNA that are described below or above the sequence. The only omitted sequence is that of the *cat* gene and is denoted by the multiple dots. The promoter was modeled on the sequence of the *recA* promoter and 5'untranslated region. Deviations from the *recA* sequence to remove SOS regulation are denoted in lowercase letters. The allele numbers of the operator mutations that remove LexA regulation are given below the line. The sequences for -10 and -35 boxes are underlined, and the transcriptional start site is denoted by an asterisk. The native *lexA* ribosomal binding site was maintained (underlined). The construction was verified by DNA sequencing.

Figure 2.2: Effect of *lexA3* and *lexA2005* on LexA and RecA protein amount



Strain	SS996	SS2385	SS13337	Δ control[c]
lexA allele	+	*3*	*2005*	Δ
recA allele	+	+	+	Δ
recAo allele	+	+	+	+
Relative LexA protein fold change[a]	1	1.68±0.23	3.83±0.31	N.D.
Relative RecA protein fold change[b]	1	0.68±0.01	0.14±0.02	N.D.

Strain numbers in the bar chart correspond with the strain numbers in the table. Western blots were performed in triplicate as described in Materials and Methods

[a] Amount of LexA measured relative to LexA in a *lexA*$^+$ strain (SS996). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean.

[b] Amount of RecA measured relative to RecA in a *recA*$^+$ strain (SS996). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean.

[c] Deletion strains were used as negative controls for each protein. SS12852 was Δ*lexA* and SS5958 was Δ*recA*. LexA and RecA proteins were not detected (N.D.) in control strains.

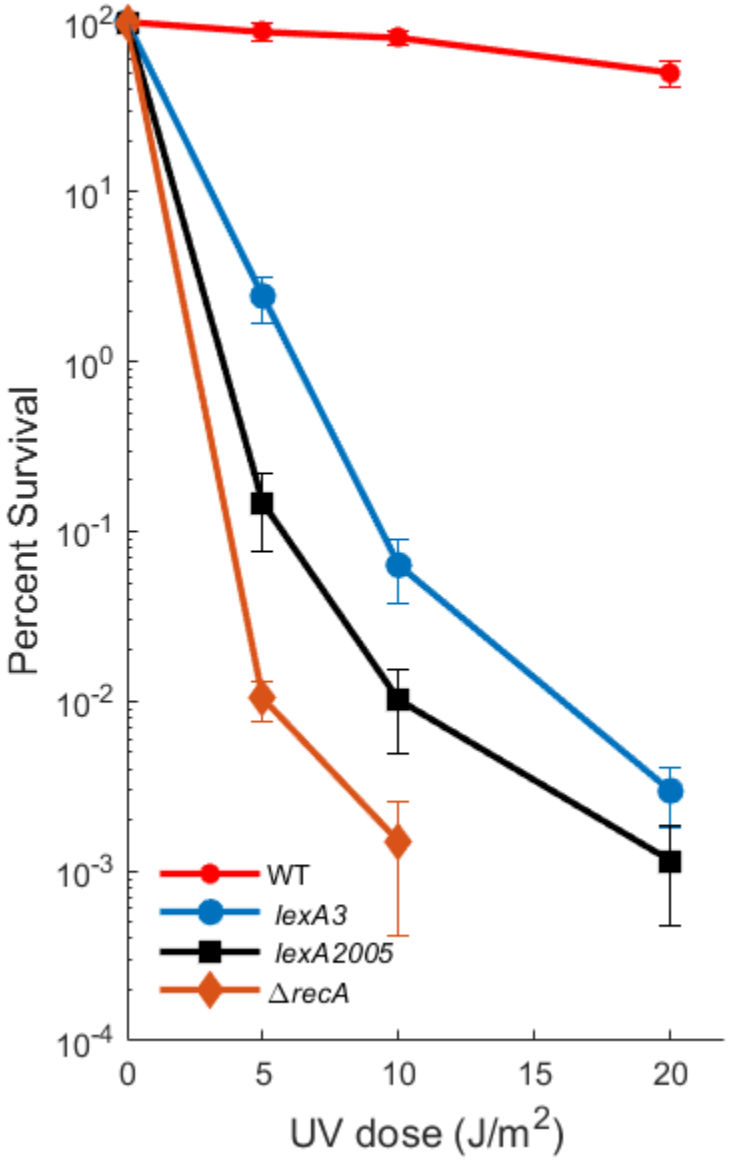
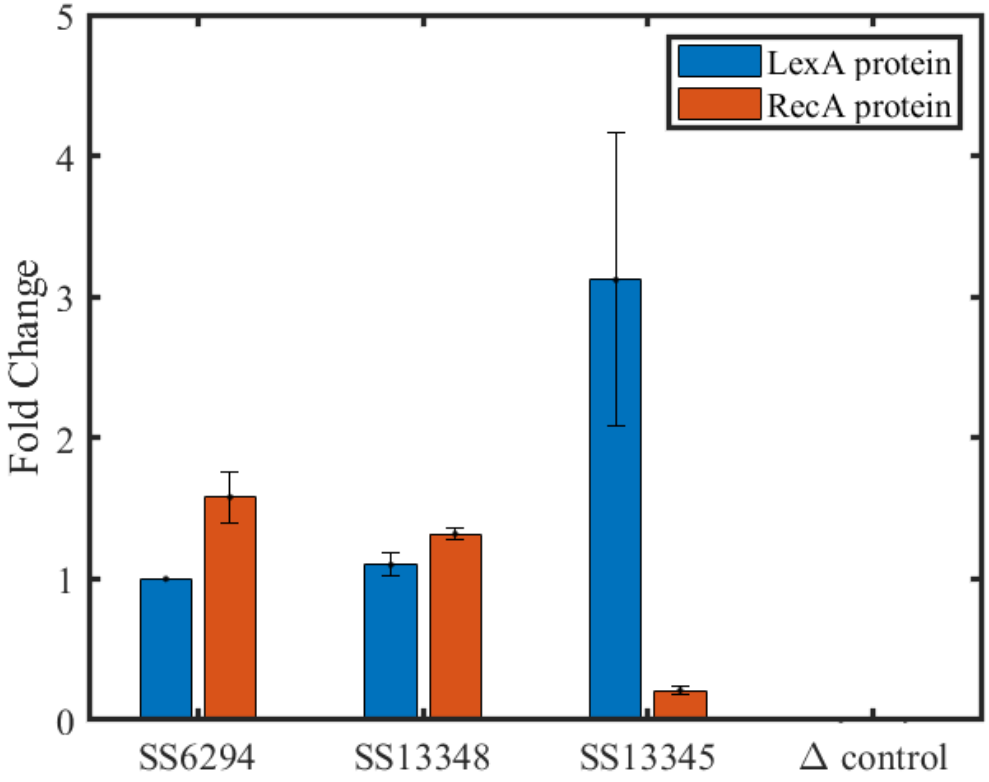


Figure 2.3: Survival curves of *lexA* mutants after exposure to ultraviolet light.

Survival of log phase cells being exposed to up to 20 joules/m^2 ultraviolet light. Overexpressing *lexA3* increases UV sensitivity compared to *lexA3* expressed at wildtype levels.

Figure 2.4: Effect of *lexA3* and *lexA2005* on LexA and RecA-GFP protein amounts



Strain	SS6294	SS13348	SS13345	Δ control[c]
lexA allele	+	*3*	*2005*	Δ
recA allele	+	+	+	Δ
recAo allele	*1403*	*1403*	*1403*	+
Relative LexA protein fold change[a]	1	1.10±0.08	3.12±1.04	N.D.
Relative RecA-GFP protein fold change[b]	1.58±0.18	1.32±0.04	0.21±0.03	N.D.

Strain numbers in the bar chart correspond with the strain numbers in the table. Western blots were performed in triplicate as described in Materials and Methods. RecA-GFP full construct is *recAo1403 recA4155,4136-gfp901*

[a] Amount of LexA measured relative to LexA in a *lexA*$^+$ strain (SS6294). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean.

[b] Amount of RecA measured relative to RecA in a *recA*$^+$ strain (SS6294). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean.

[c] Deletion strains were used as negative controls for each protein. SS12852 was Δ*lexA* and SS5958 was Δ*recA*. LexA and RecA proteins were not detected (N.D.) in control strains.

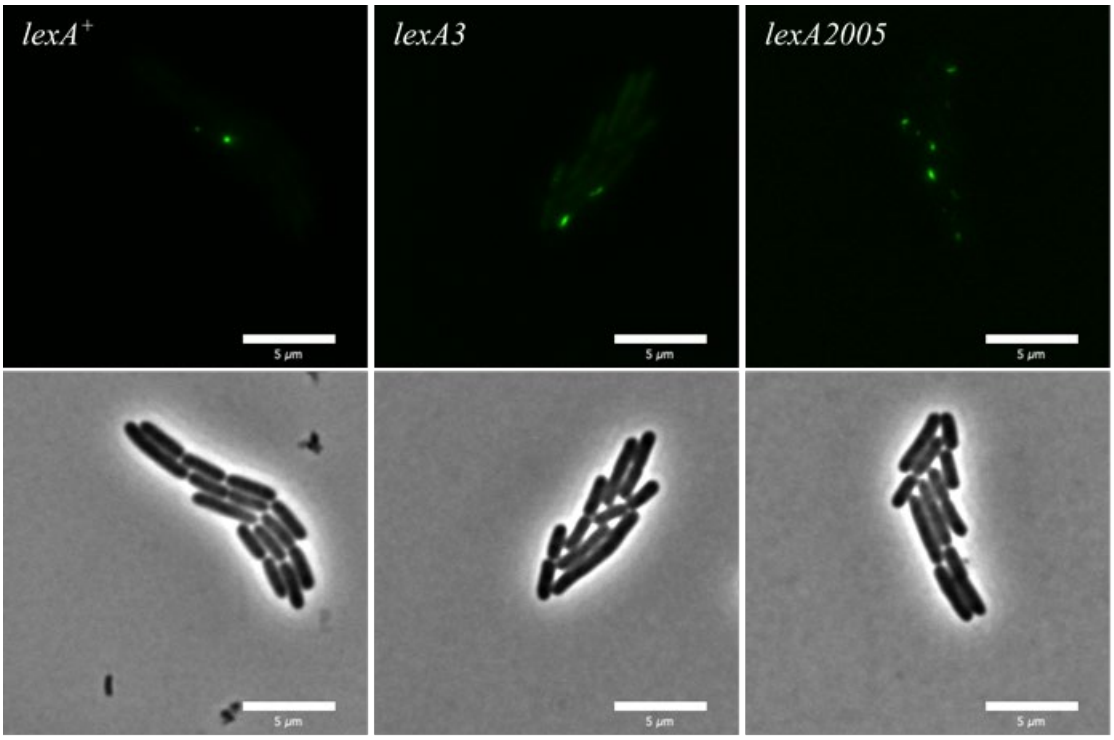
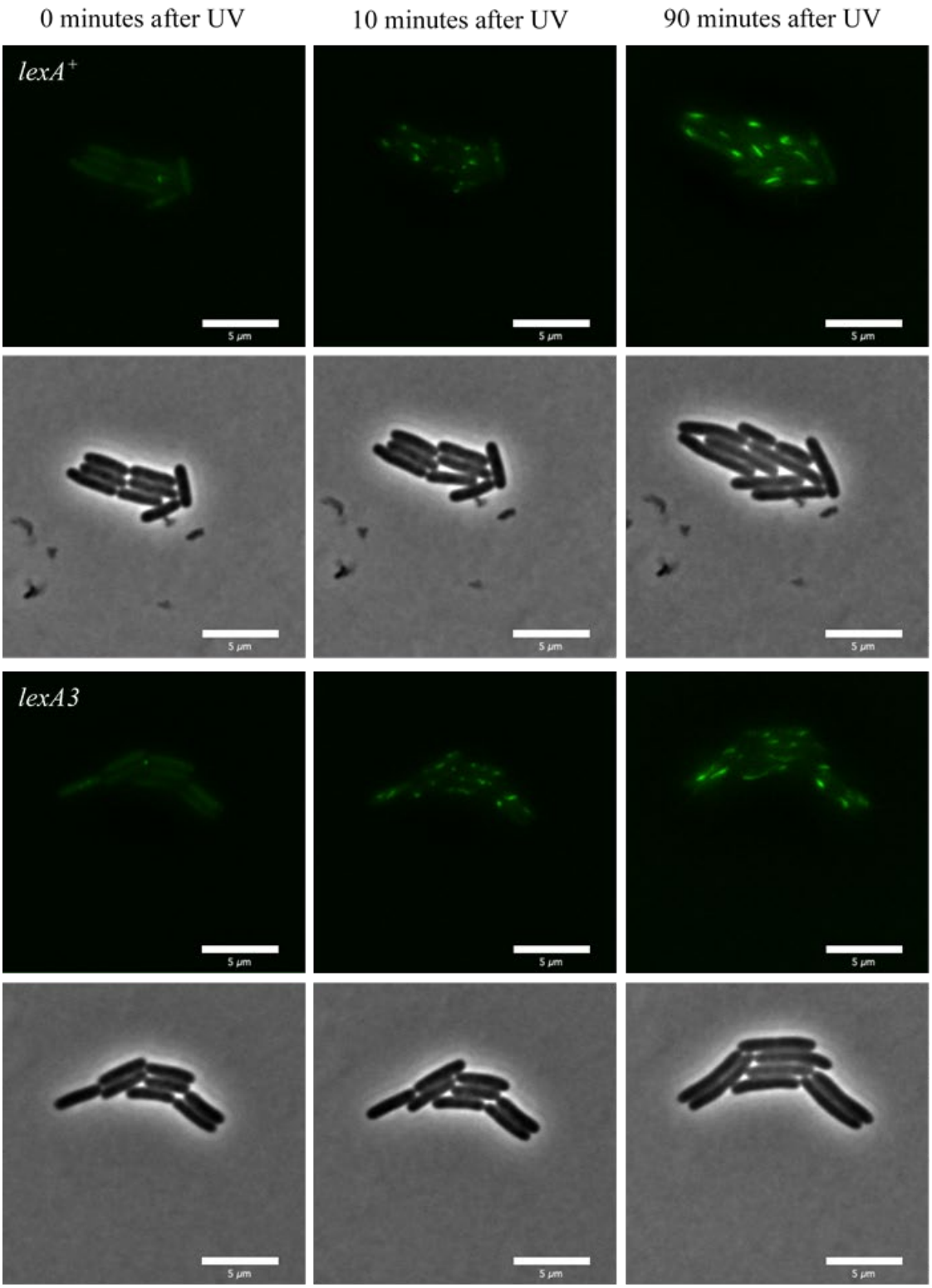


Figure 2.5: Fluorescent RecA structures in log phase *E. coli* with *recAo1403* operator.

Visualization RecA-GFP structures in log phase *E. coli* cells. Each strain contains *ygaD1::kan recAo1403 recA4155,4136::gfp* for visualization of recombination structures on the DNA. The top row is the fluorescent image of RecA-GFP. The bottom row is the phase contrast image. SS6294 is the *lexA*$^{+}$ strain. SS13348 is the *lexA3* strain. SS13345 is the *lexA2005* strain. The *lexA2005* strain shows a five-fold increase in the number of RecA-GFP structures per area of cell and a predominance of circular shaped structures over linear shaped structures.


0 minutes after UV
10 minutes after UV
90 minutes after UV
lexA+
lexA3
5 µm

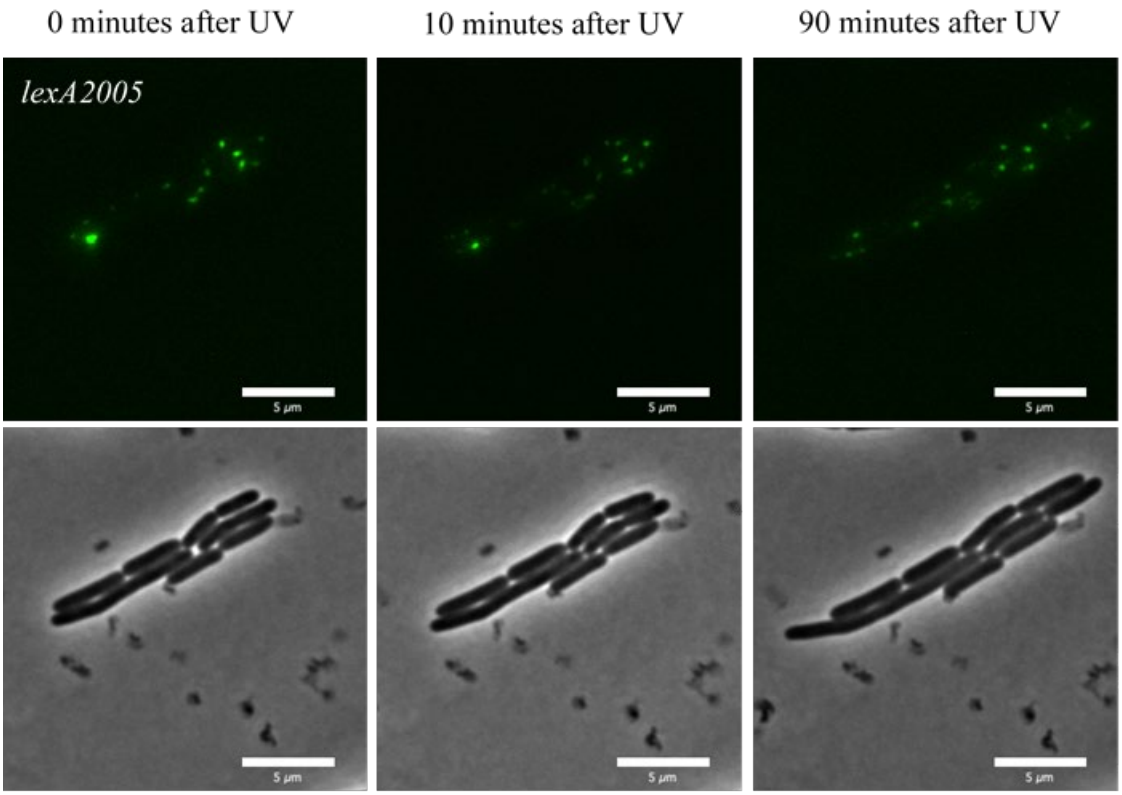
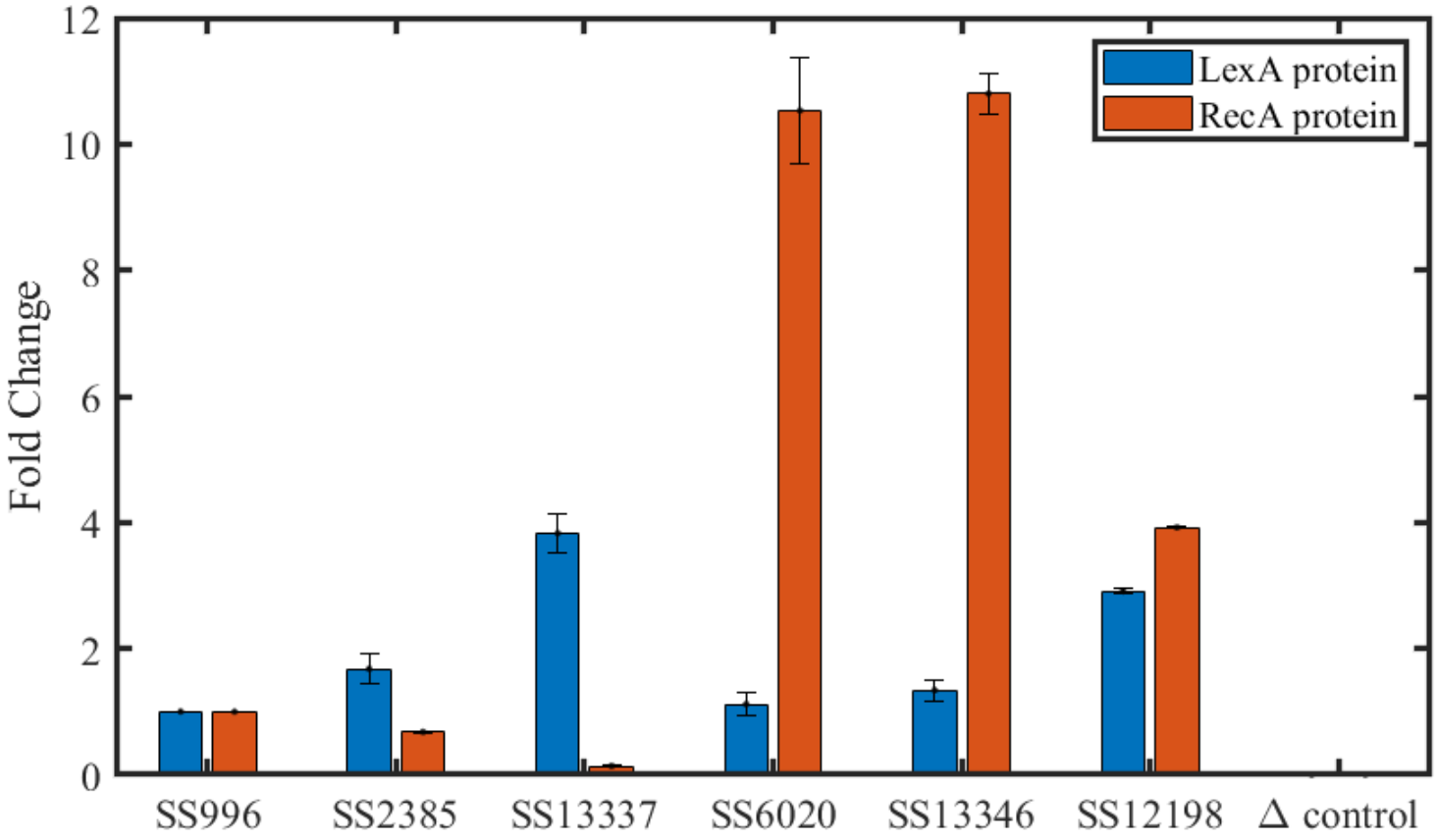


Figure 2.6: Timelapse of RecA-GFP structures after exposure to 10 J/m2 ultraviolet light in E. coli cells with recAo1403 operator**.**

Visualization RecA-GFP structures in log phase *E. coli* cells before and after ultraviolet light irradiation. Each strain contains *ygaD1::kan recAo1403 recA4155,4136::gfp* for visualization of recombination structures on the DNA. Images in the left column were taken immediately before ultraviolet light irradiation. Image in the middle column were taken ten minutes after irradiation. Images in the right column were taken ninety minutes after irradiation. The top, third and fifth rows are the fluorescent image of RecA-GFP for each strain. The second, fourth, and bottom rows are the phase contrast image for each strain. SS6294 is the *lexA*$^{+}$ strain. SS13348 is the *lexA3* strain. SS13345 is the *lexA2005* strain. All of the strains showed an increase in the number of RecA-GFP structures ten minutes after UV irradiation. At ninety minutes after UV, 20% of RecA-GFP structures in the *lexA*$^{+}$ strain are linear shaped. In contrast, at the same time point, only 2% of RecA-GFP structures in the *lexA2005* strain are linear shaped.

Figure 2.7: Combined effect of *lexA3* and *lexA2005* with *recAo281* on LexA and RecA



Strain	SS996	SS2385	SS13337	SS6020	SS13346	SS12198	Δ control[c]
lexA allele	+	*3*	*2005*	+	3	2005	Δ
recA allele	+	+	+	+	+	+	Δ
recAo allele	+	+	+	*281*	*281*	*281*	+
Relative LexA protein fold change[a]	1	1.68±0.23	3.83±0.31	1.12±0.17	1.34±0.17	2.91±0.04	N.D.
Relative RecA protein fold change[b]	1	0.68±0.01	0.14±0.02	10.53±0.85	10.80±0.33	3.92±0.01	N.D.

Columns of table are aligned with the strains in the Western blots. Western blots were performed in triplicate as described in Materials and Methods.

[a] Representative Western blot for LexA shown above. Fold-increase of LexA measured relative to LexA in a *lexA*$^+$ strain (SS996). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean.

[b] Representative Western blot for RecA shown above. Fold-increase of RecA measured relative to RecA in a *recA*$^+$ strain (SS996). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean. Real RecA levels for *recAo281* strains were determined by diluting lysates one in four and running dilution on gel alongside controls.

[c] Deletion strains were used as negative controls for each protein. SS12852 was Δ*lexA* and SS5958 was Δ*recA*. LexA and RecA proteins were not detected (N.D.) in control strains.

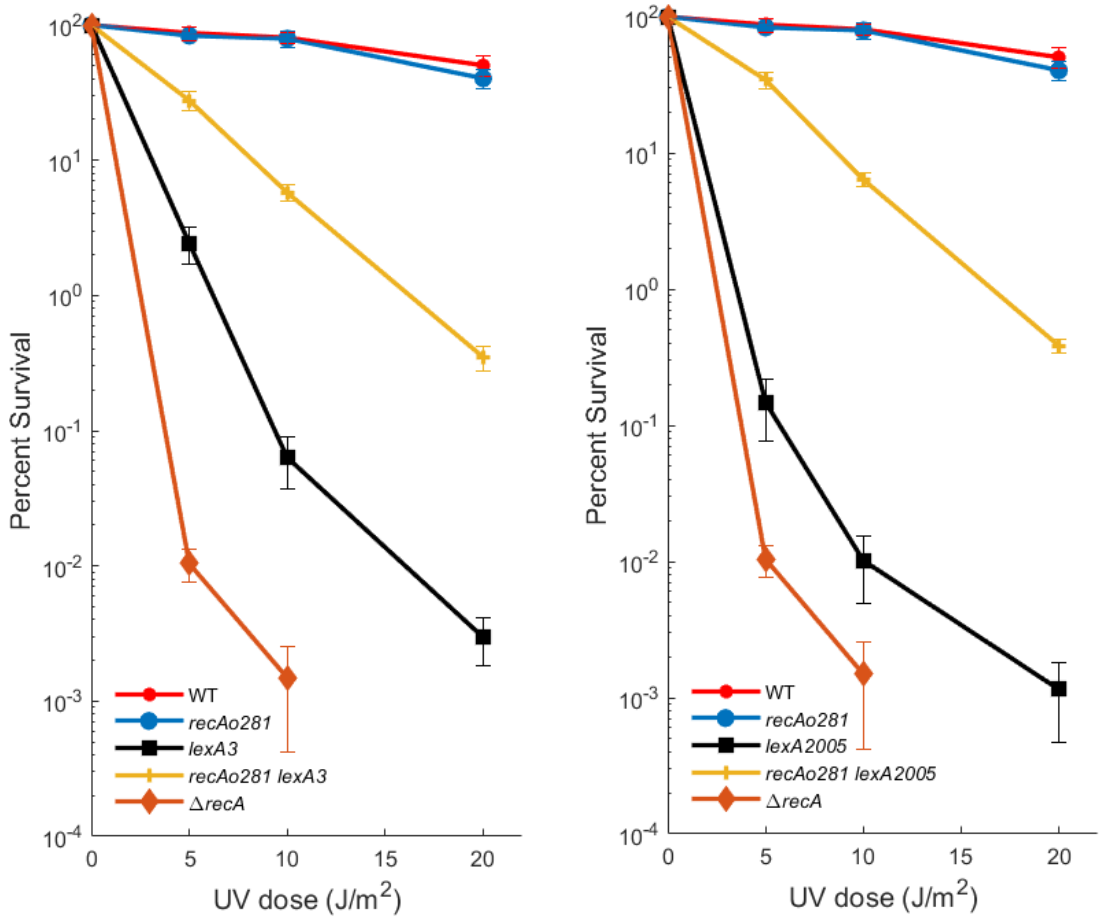
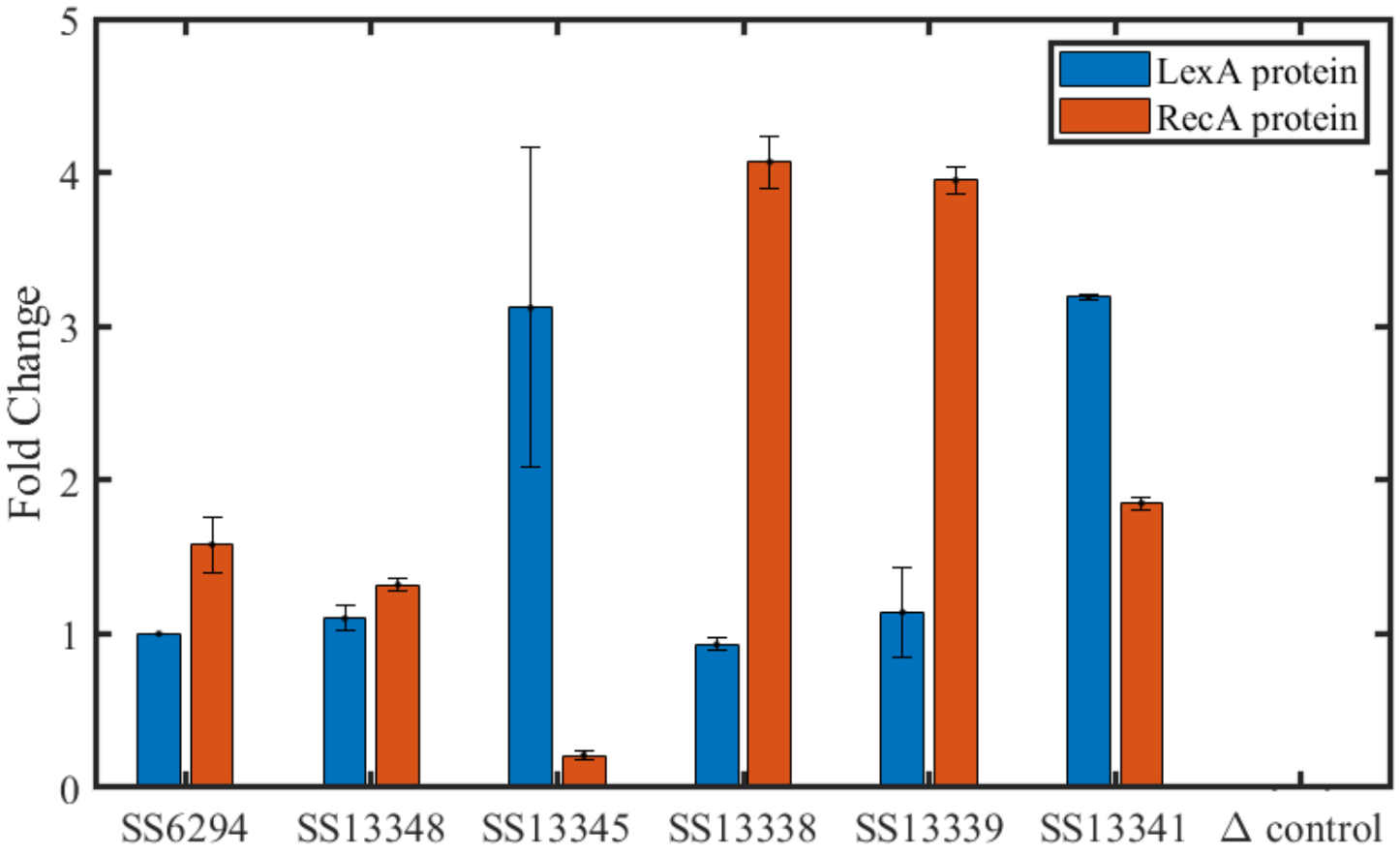


Figure 2.8: Survival curves of *lexA* mutants after exposure to ultraviolet light.

Survival of log phase cells exposed to up to 20 joules/m^2 ultraviolet light. Adding the *recAo281* operator mutation partially rescues the ultraviolet light sensitivity phenotype in both *lexA3* and *lexA3* overproducer to the same level, suggesting some other SOS gene(s) is limiting UV resistance. Additionally, *lexA3* is not inhibitory after a certain level as long as there is the amount of RecA given by the *recAo281* mutation.

Figure 2.9: Combined effect of *lexA3* and *lexA2005* with *recAo281* on LexA and RecA-GFP



Strain	SS6294	SS13348	SS13345	SS13338	SS13339	SS13341	Δ control[c]
lexA allele	+	*3*	*2005*	+	3	2005	Δ
recA allele	+	+	+	+	+	+	Δ
recAo allele	*1403*	*1403*	*1403*	*281*	*281*	*281*	+
Relative LexA protein fold increase[a]	1	1.10±0.08	3.12±1.04	0.93±0.04	1.14±0.29	3.19±0.02	N.D.
Relative RecA-GFP protein fold increase[b]	1.58±0.18	1.32±0.04	0.21±0.03	4.07±0.17	3.95±0.09	1.85±0.04	N.D.

Columns of table are aligned with the strains in the Western blots. Western blots were performed in triplicate as described in Materials and Methods. RecA-GFP full construct is *recAo1403 recA4155,4136::gfp901*. Strains with *recAo281*, replaced *recAo1403* with *recAo281*.

[a] Fold-increase of LexA measured relative to LexA in a *lexA*$^+$ strain (SS6294). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean.

[b]. Fold-increase of RecA measured relative to RecA in a *recA*$^+$ strain (SS996). Band intensities were normalized to a reference band that did not change intensity between conditions. Confidence intervals are standard error of the mean. Real RecA levels for *recAo281* strains were determined by diluting lysates one in four and running dilution on gel alongside controls.

[c] Deletion strains were used as negative controls for each protein. SS12852 was Δ*lexA* and SS5958 was Δ*recA*. LexA and RecA proteins were not detected (N.D.) in control strains

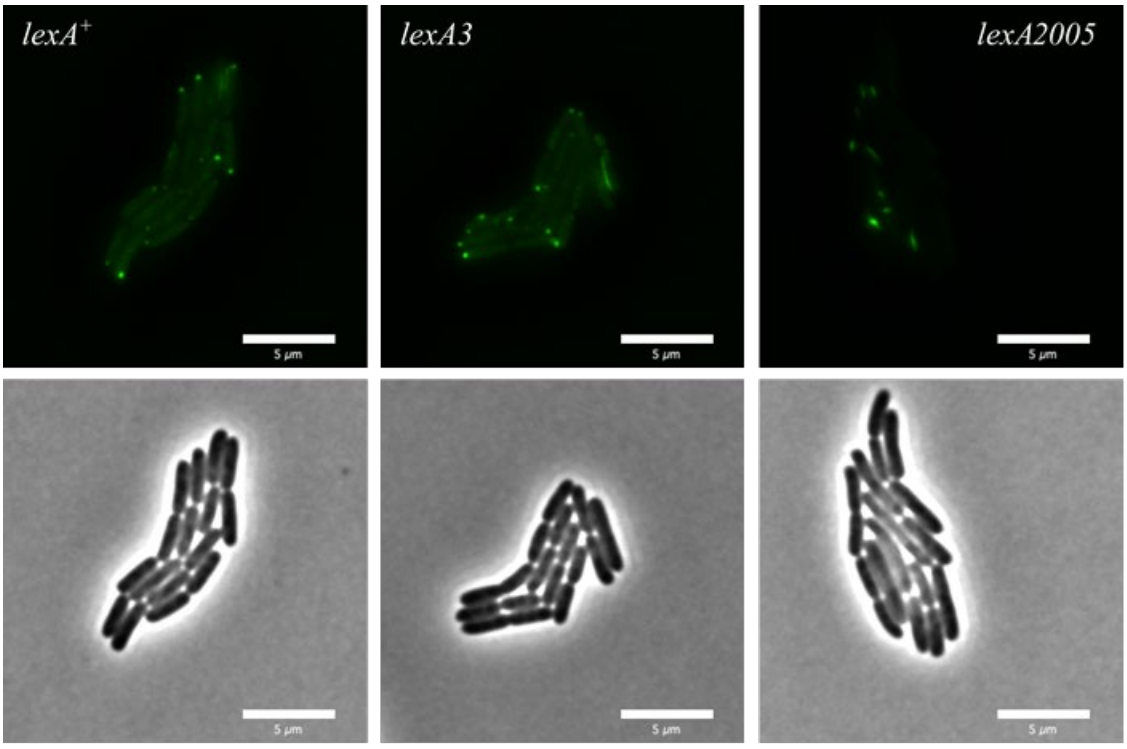
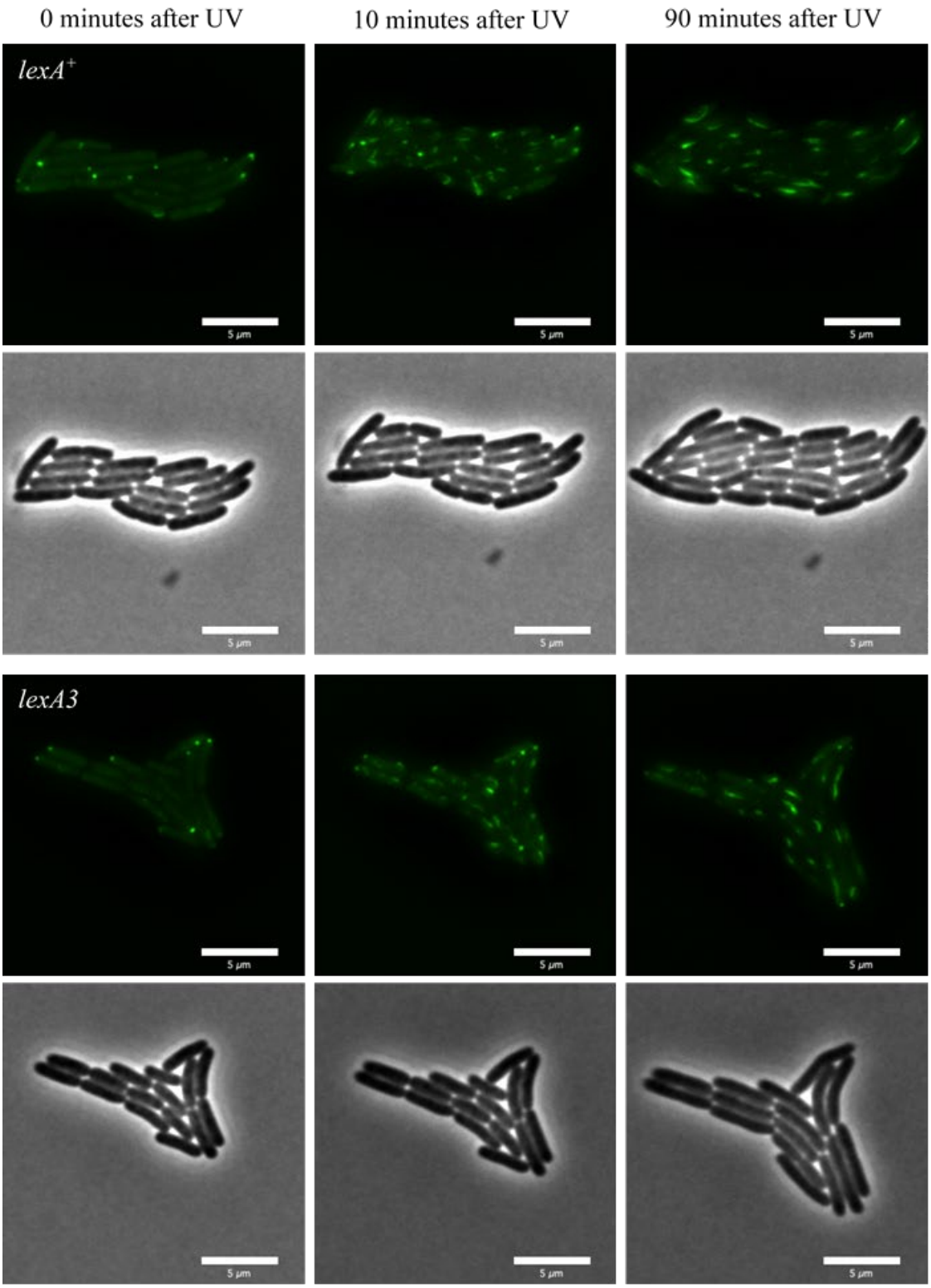


Figure 2.10: Fluorescent RecA structures in log phase *E. coli* with *recAo281* operator.

Visualization RecA-GFP structures in log phase *E. coli* cells. Each strain contains *ygaD1::kan recAo281 recA4155,4136::gfp* for visualization of recombination structures on the DNA. The top row is the fluorescent image of RecA-GFP. The bottom row is the phase contrast image. SS13338 is the *lexA*$^{+}$ strain. SS13339 is the *lexA3* strain. SS13341 is the *lexA2005* strain. In contrast to the strains with *recAo1403*, the strains with *recAo281* all have about the same number of RecA-GFP structures per area cell; however, the *lexA2005* strain has more linear structures than the *lexA*$^{+}$ strain (23% vs 4%).


0 minutes after UV
10 minutes after UV
90 minutes after UV
lexA+
lexA3
5 µm

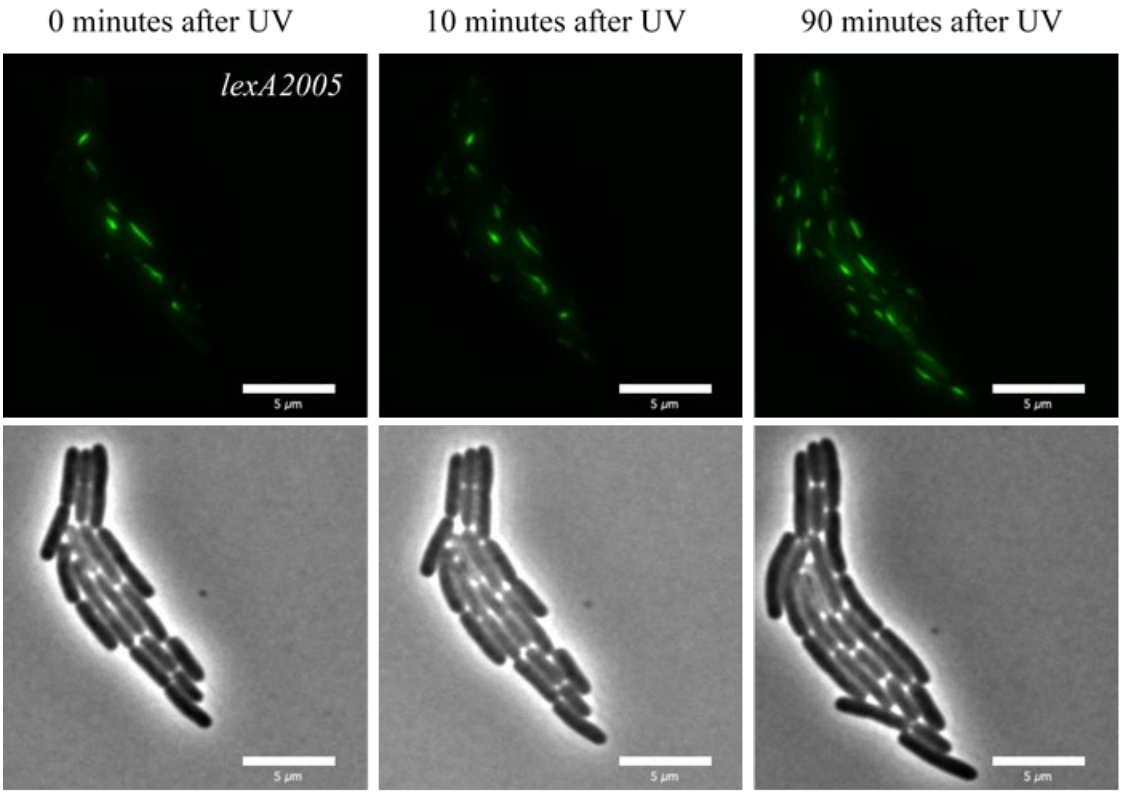


Figure 2.11: Timelapse of RecA-GFP structures after exposure to 10 J/m2 ultraviolet light in E. coli cells with recAo281 operator.

Visualization RecA-GFP structures in log phase *E. coli* cells before and after ultraviolet light irradiation. Each strain contains *ygaD1::kan recAo281 recA4155,4136::gfp* for visualization of recombination structures on the DNA. Images in the left column were taken immediately before ultraviolet light irradiation. Image in the middle column were taken ten minutes after irradiation. Images in the right column were taken ninety minutes after irradiation. The top, third and fifth rows are the fluorescent image of RecA-GFP for each strain. The second, fourth, and bottom rows are the phase contrast image for each strain. SS13338 is the *lexA*$^{+}$ strain. SS13339 is the *lexA3* strain. SS13341 is the *lexA2005* strain. All of the strains showed an increase in the number of RecA-GFP structures ten minutes after UV irradiation. The *lexA*$^{+}$ increased its percentage of linear structures with time. The *lexA3* and *lexA2005* strains saw a small shift away from the linear structures to the undecided and circular structures.

CHAPTER 3

POSITIVE CHARGES ARE IMPORTANT FOR THE SOS CONSTITUTIVE PHENOTYPE IN *RECA730* AND *RECA1202* IN *ESCHERICHIA COLI* K-12

Summary:

In *Escherichia coli* K-12, RecA binds to ssDNA created by DNA damage to form a protein-DNA helical filament that serves to catalyze LexA auto-proteolysis that induces the SOS response. SOS constitutive (SOS^C) mutations, *recA730* (E38K) and *recA1202* (Q184K), are both on the outside of the RecA-filament, opposite to the face that binds DNA. *recA730* (E38K) is also able to suppress the UV sensitivity caused by *recF* mutations. Both SOS^C expression and *recF* suppression are thought to be due to RecA730's ability to compete better for SSB-coated ssDNA than wild type. We tested whether other positively charged residues at these two positions would lead to SOS^C expression and *recF* suppression. We found that 5/6 positively charged residues were SOS^C and 4/5 of these were also *recF* suppressors. While other mutations at these two positions (and others) were *recF* suppressors, none were SOS^C. Three *recF* suppressors could be made moderately SOS^C by adding a *recA* operator mutation. We hypothesize two mechanisms for SOS^C expression: the first suggests that the positive charge at positions 38 and 184 attract negatively charged molecules that block interactions that would destabilize the RecA-DNA filament and the second causes more stable filaments through increases in mutant RecA concentration.

3.1 Introduction:

Escherichia coli K-12 induces the SOS response when the cell's DNA is damaged (Courcelle et al., 2001; Culyba et al., 2018; Little & Mount, 1982). This increases the level of transcription of approximately 40 genes that help to repair the DNA, increase the rate of mutagenesis and inhibit cell division. The signal for induction is ssDNA generated

as a result of DNA damage (Craig & Roberts, 1980; Sassanfar & Roberts, 1990). The regulation of the SOS response is governed by RecA and LexA. Both are members of the SOS regulon. LexA is a transcriptional repressor that binds to operator sites in the promoter regions of the SOS genes. RecA is the sensor for the SOS response. When bound with ATP, RecA adopts a high affinity conformation and binds (polymerizes) on ssDNA to create a protein-DNA helical filament ((Menetski & Kowalczykowski, 1985) and reviewed in (Cox, 2007; Kowalczykowski et al., 1994)). The upper and lower size limits of these filaments are not explicitly known, but are thought to range from tens to hundreds of RecA monomers. These RecA-ssDNA filaments serve two purposes. First, they act as a catalyst that increases the rate of LexA auto-proteolysis. This, in turn, decreases the level of LexA in the cell and induces the SOS response. Second, the filaments are key structures that initiate recombinational DNA repair.

The production of RecA-ssDNA filaments does not necessarily lead to induction of the SOS response. In log phase, about 15-20% of the cells have RecA filaments at any one time while about 1% are induced for the SOS response (Massoni et al., 2012; McCool et al., 2004; Renzette et al., 2005). The 15-20% group of cells are presumably using homologous recombination to fix broken replication forks and the 1% has suffered some damage that cannot be fixed by standard house-keeping mechanisms. Thus, the cell can distinguish between events of DNA damage that require the SOS response and events that do not. There are a number of *recA* mutants that express the SOS response in log phase cells when they should not. These are called SOS constitutive (SOS^{C}) mutants and how they induce the SOS response, when they should not, is the subject of this paper. Critical to this process (and normal SOS induction) is the length and stability of RecA

filaments. There are several proteins: DinI, DinD, RecX, RecOR and UvrD that affect the stability (both positively and negatively) of RecA-DNA filaments (reviewed in (Cox, 2007)). All but RecOR are all part of the SOS response.

Historically, the first *recA* constitutive mutant found was *tif-1* (for **t**hermo-**i**nducible **f**ilamentation) (Kirby et al., 1967). These cells filamented because, *sulA*, a cell division inhibitor gene, was induced as part of the SOS response (Bi & Lutkenhaus, 1990). Thus, initially only conditional RecA SOS constitutive (SOS^C) mutants could be found because constitutive expression of the SOS Response would lead to large amounts of SulA that would inhibit cell division (and thus colony formation). After much study, it as was found that *tif-1* had two missense mutations: E38K and I298V (Knight et al., 1984). The former was responsible for the SOS^C phenotype and the second was a temperature sensitive intragenic suppressor (W. B. Wang & Tessman, 1985). *tif-1* was renamed to *recA441* and the individual mutations were named *recA730* (E38K) and *recA4162* (I298V) (Long et al., 2009).

In a comprehensive review article McGrew and Knight compiled a list of many mutants of *recA* and some of their properties (McGrew & Knight, 2008). It is seen from this list that there are about 25 positions that can be mutated that lead to SOS^C expression. While the ability to polymerize on ssDNA is critical for SOS^C expression, the fact that several mutants are Rec^- reveals that RecA's other roles in recombination, that occur after the formation of the initial protein-DNA helical filament, are not required for SOS^C expression (Gruenig et al., 2008; Mirshad & Kowalczykowski, 2003; Nastri & Knight, 1994; Tessman & Peterson, 1985b). Mapping some SOS^c mutations on to the crystal structure of RecA reveals that they span the entire protein (Figure S1).

This paper focuses on two SOS^C mutations: *recA730* (E38K) and *recA1202* (Q184K) (Tessman & Peterson, 1985a; W. B. Wang & Tessman, 1986). The two residues identified by these mutations are both on a single surface of the RecA protein that is on the back side of the RecA filament as it is bound to ssDNA (Figure 3.1). It is easy to imagine that the many SOS^C mutations that map on the side of the protein that interacts with the DNA are SOS^C because they bind ssDNA better than wild type. It is less clear why these two positions, 38 and 184, on the backside of the protein, away from the DNA, allow for better ability to compete with SSB-coated ssDNA and SOS^C expression.

Why is *recA730* (E38K) able to bind to ssDNA when it should not and cause SOS^C expression? Biochemical characterization of this protein led to the finding that it is able to compete for SSB-coated ssDNA better than wild type RecA (Eggler et al., 2003; Lavery & Kowalczykowski, 1988, 1992) (and with faster kinetics in the absence of SSB (Lu et al., 2017)). It is also known that *recA730* (E38K) allows for indirect suppression of the UV^S caused by *recF* mutations (Volkert et al., 1984; Volkert & Hartke, 1984). A single hypothesis can explain both of these phenotypes: the mutant protein is better able to compete for SSB-coated ssDNA either at the replication fork or at gapped DNA after DNA damage than wild type RecA. One caveat to this hypothesis is that other mutations (*e.g., recA803* (V37M) found as an indirect suppressor to *recF*(*OR*) mutations (M. V. Madiraju et al., 1988; Volkert & Hartke, 1984)) also compete for SSB-coated ssDNA better than wild type RecA, but are not SOS^C. In a comparison, it was shown that RecA730 (E38K) is able to better compete for SSB-coated ssDNA than RecA803 (V37M), thus providing a reasonable explanation for why the former is SOS^C and the latter is not (Lavery & Kowalczykowski, 1992).

To better understand SOS^C expression for *recA730* (E38K) and *recA1202* (Q184K) we sought to address the following two questions. First, is the positive charge introduced by the above mutations critical for SOS^C expression or will other non-positively charged amino acids at these two positions suffice? We tested this by changing the amino acids at these two positions to other positively charged, negatively charged, just polar and hydrophobic amino acids. Second, is there a correlation between the ability to indirectly suppress the UV^S of *recF* mutations and the ability to be SOS^C. Our results show that there is a good correlation (5/6 mutations) between the introduction of a positive charge at either of these two positions and the ability to be SOS^C. There is also a good correlation between the ability to be SOS^C at these two positions and the ability to indirectly suppress *recF* mutations (4/5 SOS^C mutants were also *recF* suppressors). However, we also found that introduction of other mutations (non-positively charged, removal of negative charges, hydrophobic residues) in that region or in the C-terminus allow *recF* suppression, but do not cause SOS^C expression. Lastly, we found that three *recF* suppressor mutants that were not SOS^C by themselves, could be induced to be SOS^C, by the addition of a *recA* operator mutation (either *recAo281* or *recAo1403*). We suggest there are at least two mechanisms to induce SOS^C expression. One involves the introduction of a positive charge in the 38/184 region and the other requires two parts: a mutation that allows *recF* suppression and a mutation that increases the basal level of transcription (and thus concentration) of this mutant *recA* protein.

3.2 Results

This study focuses on two positions (38 and 184) of the RecA protein where the introduction of positive charges has led to SOS^C expression. The mutations at these two positions are *recA730* (E38K) and *recA1202* (Q184K) (see above). Since these two

mutations were not comparable in a direct sense because they were isolated at different locations on the chromosome (*recA730* (E38K) was at the *recA* locus and *recA1202* (Q184K) was on a λ transducing phage), we transferred all mutations in this study to the *recA* locus for characterization. Four assays were used to characterize the *recA* mutations. 1) The ability to constitutively express the SOS regulon as determined by a SOS reporter gene, *sulAp-gfp*, inserted at the lambda attachment site (*attB*) (McCool et al., 2004). SOS expression is expressed as the fold increase in fluorescence above background in Table 1. 2) Their survival to UV irradiation in a *recF*$^+$ strain. This is a combined measure of recombinational DNA repair function and the ability to induce the SOS response. This is expressed as the percent survival at a dose of 60 J/m^2 in Table 1. Full UV survival curves are shown in Figure S2. 3) Their survival to UV irradiation in a *recF4115* mutant is a measure of its ability to suppress the UV sensitivity of a *recF* mutation. It is reported in a similar way as the UV survival in a *recF*$^+$ strain. Table 1 also calculates the **E**fficiency **o**f *recF* **S**uppression (EoS) for each *recA* mutant. 4) The amount of RecA protein was determined by Western Blot using log phase cultures and is normalized to the amount found in wild type cells in Table 1. All of these experiments were also done in strains with a *sulB103* mutation that inhibits SOS-induced cellular filamentation.

3.2.1 All positive charges at position 38 lead to SOS^C expression.

We first tested if other positive charges at position 38 would lead to SOS^C expression. Table 1 shows that changing the glutamic acid residue to either lysine (*recA730*), arginine (*recA4184*) or histidine (*recA4187*), all led to high levels of SOS^C expression. The levels of SOS^C expression for *recA730* (E38K) and *recA4184* (E38R) were virtually identical and the level for *recA4187* (E38H) was about 12% less. The levels of mutant RecA protein correlated with the level of SOS expression (Table 3.1). It

is possible that while all of these mutations had SOS^C expression, some might have defects in normal RecA function. To test this, we measured the UV survival of strains carrying these mutations. Table 1 showed that strains with *recA730* (E38K) or *recA4187* (E38H) had slightly higher levels of UV survival than the wild type strain. *recA4184* (E38R), however, had 50-fold less survival after UV treatment than wild type indicating that while the mutation allowed SOS^C expression, it interfered with some other aspect of RecA function needed during recombinational DNA repair. Lastly, we tested the ability of the *recA* allele to indirectly suppress a *recF4115* mutation. *recF4115* is a well characterized *recF* mutation that has no measurable *recF* activity, but still produces a *recF* protein (Sandler, 1994). This test was accomplished in two stages. The first step combined the *recA* and *recF* mutations into a single strain and the second measured the UV survival of that strain. This number was then used to calculate the "Efficiency of Suppression" (EoS). Table 3.1 shows that *recF* derivatives of *recA730* (E38K) and *recA4187* (E38H) had a level of UV survival that was about 50-fold greater than wild type and this led to an EoS of about 50%. As expected, *recF4115 recA4184* (E38R) double mutant was about 500-fold more UV sensitive than wild type and led to an EoS of -83%.

We then placed five other mutations on the chromosome that changed the amino acid at position 38 to an aspartic acid (*recA4200*), glutamine (*recA4186*), asparagine (*recA4201*), serine (*recA4185*) and alanine (*recA4199*). None of these changes produced a strain with SOS^C expression. All showed levels of mutant RecA protein that were about equal to wild type levels (Table 3.1). With the exception of *recA4200* (E38D), all of the other four strains were approximately as UV resistant as wild type. *recA4200* (E38D)

was about 2-fold less UV^R and all but *recA4200* (E38D) and *recA4199* (E38A) had substantial EoS in the range of 34% to 57%. *recA4199* (E38A) had an EoS about like wild type (-1%). Interestingly, *recA4200* (E38D) *recF4115* strain was much more UV sensitive than either of the single mutants suggesting that this *recA* mutant had an additional defect. It had an EoS of -60% and will be discussed more below.

From these experiments, we conclude that a positive charge at position 38 will lead to SOS^C expression, but that it does not necessarily lead to *recF* suppression although there is a high correlation between the two. Other types of mutations at this position do not allow SOS^C expression, but often allow *recF* suppression.

3.2.2 Some positive charges at position 184 allowed SOS^C expression.

We then applied this methodology to the study of charges at position 184. Our reference mutation is *recA1202* (Q184K). It introduces a positive charge and is close to position 38 in three-dimensional space according to the crystal structure. As mentioned above, this mutant was isolated on a λ specialized transducing phage. Therefore, we first transferred this allele to the *recA* locus on the chromosome and characterized it for SOS^C expression, UV survival and *recF* suppression. Like *recA730* (E38K) we found that *recA1202* (Q184K) was SOS^C. The level was slightly higher (about 10%) than *recA730* (E38K) and one saw a corresponding amount of RecA produced using Western Blots. It was about as UV^R and had about the same ability to suppress the UV sensitivity of *recF* mutations (EoS) as *recA730* (E38K). We then changed the amino acid at this position to arginine (*recA4204*) and histidine (*recA4205*). Table 1 shows that *recA4204* (Q184R) was highly SOS^C, but that *recA4205* (Q184H) was not. Once again, the amount of RecA produced was commensurate with the amount of SOS expression. Both alleles were fairly

UV^R (*recA4205* (Q184H) a little more UV^R than wild type and *recA4204* (Q184R) had a little less UV^R than wild type) and both had the ability to indirectly suppress the UV sensitivity caused by *recF* mutations. Changing the amino acid at position 184 to glutamic acid (*recA4206*) or asparagine (*recA4207*) did not lead to SOS^C expression and had differential effects on the ability of *recA* to repair DNA and suppress the UV sensitivity caused by *recF* mutations. *recA4206* (Q184E) was more UV^S than wild type and had a poor ability to suppress *recF* mutations while *recA4207* (Q184N) was very similar to wild type in both regards.

From these experiments, we conclude that a positive charge at position 184 always leads to *recF* suppression, but only leads to SOS^C expression in 2/3 cases. For the other mutations tested at this position, none allow SOS^C expression or *recF* suppression.

3.2.3 A positive charge at position 36 does not cause SOS^C expression, but does allow *recF* suppression.

The positions at 38 and 184 are part of a larger surface that includes positions 36 and 37. Position 37 contains a nonpolar valine residue and is the site of the *recA803* (V37M) mutation (M. V. Madiraju et al., 1988). This allele has been characterized elsewhere (Volkert & Hartke, 1984) and is shown in Table 3.1 to have 40% lower than wild type levels of RecA, but is as UV resistant as wild type. Hence the decrease in RecA levels does not affect its abilities in DNA repair. Table 3.1 also shows *recA803* (V37M) not to be SOS^C but is able to suppress the UV sensitivity of *recF* mutations (Volkert & Hartke, 1984). Position 36 has a negatively charged aspartic acid residue. Its orientation is similar to amino acids at positions 38 and 184 in that it points away from the surface. We therefore tested whether introducing a positive charge, a lysine (*recA4197* (D36K)), at this position would result in SOS^C expression. Table 3.1 shows it does not. It does,

however, allow the RecA protein to provide UV resistance like wild type even though its RecA levels are about 20% lower than wild type. We note it has some ability, more than wild type, but less than *recA730* (E38K), to indirectly suppress the UV^S caused by *recF* mutations. From these experiments, we conclude that a change of a negative to positive charge, a lysine, at position 36 does not lead to SOS^C expression, but does allow *recF* suppression.

3.2.4 Deletion of the last 17 amino acids (*recA4161* (CΔ17)) of RecA does not allow SOS^C expression, but does allow *recF* suppression.

The C-terminus of *recA* has a preponderance of negatively charged residues. It has been shown that the deletion of the last 17 amnio acids of *recA*, removing seven negative charges, results in a protein that can displace SSB from ssDNA better than wild type *in vitro* like RecA730 (E38K) (Eggler et al., 2003). Therefore, to test its ability to produce the SOS^C phenotype, we transferred this mutation to the *recA* locus on the chromosome. Table 3.1 shows that it has no SOS^C activity. Its survival to UV irradiation is like wild type and its ability to indirectly suppresses *recF4115* was better than *recA730* (E38K) even though its level of RecA is about 50% lower than wild type. It was concluded that removal of the last 17 amino acids of RecA, removing seven negative charges, did not cause SOS^C expression, but did result in high levels of *recF* suppression.

3.2.5 Adding *recA* operator mutations increased SOS^C expression of some mutants that allowed *recF* suppression.

It has been shown that overproduction of RecA protein in cells does not lead to SOS^C expression (Uhlin & Clark, 1981). This previous work was done both with a plasmid overexpressing RecA and with *recA* operator mutations that overexpressed *recA* from the chromosome (Ginsburg et al., 1982). In agreement with this previously published work, Table 3.2 shows that the addition of *recA* operator mutations, *recAo1403*

(Wertman & Mount, 1985) and *recAo281* (Uhlin et al., 1982; Volkert et al., 1981), that increase the basal level of transcription 2-3 fold and 10-fold resulting in a 3.4 and 9.6 fold increase in RecA respectively, did not result in any increase in SOS^C expression (Ginsburg et al., 1982; Long et al., 2008). It has also been shown that *recAo*$^+$ *recA4142* (F217Y) had no SOS^C expression (Long et al., 2008). However, when *recAo1403* was added, *recA4142* (F217Y) yielded SOS^C expression in a range that was nearly equivalent to a strain containing *recA730* (E38K) (Long et al., 2008). From these results, it can be hypothesized that some other attribute of RecA must be modified in addition to increased transcription to result in SOS^C expression. It was speculated that *recA* mutants with a high EoS, but low SOS^C expression, would then show higher SOS^C expression if coupled with a *recA* operator mutation like *recA4142* (F217Y).

We first tested if the addition of *recAo1403* would increase the SOS^C expression of *recA4197* (D36K). Table 3.2 show that the addition of *recAo1403* resulted in about a 9 to 10-fold increase in SOS^C expression with an 12 fold increase in the amount of RecA produced. Table 3.2 also shows that the addition of *recAo1403* to *recA4161* (CΔ17) also yielded an increase, approximately 7-fold, in SOS^C expression with a 5-fold increase in the amount of RecA produced. Lastly, we tested if the addition of *recAo1403* to *recA803* (V37M) would yield an increase in SOS^C expression. Table 3.2 shows that with a 7-fold increase in the amount of protein produced, no increase in SOS^C expression was seen. It was then tested if increasing the basal level of expression even more with *recAo281*, would lead to an increase SOS^C expression. Table 2 shows that combining *recAo281* with *recA803* (V37M) has a small, 2 to 3-fold increase in SOS^C expression compared to wild type even though the amount of protein was increased 16-fold. We conclude that

increasing the basal level of transcription and protein production of some *recA* mutants that show the ability to indirectly suppress the UV sensitivity of *recF4115* is a viable strategy to increase the level of SOS^C expression. The results, however, are variable and do not reach the level of SOS^C expression of mutants like *recA730* (E38K) or *recA1202* (Q184K).

3.2.6 Efficiencies of SOS induction after UV irradiation of the non-SOS^C *recA* mutations.

It is possible that the reason why some of the *recA* mutations that did not yield SOS^C expression is that they were deficient in SOS induction. This could be due to a deficiency in binding to ssDNA and or an inability to accelerate cleavage of LexA. This seemed unlikely for most of the *recA* mutants as they were shown to be fully UV resistant and some were additionally able to indirectly suppress the UV sensitivity caused by *recF* mutations (Table 3.1). There was one, however, *recA4206* (Q184E) that was fairly UV sensitive as a single mutant. To test if SOS induction was impaired in any of these mutants, we measured the levels of SOS induction in all non-SOS^C *recA* mutants. Table 3.3 shows that all mutants were able to induce SOS expression to some degree. Most were able to induce SOS within 30% of wild type levels. Only *recA4206* (Q184E) showed levels that were decreased by 66% relative to wild type. Thus, the hypothesis that the *recA* mutants that were not SOS^C because they could not induce the SOS response is not supported. This conclusion is tempered by the observation that some of the mutants were mildly perturbed in their ability to induce SOS after UV irradiation.

3.3 Discussion

In this paper we tested whether introduction of positive charges on a surface of the RecA protein defined by positions 38 and 184, are important for SOS^C expression and

whether these changes correlate with the ability to indirectly suppress the UV^S of *recF* mutations. There is a high degree of correlation for both. 5/6 of the introduced positive charges led to SOS^C expression and 4/5 of those mutations also led to the ability to suppress *recF* mutations. Thus in 4/6 cases the mutants showed both high SOS^C expression and *recF* suppression. The one case where the SOS^C mutant was not also a *recF* suppressor, *recA4184* (E38R), is likely exceptional because the mutation affected normal RecA activity (the strain is very UV^S in a $recF^+$ background) and so it was not able to suppress the UV sensitivity of *recF* mutations. Another exception was *recA4205* (Q184H). Here the introduced positively charged amino acid did not provide SOS^C expression, but it did provide *recF* suppression. While the reason for this is not clear, it may be due to the possibility that the histidine, an aromatic amino acid, was not sufficiently protonated at the cellular pH or it is not optimally oriented for the SOS^C phenotype. Several mutations at the 38/184 positions and other positions tested did lead to *recF* suppression, but not to SOS^C expression. Taken together, these results suggest that while many types of mutations seem to be able to lead to *recF* suppression, something else is necessary for the mutation to also lead to SOS^C expression.

There have been several mechanisms postulated to explain SOS constitutive expression. These mechanisms are often thought of as deviations of the processes that wild type RecA goes through to induce SOS expression. The mutants potentiate some aspect of this process in log phase, undamaged cells when they should not and are thus SOS^C. As mentioned above, the ability to bind ssDNA at the replication fork on the exposed lagging strand template is one mechanism. If, the ability to suppress the UV sensitivity of *recF* mutations is a good proxy for the ability to bind SSB-coated ssDNA

better than wild type, then it is tempting to speculate that 4/6 mutations that were both SOS^C and *recF* suppressors have better ability to compete for SSB-coated ssDNA. One needs to temper this thought, however, with the knowledge that SOS^C expression also leads to higher concentrations of RecA in the cell and many other DNA repair proteins (because the SOS response is induced). Hence, the mechanism of *recF* suppression in SOS^C strains may be different from strains with mutations like *recA803* (V37M) that are not SOS^C. A second mechanism for SOS^C expression has been proposed based on the structure of RecA in crystals. This hypothesis suggest that there may be higher order structures involving associations between multiple RecA filaments and this is the key structure that leads to SOS^C expression (Liu et al., 1993). A third mechanism is suggested by the ability of *recA730* to directly suppress the SOS expression defects of a RecA ATPase mutant (*recA2201* (K72R)) (Gruenig et al., 2008). This may involve the ability of RecA730 to adopt an extended conformation and produce longer filaments. In none of these models is a positive charge a critical part of the model. Therefore, below we suggest a new model for SOS^C mutants where a positive charge at the 38/184 positions is a critical part of the mechanism.

Remembering that the 38/184 surface lies on the opposite face to the one that bind ssDNA, we suggest that the positive charge at positions 38/184 is important for an interaction with a negatively charged molecule that then sterically blocks other interactions that would normally destabilize the RecA-ssDNA filament preventing SOS expression. These other interactions could be an interaction with some other part of the RecA protein or other proteins (possibly RecX, DinI, DinD, RecOR or UvrD). Supporting this idea is that *recA730* SOS^C expression is independent of *recBCD*, *recF*

and *dinI* whereas *recAo1403 recA4142* SOS^C expression is either completely or partially dependent on *recBCD*, *recF* and *dinI* (Long et al., 2008). We further suggest that the specific identity of the negatively charged molecule may not be important as long as it blocks the interaction. It is possible that just the positive charge alone is sufficient to block this interaction. We think this less likely, however, since the charges or sizes of other residues tested that would have likely sterically inhibited binding of this unidentified interaction, do not cause SOS^C expression. If this positive charge hypothesis is true, then it is possible that the surface in the RecA filament defined by 38/184 may be important for the wild type RecA protein as a site for interactions that regulate filament stability.

A second group of mutants have the ability to suppress the UV^S of *recF* mutations, but they produce no SOS^C expression under normal levels of transcription (*recAo*$^+$). They only show SOS^C expression if the basal level of transcription is increased by either a *recAo1403* or *recAo281* mutation. This group includes *recA4142* (F217Y), *recA4197* (D36K), *recA4161* (CΔ17) and *recA803* (V37M). For this group, the explanation of SOS^C expression is that the increased *recA* transcription leads to higher concentrations of mutant RecA in the cell and mass action leads to more RecA filaments or more stable RecA filaments. These in turn lead to a lower LexA concentration. The increase in RecA concentration is also likely to be concurrent with an increase in RecX concentration since the two genes are co-transcribed. The increase in RecA transcription is independent of other SOS genes like *dinI*, *dinD* and *uvrD* that are known to affect RecA filament stability. It is clear, however, that in cells where the population has achieved high levels of SOS^C expression, that the amounts of RecA, RecX, DinI, DinD

and UvrD all increase and that their balance is critical for whether the cell remains with high SOS^C levels or decreases back to wild type levels.

It is interesting that 11/16 mutations used in this study led to suppression of the UV^S caused by *recF* mutations given that these were constructed more so to test for their ability to produce SOS^C expression than *recF* suppression. It is known that mutations elsewhere in the protein also cause *recF* suppression (T. C. V. Wang et al., 1991). The ability to better compete for ssDNA is still an attractive idea for why these types of mutant *recA* proteins may indirectly suppress *recF* mutations. The fact that many different types of changes and locations can become *recF* suppressors suggests that there may be multiple ways to improve the ability to better compete for SSB-coated ssDNA.

One mutant, *recA4200* (E38D), was very UV sensitive when combined with *recF4115*. This was unexpected, as it is nearly as UV resistant as wild type (down about 2-fold) and had about 80% normal SOS activity as a single mutant. It would seem to be different than *recA4185* (E38R) that was very UV sensitive and deficient in SOS expression as a single mutant. *recA4200* (E38D) was, however, very UV sensitive when combined with *recF4115*. This suggests that it not only does not have the ability to indirectly suppress the UV sensitivity of *recF* mutants, but may be deficient in some activity. Perhaps this activity is used the RecBCD pathway. This pathway is the only other active recombination pathway that is available in a *recF* mutant in these cells.

The surface defined by positions 36, 37, 38 and 184 is along the outside of the RecA-DNA filament, opposite to the surface that binds ssDNA. No studies have yet identified a role for this surface in RecA's normal roles in recombination and induction of the SOS response. This work shows that there is a high degree of correlation between the

introduction of mutations on this surface with SOS^C expression and *recF* suppression. Further work will be need to define a role for this surface in normal RecA function and how mutations in this region lead to these specific mutant phenotypes.

3.4 Materials and Methods

3.4.1 Bacterial strains and growth conditions.

All bacterial strains are derivatives of *E. coli* K-12 and are characterized in Table S1. Strains were generated using either linear transformation (Datsenko & Wanner, 2000b) or P1 transduction (Willetts & Clark, 1969). Transformants and transductants were selected on 2% agar plates containing either Luria broth (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) or 56/2 minimal medium supplemented with 0.2% glucose, 0.001% thiamine, 0.02% arginine, 0.005% histidine, 0.02% proline, 0.01% leucine, 0.01% threonine (Willetts & Clark, 1969). When appropriate, ampicillin was used at 50 mg/ml, chloramphenicol at 25 mg/ml, kanamycin at 50 mg/ml, and tetracycline at 10 mg/ml. The cells were purified on the same type of media on which they were selected and grown at 30° or 37°. L-arabinose was used for induction of λ Red expression from pKD46 at a final concentration of 0.5% (w/v).

3.4.2 Plasmid construction and transfer of *recA* alleles onto the chromosome.

DNA oligonucleotide primers and plasmids are described in Tables S2 and S3, respectively. The mutations at position 36 and 38 were generated using mutagenic primers described in Table S2. Using wildtype genomic DNA as a template, the mutations were generated using PCR with the mutagenic oligonucleotides and prSJS508. These fragments with the mutations were then cloned into pNR117 (Long et al., 2009). For mutants at position 38, both the PCR fragments and the plasmids were digested with *Nco*I and *Pme*I and for position 36 the fragment and plasmid were digested with *Sac*II and *Rsr*II. Properly digested fragments and plasmids were treated with T4 ligase and used to transform competent cells. Mutant *recA* genes were identified by restriction site polymorphisms. The mutations at position 184 were constructed using fragments

synthesized by Twist Biosciences. These fragments were PCR amplified using prSJS453 and prSJS508. Both the fragments and pNR117 were digested with *Rsr*II and *Pme*I. The fragments also contain a silent mutation generating a *Hind* III site used for screening successful clones. Ligation mixtures were used to transform SS10168. Kanamycin and ampicillin-resistant colonies were screened for restriction site polymorphisms. *recA* mutations were transferred to the chromosome using λ Red recombination method (Datsenko & Wanner, 2000b). The plasmids were linearized by digestion with *Bam* HI and *Bsa*I enzymes followed by PCR with prSJS816 and prSJS1096. Column purified DNA fragments were used to transformed SS10191 electrocompetent cells expressing λ Red recombinase from the pKD46 plasmid. Successful transformants were selected on LB-Kan, screened for Amp sensitivity and the presence of a *recA* gene with prSJS507 and prSJS508. Presence of the correct *recA* mutations were verified by DNA sequencing. To combine the *recAo1403* mutation with *recA4197* (D36K), a DNA fragment containing *ygaD::cat recAo1403* was generated by PCR with prSJS816 and prSJS1719 using SS6060 as template. SS13841 was transformed with pKD46 to make SS13238. This strain was used to transform a DNA fragment encoding *ygaD::cat recAo1403* DNA. Chloramphenicol resistant clones were selected. These were screened for Kan sensitivity and one was saved and named SS13244. To construct the *recAo281 recA803* double mutant, SS12727 was used as a template using oligos prSJS816 and prSJS1719 to amplify *ygaD1::kan recAo281*. This PCR product was then used to transform SS6095 (*recA803* and pKD46). Kanamycin resistant clones were selected. The presence of the *recAo281* operator mutation was screened for by PCR using prSJS1225 and prSJS508 followed with digestion by *Sph*I to test for addition of *Sph*I site in *recAo281*. One of these

was saved and named SS13900. All *recA* mutations were then transduced into SS996 and SS1426 for characterization.

3.4.3 Preparation of cells for microscopy.

Cells were grown in 56/2 minimal media overnight at 37°C with shaking. 200µl of the overnight culture was diluted into 3 ml of 56/2 minimal media and grown for 3 hours at 37°C with shaking into early log phase. 3–5 µl of the log phase culture was loaded onto a 2% agarose pad prepared from 56/2 minimal medium and low-melting agarose. Placing a coverslip on top the inoculated agarose pad, the slides were incubated for 3–4 hours at 37°C before imaging. Cells were visualized using a Nikon E600 microscope equipped with automated filter wheels, shutters, Cool LED light source and an ORCA-ER camera. Phase contrast and fluorescence images were taken for at least 18 different fields of view (six fields on three different days) under total magnification of 600x. Number of cells analyzed for each strain ranged between 2,000 and 4,000 cells.

3.4.4 Analysis of microscopic images.

Micrographs were analyzed with the following software: I-Vision (BioVision Technologies), SuperSegger (Stylianidou et al., 2016), and MATLAB R2016a and MATLAB R2019a (MathWorks). Individual cells were outlined using SuperSegger. Strains were analyzed for number of cells and fluorescent intensity using specially written MATLAB programs. Statistical analysis of average relative fluorescence intensity (RFI) was performed with Student's T-test.

3.4.5 UV survival assay.

The strains were grown in LB broth overnight at 37°C in shaking water bath. 200 µl of the cultures was diluted in 10 ml LB broth and grown for 3 hours into log phase at

37°C with shaking. At the end of 3 hours, the cells were harvested by centrifugation and resuspended in 10 ml of 56/2 buffer. Cells were transferred to a sterile 10 mm petri dishes and irradiated by UV light from two General Electric 15W germicidal lamps at a rate of 1 J/m^2/sec. 1 ml aliquots were taken at time intervals and serially diluted in 56/2 buffer. 5 µl of each dilution were spotted in duplicate on LB agar and incubated overnight at 37°C and then colonies were counted. Survival at a certain dose was measured as a ratio between the number of colony forming units in the radiated sample and nonirradiated control. Efficiency of Suppression (EoS) has been described elsewhere (Volkert & Hartke, 1984). Briefly, the EoS (%) =100*(1-(log(fraction surviving of strain with suppressor)/log(fraction surviving of strain without suppressor))). This number is expressed as a percentage. Each experiment was repeated three times. Confidence intervals are standard error.

3.4.6 Western Blots.

Overnight cultures were grown in LB at 37°C in shaking water bath. 200 µl of overnight culture was diluted into 10 ml of LB for log phase culture. Cells were grown in LB to a OD_{600} = 0.2 - 0.3. Two ml of cells normalized to OD_{600} = 0.2 were spun down and resuspended in 74 µl 1X TE buffer (10mMTris-HCl 1mM EDTA). To this 1 µl of 100x PMSF protease inhibitor was added. 25 µl of 4x Laemmli buffer from BIO-RAD prepared with 355mM Beta-mercaptoethanol for a final volume of 100 µl. For *recA* that were highly expressed, the samples were diluted 1:4 in crude lysate from a Δ*recA* strain. Samples were boiled for 10 minutes immediately before loading SDS-PAGE. 20 µl were run on a 4%-20% gradient polyacrylamide gel at 120V for 60 minutes. Protein was transferred to PVDF membranes using BIORAD Trans-Blot Turbo Transfer system at 1.3 Amps and 25V for 10 minutes. Membranes were blocked for 1 hour with 5% milk in

TBST (50mMTris-HCL 150mMNaCl with 0.2% Tween 20). Commercially available primary antibodies (Abcam) were incubated overnight in 5% milk in TBST diluted 1:10,000. Commercially available secondary antibodies (Li-Cor) were incubated for 1 hour in 5% milk in TBST diluted 1:10,000. Membranes were imaged using Li-Cor Odyssey CLx Imaging System. Images were quantitated using ImageJ. Band intensities of RecA were normalized to a loading control. Given values for RecA protein amounts are in terms of fold change in relation to wildtype RecA.

3.4.7 Determination of SOS level after UV irradiation.

Overnight cultures were grown in 56/2 minimal media at 37ºC in a shaking water bath. 667 µl of overnight culture was diluted into 10 ml of 56/2 minimal media. The log phase culture was grown at 37ºC for three hours. The culture was divided into two 5 ml aliquots. One aliquot was placed in a petri dish and irradiated at a dose of 20 J/m^2. The other aliquot served as an unirradiated control. Both irradiated and unirradiated aliquots were transferred to tubes and allowed to grow at 37ºC in a shaking water bath for 90 minutes. At the end of the 90 minutes, 3 µl of culture was placed on 2% low melting agarose pads and incubated at 37ºC for 30 minutes before imaging. Images were captured using 600X total magnification. 3-6 fields of view were captured for three replicates. Total number of cells for each strain was between 1000 and 2000 cells. Images were processed and fluorescent intensity was measured as described above.

3.5 Tables

Table 3.5.1

Summary of characteristics of *recA* mutations studied

Allele and position of mutation tested	Character of changed aa	SOS [a]	Rel. Amt. RecA [b]	% UV Survival at 60 J/m^2		Efficiency of suppression [c]
				recF$^+$	*recF4115*	
wt		2	1	15 ± 3	0.05 ± 0.01	0
recA730 (E38K)	Positive	86	9.8 ± 0.4	23 ± 1	2 ± 0.4	49 ± 3
recA4184 (E38R)	"	85	10.6 ± 0.6	0.3 ± 0.03	0.0001 ± 0.00006	-83 ± 9
recA4187 (E38H)	"	75	11.0 ± 0.8	22 ± 2	3 ± 1	55 ± 5
recA4200 (E38D)	Negative	2	0.8 ± 0.1	8 ± 1	0.0006 ± 0.0001	-60 ± 7
recA4186 (E38Q)	Polar	2	1.1 ± 0.2	13 ± 6	0.7 ± 0.1	34 ± 3
recA4201 (E38N)	Polar	2	1.1 ± 0.1	20 ± 4	2 ± 0.5	44 ± 4
recA4185 (E38S)	Polar	3	1.0 ± 0.1	22 ± 4	4 ± 0.4	57 ± 2
recA4199 (E38A)	Nonpolar	2	1.0 ± 0.1	21 ± 9	0.05 ± 0.04	-1 ± 10
recA1202 (Q184K)	Positive	96	12.3 ± 0.7	56 ± 21	5 ± 1	60 ± 3
recA4204 (Q184R)	"	74	8.7 ± 0.1	5 ± 2	2 ± 0.5	47 ± 4

recA4205 (Q184H)	"	2	1.2 ± 0.1	27 ± 4	1 ± 0.3	37 ± 5
recA4206 (Q184E)	Negative	2	1.1 ± 0.1	0.04 ± 0.008	0.00005 ± 0.00005	-80 ± 6
recA4207 (Q184N)	Polar	2	1.2 ± 0.1	24 ± 9	0.1 ± 0.05	4 ± 9
recA4197 (D36K)	Positive	3	0.8 ± 0.1	14 ± 2	0.3 ± 0.1	23 ± 6
recA803 (V37M)	Nonpolar	2	0.6 ± 0.1	17 ± 5	0.5 ± 0.2	30 ± 7
recA4161 (ΔC17)	See footnote[d]	2	0.5 ± 0.1	18 ± 1	6 ± 1	63 ± 3

[a] SOS expression was measured using a *sulAp-gfp* transcriptional reporter inserted into the λ attachment site (McCool et al., 2004). SOS expression was quantified as an average relative fluorescence intensity (RFI) of pixels for the entire population of cells normalized to the average fluorescence intensity of the background of the images. Statistical analysis was done using the Student's T-test. Values that are different by more than 5 have a *p* value less than 0.001.

[b] The relative fold change is a measure of the amount of RecA protein relative to the wildtype strain. Western blots were performed as described in Materials and Methods. Each value is the average of three biological replicates. Confidence intervals are standard error of the mean. A strain carrying a *recA* deletion was undetectable in this assay.

[c] Efficiency of suppression has been described elsewhere(Volkert & Hartke, 1984).Briefly, efficiency of suppression is calculated as (in percent) =100*(1-(log(fraction surviving of strain with suppressor)/log(fraction surviving of strain without suppressor))) Values were taken at 60 J/m^2 point.

[d] The amino acid sequence that is deleted is DFSVDDSEGVAETNEDF.

Table 2

The effect of *recA* operator mutations on the properties of some *recA* mutations that indirectly suppress the UV sensitivity of *recF* mutations [a]

recAo	*recA*	SOS	Relative Protein
+	+	2	1
1403	+	2	3.4 ± 0.2
281	+	2	9.6 ± 0.5
+	*4197* (D36K)	3	0.8 ± 0.05
1403	*4197* (D36K)	29	9.4 ± 0.5
+	*4161* (ΔC17)	2	0.5 ± 0.1
1403	*4161* (ΔC17)	14	2.6 ± 0.2
+	*803* (V37M)	2	0.6 ± 0.1
1403	*803* (V37M)	2	4.3 ± 0.6
281	*803* (V37M)	5	9.6 ± 0.4

[a] Same as Table 3.1.

Table 3

The effect of non-constitutive SOS *recA* mutations to SOS Induction after UV-irradiation

		Relative SOS [a]	
recA	UV	-	+
+		2	38
del		1	1
recA803 (V37M)		1	27
recA4161 (Δ17)		1	32
recA4201 (E38N)		2	41
recA4200 (E38D)		2	30
recA4199 (E38A)		2	36
recA4186 (E38Q)		2	32
recA4197 (D36K)		2	31
recA4185 (E38S)		2	26
recA4207 (Q184N)		1	27
recA4206 (Q184E)		2	14
recA4205 (Q184H)		1	31

[a] SOS expression was measured using a *sulAp-gfp* transcriptional reporter inserted into the λ attachment site. SOS expression was quantified as an average relative fluorescence intensity (RFI) of pixels for the entire population of cells normalized to the average fluorescence intensity of the background of the images. Statistical analysis for the populations was done using the Student's T-test. Values that are different by more than 3 have a *p* value less than 0.01.

Table 3.4: Strains used in this study

Strain	*recA*	*recF*	Other relevant genotype	Source or derivation
SS996	+	+		McCool et al. 2004
SS1426	+	*4115*	*tna300::Tn10*	McCool et al. 2004
SS4629	*recA730* (E38K)	+		Long et al. 2008
SS4645	*recA730* (E38K)	*4115*	*tna300::Tn10*	Long et al. 2008
SS4656	*recA803* (V37M)	+	*srlC300::Tn10*	Laboratory stock
SS6061	*recAo1403 4161* (ΔC17)	+		Laboratory stock
SS6067	*recAo1403 4161* (ΔC17)	*4115*	*tna300::Tn10*	Laboratory stock
SS6088	*recAo1403*	+		Long et al. 2008
SS6131	*recAo1403 803* (V37M)	+		Laboratory stock
SS6133	*recAo1403 803* (V37M)	*4115*	*tna300::Tn10*	Laboratory stock
SS6428	*recA4161* (ΔC17)	+		Laboratory stock
SS10168	*ΔrecA200::frt*	+	*lexA3 malE::Tn10-9 (cam)*	Laboratory stock
SS10191	*ΔrecA200::frt*	+	pKD46 *lexA3 malE::Tn10-9 (cam)*	Laboratory stock
SS13218	*recA4207* (Q184N)	*4115*	*tna300::Tn10*	SS13883→SS1426[a]
SS13219	*recA4206* (Q184E)	*4115*	*tna300::Tn10*	SS13884→SS1426[a]
SS13220	*recA4205* (Q184H)	*4115*	*tna300::Tn10*	SS13885→SS1426[a]
SS13238	*recA4197* (D36K)	+	pKD46	SS13841[d]
SS13253	*recAo1403 recA4197* (D36K)	+		SS13244→SS996[b]
SS13254	*recAo1403 recA4197* (D36K)	*4115*	*tna300::Tn10*	SS13244→SS1426[b]
SS13261	*recA803* (V37M)	*4115*	*srlC300::Tn10 tnaA::miniTn5 cam*	MVM10→SS4645[c]

SS13263	*recA4161* (ΔC17)	*4115*	*tna300::Tn10*	SS6422→SS1426 [a]
SS13264	*recAo1403*	*4115*	*tna300::Tn10*	SS6087→SS1426 [a]
SS13265	*recA4185* (E38S)	*4115*	*tna300::Tn10*	SS13359→SS1426 [a]
SS13364	*recA4201* (E38N)	+		SS13356→SS996 [a]
SS13365	*recA4184* (E38R)	+		SS13357→SS996 [a]
SS13369	*recA4200* (E38D)	+		SS13358→SS996 [a]
SS13373	*recA4187* (E38H)	+		SS13161→SS996 [a]
SS13374	*recA4187* (E38H)	*4115*	*tna300::Tn10*	SS13161→SS1426 [a]
SS13376	*recA4201* (E38N)	*4115*	*tna300::Tn10*	SS13356→SS1426 [a]
SS13377	*recA4184* (E38R)	*4115*	*tna300::Tn10*	SS13357→SS1426 [a]
SS13378	*recA4200* (E38D)	*4115*	*tna300::Tn10*	SS13358→SS1426 [a]
SS13835	*recA4199* (E38A)	+		SS13821→SS996 [a]
SS13836	*recA4186* (E38Q)	+		SS13822→SS996 [a]
SS13841	*recA4197* (D36K)	+		SS13827→SS996 [a]
SS13859	*recA4197* (D36K)	*4115*	*tna300::Tn10*	SS13827→SS1426 [a]
SS13860	*recA4186* (E38Q)	*4115*	*tna300::Tn10*	SS13822→SS1426 [a]
SS13861	*recA4199* (E38A)	*4115*	*tna300::Tn10*	SS13821→SS1426 [a]
SS13862	*recA4185* (E38S)	+		SS13359→SS996 [a]
SS13867	*recA1202* (Q184K)	+		SS13866→SS996 [a]
SS13869	*recA1202* (Q184K)	*4115*	*tna300::Tn10*	SS13866→SS1426 [a]
SS13881	*recA4204 (*Q184R)	+		SS13844→SS996 [a]
SS13882	*recA4204 (*Q184R)	*4115*	*tna300::Tn10*	SS13844→SS1426 [a]
SS13893	*recA4207* (Q184N)	+		SS13883→SS996 [a]
SS13894	*recA4206* (Q184E)	+		SS13884→SS996 [a]
SS13895	*recA4205* (Q184H)	+		SS13885→SS996 [a]

All strains have a JC13509 background. JC13509 is derived from SK362 strain and has the following genotype: F^- *lacMS286* Φ*80dIIlacBK1 sulB103 argE4 his-4 thi-1 xyl-5 mtl-1* Sm^R $T6^R$. The *lacMS286* Φ*80dIIlacBK1* codes for two partial non-overlapping deletions of the lac operon (Konrad, 1977; Zieg & Kushner, 1977). Unless notated otherwise, all *recA* mutants generated in this study have *ygaD1::kan* in addition to noted mutations (Renzette et al., 2005).

[a] Select for kanamycin resistance and then screen by phenotypic marker

[b] Select for chloramphenicol resistance and then screen by either UV sensitivity or red or green cells

[c] Select for tetracycline resistance and then screen UV sensitivity

[d] Select for ampicillin resistance at 30ºC

Strain	Description
SS13161	Transformant of chromosomally integrated *recA4187* (E38H) allele at *recA* locus
SS13244	Transformant of chromosomally integrated *recAo1403* allele at *recA* locus in strain with *recA4197* (SS13238)
SS13356	Transformant of chromosomally integrated *recA4201*(E38N) allele at *recA* locus
SS13357	Transformant of chromosomally integrated *recA4184* (E38R) allele at *recA* locus
SS13358	Transformant of chromosomally integrated *recA4200* (E38D) allele at *recA* locus
SS13359	Transformant of chromosomally integrated *recA4185* (E38S) allele at *recA* locus
SS13821	Transformant of chromosomally integrated *recA4199* (E38A) allele at *recA* locus
SS13822	Transformant of chromosomally integrated *recA4186* (E38Q) allele at *recA* locus
SS13827	Transformant of chromosomally integrated *recA4197* (D36K) allele at *recA* locus
SS13844	Transformant of chromosomally integrated *recA4204* (Q184R) allele at *recA* locus
SS13866	Transformant of chromosomally integrated *recA1202* (Q184K) allele at *recA* locus
SS13883	Transformant of chromosomally integrated *recA4207* (Q184N) allele at *recA* locus
SS13884	Transformant of chromosomally integrated *recA4206* (Q184E) allele at *recA* locus
SS13885	Transformant of chromosomally integrated *recA4205* (Q184H) allele at *recA* locus

All *recA* mutant strains, except SS13244, were generated by selecting for kanamycin resistance on LB kanamycin at 37^{o}C. SS13244 was generated by selecting for chloramphenicol resistance on LB chloramphenicol.

Table 3.6: Plasmids used in this study

Plasmid	**Description**	Strains carrying plasmid	**Reference**
pKD46	expression of the λ Red system under control of arabinose-inducible promoter, temperature sensitive replication, Amp^R	SS10191	(Datsenko & Wanner, 2000a)
pNR117	pBR322 derivative with a *Bam*HI fragment containing *ygaD1*::*kan recA*$^+$ with upstream and downstream chromosomal sequences, Amp^R, Kan^R	SS5695	Lab stock
pSJS1642	Derivative of pNR117 replacing *recA*$^+$ with *recA4185* (E38S)	SS13159	This study
pSJS1643	Derivative of pNR117 replacing *recA*$^+$ with *recA4186* (E38Q)	SS13151	This study
pSJS1644	Derivative of pNR117 replacing *recA*$^+$ with *recA4187* (E38H)	SS13152	This study
pSJS1645	Derivative of pNR117 replacing *recA*$^+$ with *recA4184* (E38R)	SS13153	This study
pSV39	Derivative of pNR117 replacing *recA*$^+$ with *recA4201* (E38N)	SS13361	This study
pSV41	Derivative of pNR117 replacing *recA*$^+$ with *recA4200* (E38D)	SS13352	This study
pSV43	Derivative of pNR117 replacing *recA*$^+$ with *recA4197* (D36K)	SS13350	This study
pSV44	Derivative of pNR117 replacing *recA*$^+$ with *recA4199* (E38A)	SS13382	This study
pSV52	Derivative of pNR117 replacing *recA*$^+$ with *recA1202* (Q184K)	SS13390	This study
pSV53	Derivative of pNR117 replacing *recA*$^+$ with *recA4204* (Q184R)	SS13394	This study
pSV57	Derivative of pNR117 replacing *recA*$^+$ with *recA4205* (Q184H)	SS13395	This study
pSV58	Derivative of pNR117 replacing *recA*$^+$ with *recA4206* (Q184E)	SS13399	This study

pSV62	Derivative of pNR117 replacing *recA*$^{+}$ with *recA4207* (Q184N)	SS13354	This study

All plasmids carrying *recA* were selected for ampicillin and kanamycin resistance. Screen by PCR and restriction site polymorphism. Correct mutation was confirmed by DNA sequence analysis.

Table 3.6: Oligonucleotides used in this study

Name	Sequence (5' to 3')	Specificity
prSJS453	GAAATCTACGGACCGGAATCTTC CGG	Forward primer in *recA* overlapping *RsrII* site
prSJS507	TTTGCCACTGCCCGCGGTGAAGG	Forward primer upstream of *recA* overlapping *SacII* site
prSJS508	AGAGGATCTGGAATTCAGCC	Reverse primer in *recA* downstream of *PmeI* site
prSJS540	GTGGTTTTACCGGAAGATTCC	Reverse primer in *recA* downstream of *RsrII* site
prSJS816	GCCGGATCCTGACGAAAGTGCTA TCTTGTCCGG	Forward primer upstream of *ygaD*
prSJS1096	CCATCGGTCGACAAATCTCCTGG ATATCTTCC	Reverse primer downstream of *recX*
prSJS1688	CGTTCCATGGATGTGTCTACCAT CTCTACCGGTTCGC	Forward primer makes E38S removes *BstXI* site
prSJS1689	CGTTCCATGGATGTGCAGACCAT CTCTACCGGTTCGC	Forward primer makes E38Q removes *BstXI* site
prSJS1690	CGTTCCATGGATGTGCATACCAT CTCTACCGGTTCGC	Forward primer makes E38H removes *BstXI* site
prSJS1714	CGTTCCATGGATGTGAACACCAT CTCTACCGGTTCGC	Forward primer makes E38N removes *BstXI* site
prSJS1715	CGTTCCATGGATGTGGATACCAT CTCTACCGGTTCGC	Forward primer makes E38D restores *BstXI* site
prSJS1716	CGTTCCATGGATGTGGCGACCAT CTCTACCGGTTCGC	Forward primer makes E38A restores *BstXI* site
pSJS1719	CCAAATTGTTTCTCAATCTGGCC CAGTGC	Reverse primer in *recA* downstream of ATG

3.6 Figures

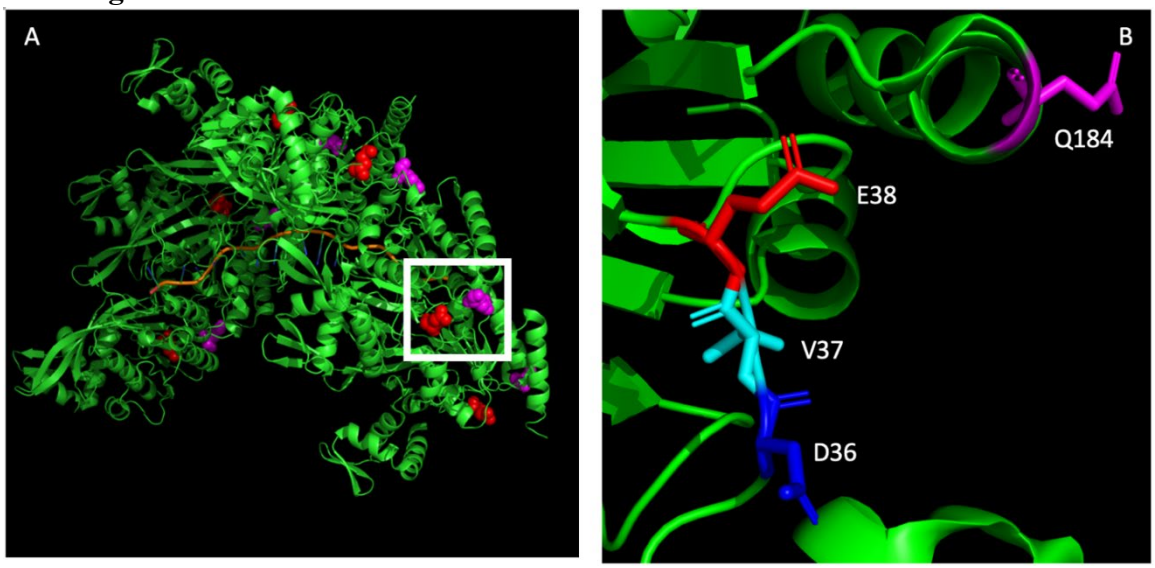


Figure 1: Location of mutations on RecA filament crystal structure:

A: Crystal structure of the RecA filament (green) on single stranded DNA (orange). Positions for mutants that constitutively express the SOS response, *recA730* (red) and *recA1202* (magenta), are shown as spheres. The colored spheres repeat with each monomer in the filament. B shows a blown up view (white box from panel A) of the surface of the positions used in this study. The locus for *recA730* (E38K) is in red. The locus for *recA1202* (Q184K) is in magenta. The locus for *recA803* (V37M) is in cyan. The locus for *recA4197* (D36K) is in blue. The locus of *recA4161* (deletion of the last seventeen amino acids) is in the very C-terminal domain and is not shown because the electron density of residues 34-352 are too faint to be able to be used to see their structure. The structures shown are from the Protein Data Base ((A) 3CMU (B) 2REB).

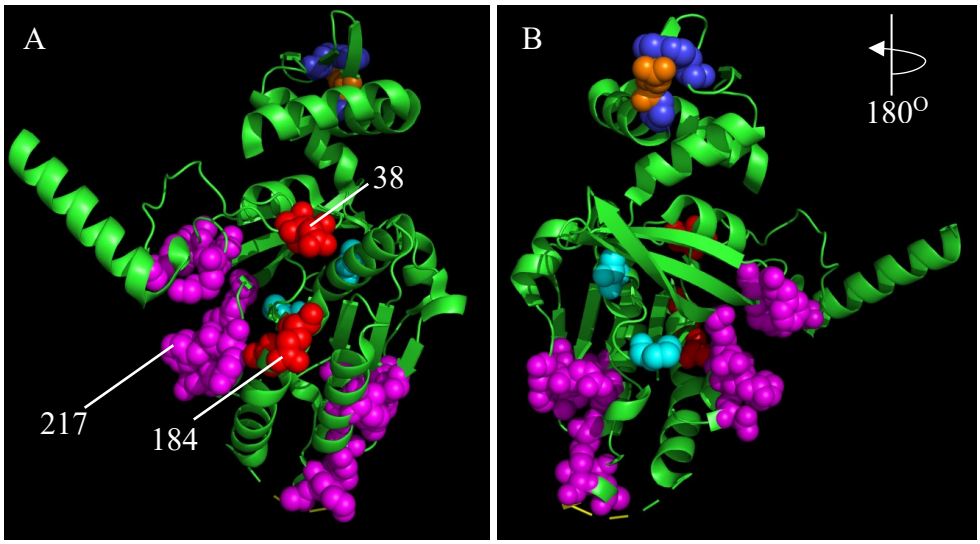
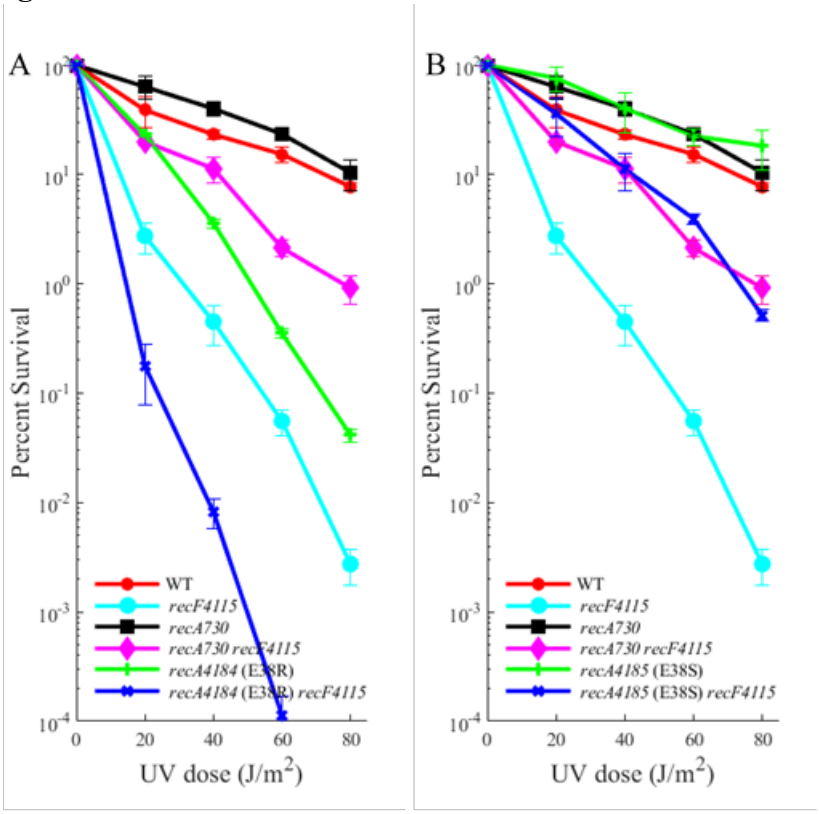
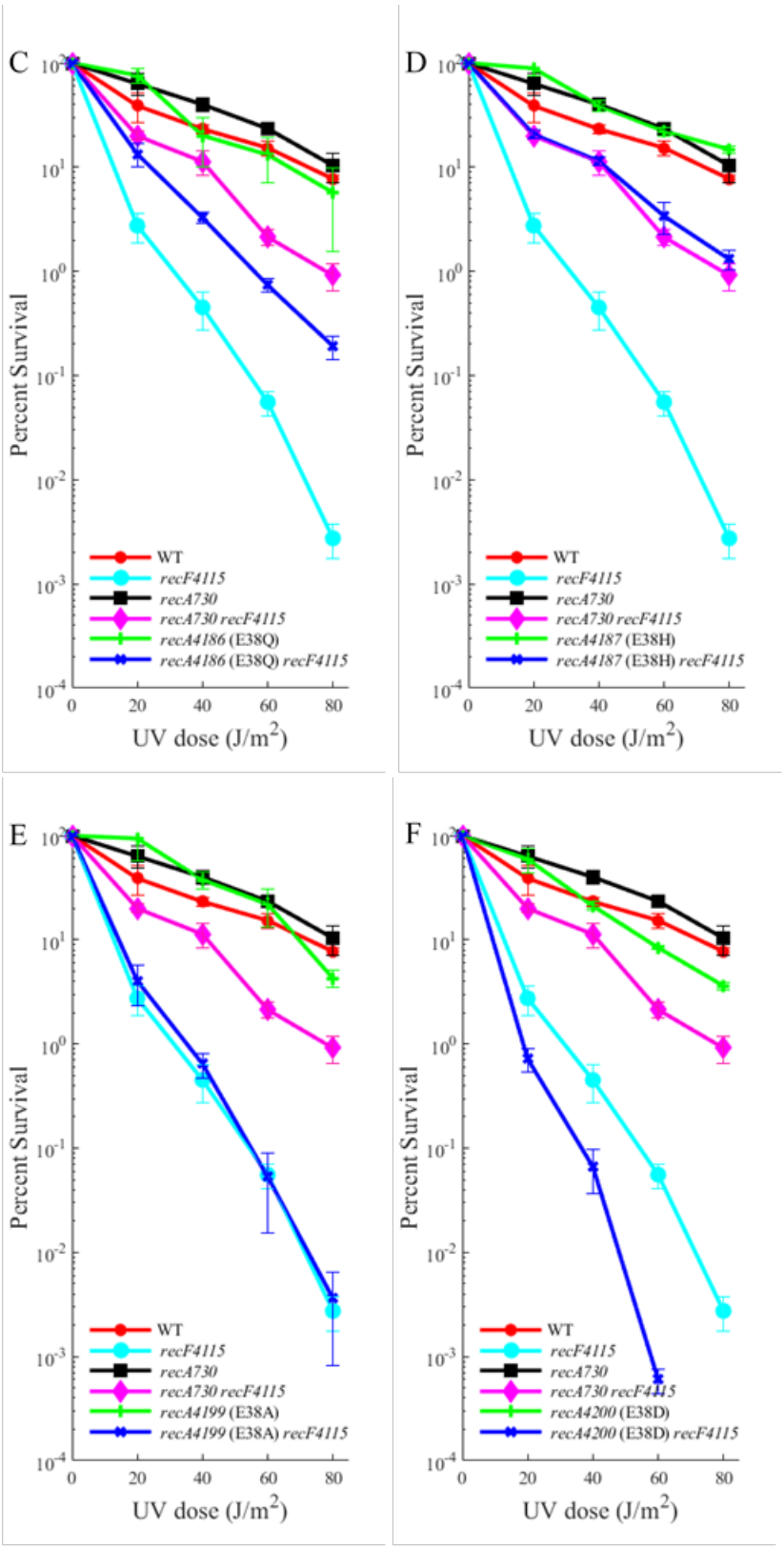


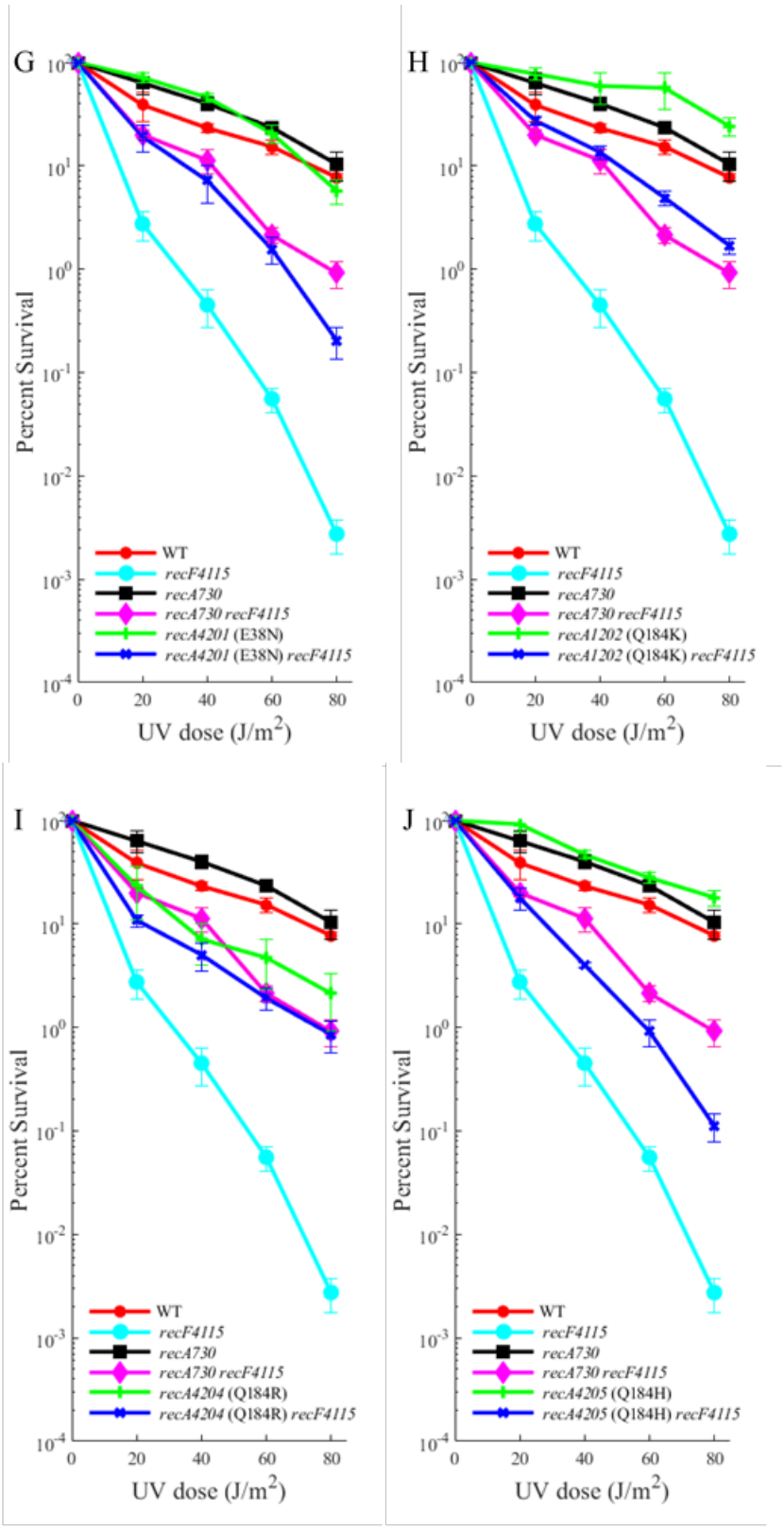
Figure S1: RecA mutants with constitutive SOS response expression.

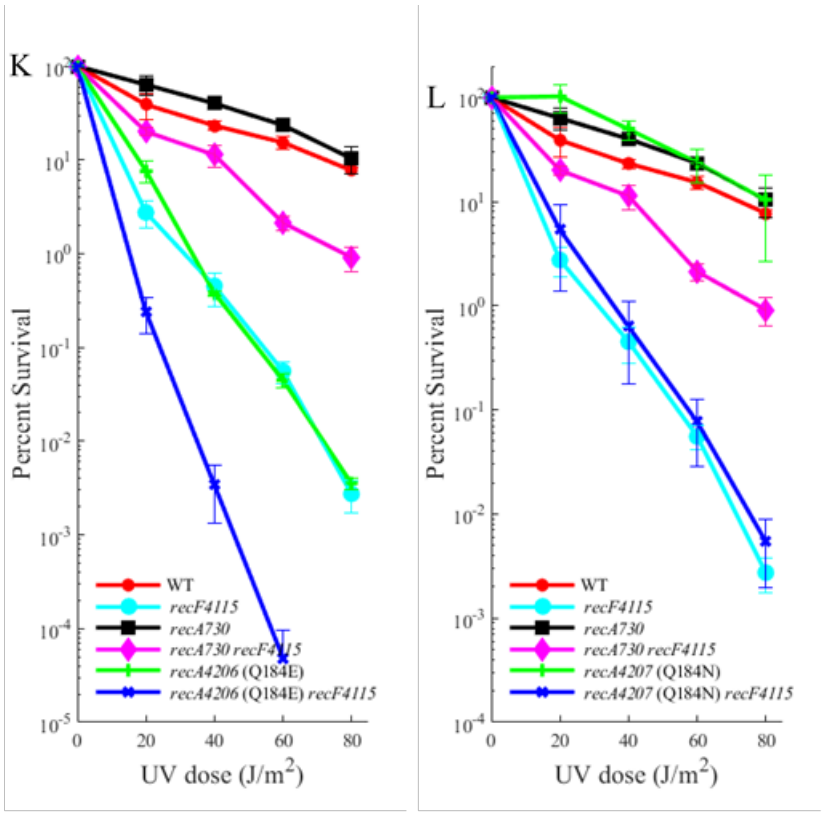
Highlighted in spheres are the positions that lead to constitutive SOS response expression as reported in McGrew and Knight 2008. Panel B is panel A rotated 180 degrees. The magenta spheres are in the oligomerization domain. The cyan spheres are in the Walker A motif. The orange spheres are associated with increased cleavage of the CI repressor and UmuD. The blue spheres are in the C-terminal domain which plays a role in regulating the binding of a second DNA molecule. The yellow residues (bottom panel B), are in Loop 1. The red spheres are in a domain of unknown function. Certain mutations in the oligomerization domain leads to increased cooperativity as seen with *recA4142* (F217Y). Positive charged residues at position 38 and 184 in the unknown function domain leads to constitutive SOS response expression as seen with *recA730* (E38K) and *recA1202* (Q184K). (PDB accession code: 2REB)

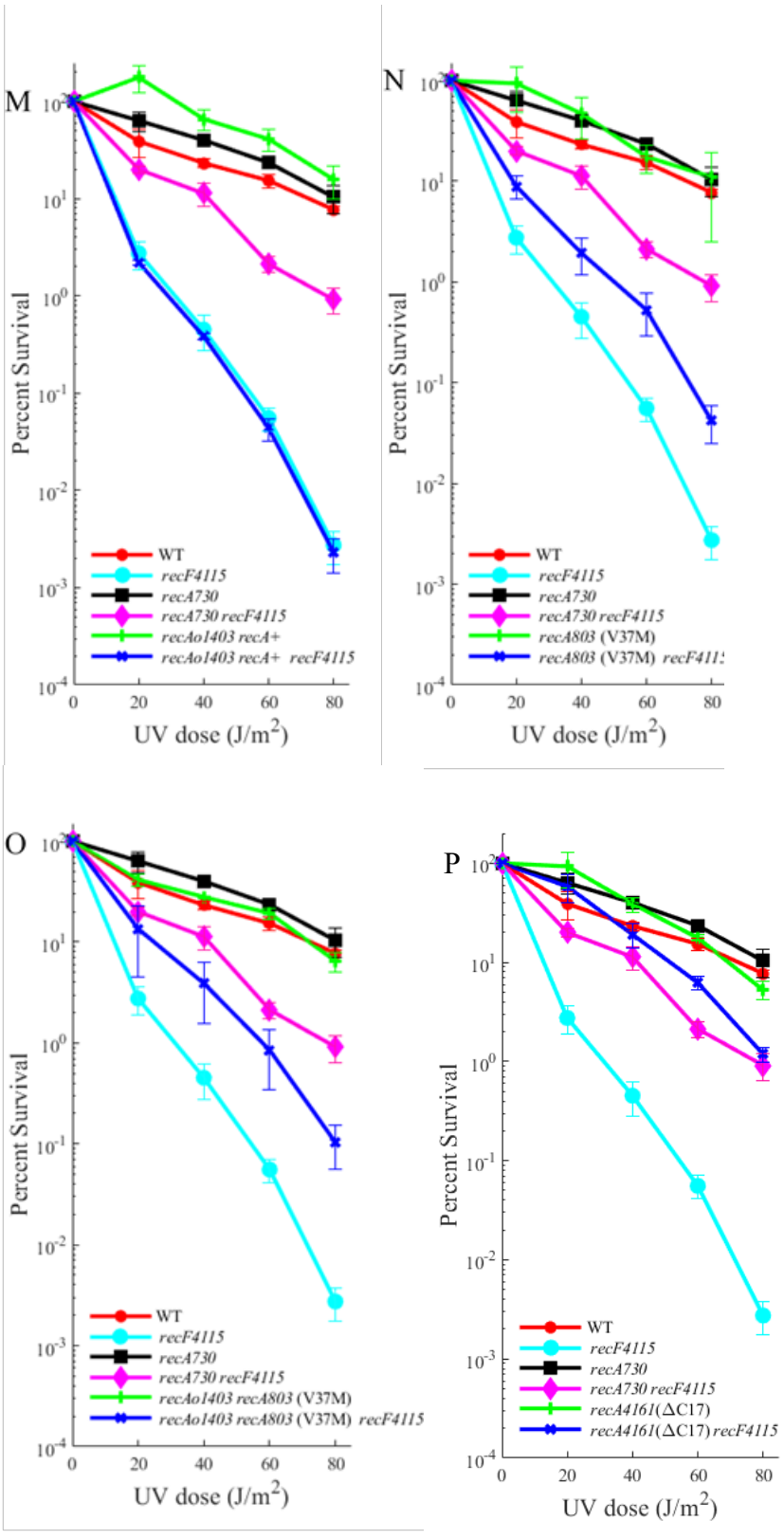
Figure S2: UV survival curves for *recA* mutations used in this study

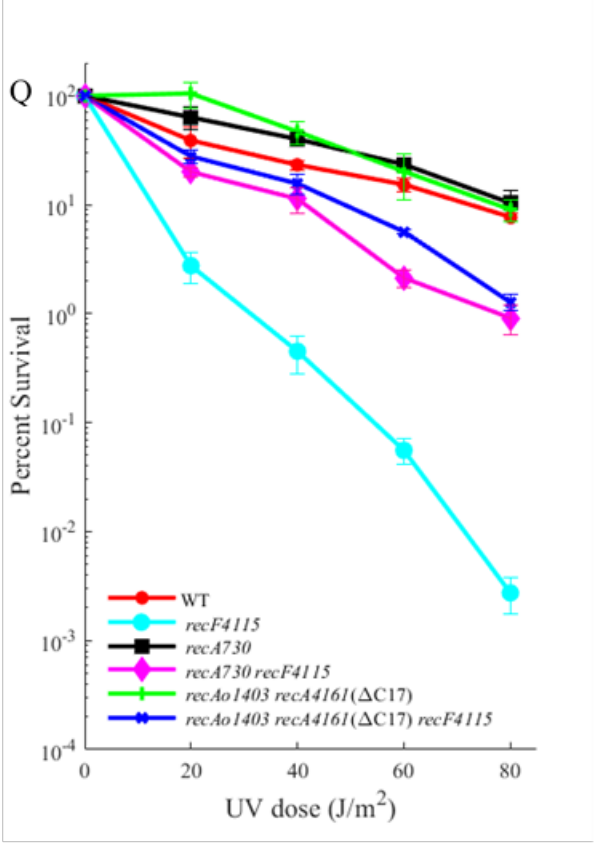


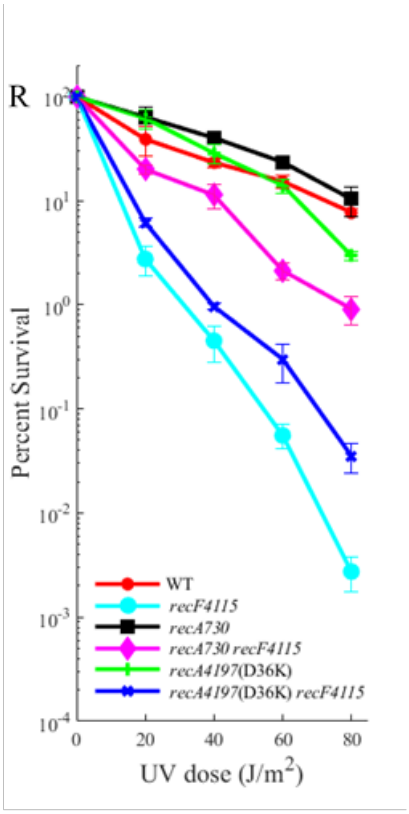

C
D
E
F
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA4186 (E38Q)
recA4186 (E38Q) recF4115
recA4187 (E38H)
recA4187 (E38H) recF4115
recA4199 (E38A)
recA4199 (E38A) recF4115
recA4200 (E38D)
recA4200 (E38D) recF4115


G
H
I
J
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA4201 (E38N)
recA4201 (E38N) recF4115
recA1202 (Q184K)
recA1202 (Q184K) recF4115
recA4204 (Q184R)
recA4204 (Q184R) recF4115
recA4205 (Q184H)
recA4205 (Q184H) recF4115


K
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA4206 (Q184E)
recA4206 (Q184E) recF4115
L
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA4207 (Q184N)
recA4207 (Q184N) recF4115


M
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recAo1403 recA+
recAo1403 recA+ recF4115
N
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA803 (V37M)
O
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recAo1403 recA803 (V37M)
recAo1403 recA803 (V37M) recF4115
P
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA4161(ΔC17)
recA4161(ΔC17) recF4115


Q
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recAo1403 recA4161(ΔC17)
recAo1403 recA4161(ΔC17) recF4115


R
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recA4197(D36K)
recA4197(D36K) recF4115

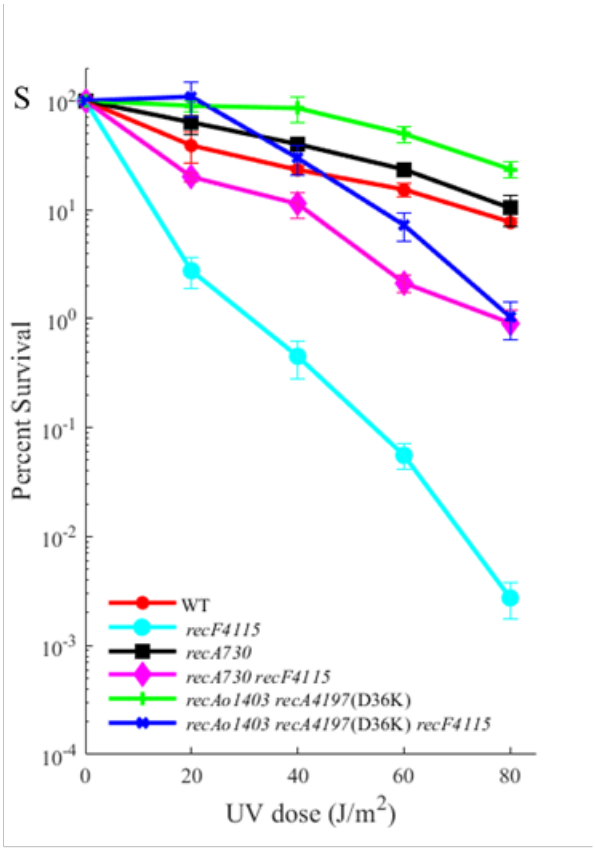

S
Percent Survival
UV dose (J/m2)
WT
recF4115
recA730
recA730 recF4115
recAo1403 recA4197(D36K)
recAo1403 recA4197(D36K) recF4115

Figure S2: UV survival assay performed as described in Materials and Methods. Survival of the *recA* mutants after UV irradiation. The strains in all of the figures are SS996 (wildtype), SS1426 (*recF4115*), SS4629 (*recA730*), SS4645 (*recA730 recF4115*). Strains unique to the plot are given as follows: (A) SS13365 (*recA4184* (E38R)) and SS13377 (*recA4184* (E38R) *recF4115*) (B): SS13862 (*4185* (E38S)) SS13265 *4185* (E38S) *recF4115*) (C): SS13836 (*4186* (E38Q)) SS13860 (*4186* (E38Q) *recF4115*) (D): SS13373 (*recA4187* (E38H)) SS13374 (*recA4187* (E38H) *recF4115*) (E): SS13835 (*recA4199* (E38A)) SS13861 (*recA4199* (E38A) *recF4115*) (F): SS13369 (*recA4200* (E38D)) SS13378 (*recA4200* (E38D) *recF4115*) (G): SS13364 (*recA4201* (E38N)) SS13376 (*recA4201* (E38N) *recF4115*) (H): SS13867 (*recA1202* (Q184K)) SS13869 (*recA1202* (Q184K) *recF4115*) (I): SS13881 (*recA4204* (Q184R)) SS13882 (*recA4204* (Q184R) *recF4115*) (J): SS13895 (*recA4205* (Q184H)) SS13220 (*recA4205* (Q184H) *recF4115*) (K): SS13894 (*recA4206* (Q184E)) SS13219 (*recA4206* (Q184E) *recF4115*) (L): SS13893 (*recA4207* (Q184N)) SS13218 (*recA4207* (Q184N) *recF4115*) (M): SS6088 (*recAo1403 recA*$^{+}$) SS13264 (*recAo1403 recA*$^{+}$ *recF4115*) (N): SS4656 (*recA803* (V37M)) SS13261 (*recA803* (V37M) *recF4115*) (O): SS6131 (*recAo1403 recA803* (V37M)) SS6133 (*recAo1403 recA803* (V37M) *recF4115*) (P): SS6428 (*recA4161* (ΔC17)) SS13263 (*recA4161* (ΔC17) *recF4115*) (Q): SS6061 (*recAo1403 recA4161* (ΔC17)) SS6067 (*recAo1403 recA4161* (ΔC17) *recF4115*) (R): SS13841 (*recA4197* (D36K)) SS13859 (*recA4197* (D36K) *recF4115*) (S): SS13253 (*recAo1403 recA4197* (D36K)) SS13254 (*recAo1403 recA4197* (D36K) *recF4115*). These strains are derivatives of SS996 which has Δ*attB:: sulAp-gfp* (McCool et al. 2004) WT, wild type.

CLOSING REMARKS

Siddhartha Mukherjee M.D. Ph.D., author of the books, The Emperor of All Maladies and The Gene, wrote "Life may be chemistry, but it's a special circumstance of chemistry. Organisms exist not because of reactions that are possible, but because of reactions that are *barely* possible." While this excerpt is in the context of maintaining metabolism in a careful balance, the same principle applies to DNA repair. The fine-tuned tools that all of life uses to maintain and protect the precious resource that is its genome is a proverbial tight rope walk, where on one side an insufficient reaction to DNA insult will lead to certain death, where on the other side an excessive reaction can lead to a proverbial "hoisting with [the cell's] own petard." One of the fundamental ways the cell navigates this difficult, high-stakes challenge is through an abundant DNA damage sensor, RecA, which, when activated, rapidly triggers an inducible response modulated by LexA. This dissertation describes two projects aimed to examine the complex relationship between LexA and RecA.

Chapter 2 examines the effect of overexpression of a noncleavable mutant of *lexA*, *lexA3*, on RecA-GFP structures and ultraviolet damage repair phenotypes. We show that a fourfold overexpression of *lexA3* is able to drive down expression of *recA* tenfold. This in turn leads to an increased sensitivity to UV light compared to *lexA3* expression at wildtype levels. Adding an operator mutation in *recA*, *recAo281*, led to a partial suppression of the UV sensitive phenotype. Lastly, we were able to show that the decreased amount of RecA had a phenotype when recombination structures were assayed using RecA-GFP. Strains overexpressing *lexA3* had three times as many RecA-GFP structures compared to $lexA^+$ strains. Furthermore, after UV irradiation the recombination

structures which typically form elongated filaments by 90 minutes were absent in the *lexA3* overexpression strain. Rather, the structures were kept in a punctate focus.

Chapter 3 examines the effect of a positive charge at positions 38 and 184 in the RecA protein. We demonstrated that amino acids with a positive charge at position 38 and 184 are highly correlated with constitutive SOS response expression. Additionally, polar residues at these positions can lead to suppression of the UV sensitivity conferred by a *recF* mutation. The idea of constitutive SOS expression was further explored by the combining of the *recAo1403* operator mutation with certain *recA* mutants that could suppress *recF* but did not constitutively express the SOS response. When combined, these mutants led to constitutive SOS response expression. Interestingly, the removal of the last seventeen amino acids in the C-terminus also showed this phenotype. This suggests that not only is the positive charge important, but perhaps the removal of negative charges plays a role.

In conclusion, these two chapters focus on the complex relationship between LexA and RecA and how this complexity contributes to the function and dynamism of the SOS response. Despite nearly sixty years of study, the SOS response still has unplumbed depths yet to be discovered.

APPENDICES

5.1 A. LEXA3OP SUPPRESSOR MUTANT ISOLATION

DNA damage is a stress that all of life needs to deal with. Not being able to repair DNA damage can lead to decreased fitness and cell death in lower order organisms and can lead to cancer in higher order organisms. How organisms respond to DNA damage will give differing survival outcomes. In most organisms, there is a DNA damage inducible response. The SOS response in *Escherichia coli* serves as an archetype of DNA damage inducible responses. Comprising of over forty genes involved with regulating homologous recombination, nucleotide excision repair, and mutagenesis, the SOS response regulon is regulated by the repressor LexA. Under conditions with no DNA damage, LexA binds to the operators of those genes and maintains them at the basal level of transcription. Under conditions with DNA damage, LexA undergoes an autocleavage reaction and is degraded by Lon and ClpXP (Neher et al., 2003). Most of the LexA in the cell is depleted after around five minutes after DNA damage exposure. LexA is autoregulated so when the amount of LexA in the cell is depleted, more LexA is produced by increased transcription. When the DNA damage has been repaired, the amount of LexA increases again to turn off the SOS response thereby creating a negative feedback loop.

One of the genes in the SOS response is *recA* (Little et al., 1981). The relationship between LexA and RecA is complex. RecA is a DNA repair protein that is central to homologous recombination. To initiate homologous recombination, the cell uses RecA loading factors in either the RecBCD pathway or the RecFOR pathway. RecA is loaded onto the DNA and forms a helical nucleoprotein filament that then searches the genome for a homologous sequence to perform strand exchange. The DNA is centrally located in

the RecA filament (Egelman & Yu, 1989). The filament will sample the double-stranded DNA by allowing the DNA to enter into the filament groove. LexA competes with double-stranded DNA for the RecA filament groove (Harmon et al., 1996; Rehrauer et al., 1996). The RecA filament stabilizes the cleavable form of LexA and promotes the autocleavage reaction. Cleavage of LexA then allows for a higher expression of RecA.

LexA is largely a dimer in the cell (Giese et al., 2008). LexA has two main domains, the N-terminal DNA binding domain and the C-terminal dimerization/catalytic site domain (Luo et al., 2001). The N-terminus has a helix-turn-helix motif that is commonly used for DNA binding. Mutations in the N-terminal domain can decrease LexA's affinity for DNA and lead to constitutive SOS expression (Oertel-Buchheit et al., 1990). The C-terminal domain is where the catalytic domain is located. Mutations in the C-terminal domain can disrupt dimerization. Since LexA needs to dimerize in the cell to bind to DNA (Thliveris et al., 1991), mutants that prevent dimerization leads to constitutive SOS expression. LexA exists in two forms in the cell. A cleavable conformation and a non-cleaving conformation (Luo et al., 2001). LexA goes between these two conformations and cleaves itself in the cleavable conformation. Three residues are important for the cleavage reaction G85, S119, and K156. The Ser119 and Lys156 are the two catalytic residues of the serine-lysine protease dyad (Slilaty & Little, 1987). The peptide bond between Ala84 and Gly85 is the target of the cleavage reaction (Little, 1993). The catalytic site is made up of mostly hydrophobic residues with the two catalytic residues at the very end. Cleavage of the peptide bond between Ala84 and Gly85 leads to separation of the N-terminal DNA binding domain and the C-terminal dimerization domain. Mutants such as G85D (*lexA3*), S119A, or K156A leads to a LexA

protein that cannot undergo a cleavage reaction (Little, 1991; Mo et al., 2016; Slilaty & Little, 1987). LexA with an inability to undergo autocleavage prevents the cell from inducing the SOS response. As a result, the cell becomes more sensitive to DNA damaging agents.

The *lexA3* (G85D) mutant has been the most studied of the SOS (Ind^-) mutants (Ginsburg et al., 1982; Mount et al., 1972). Overexpression of this LexA mutant from a plasmid made the strain more sensitive to ultraviolet light than when *lexA3* was expressed at wildtype levels (Mount et al., 1980). The authors have speculated that the increased amount of LexA3 could decrease the amount of RecA in the cell. This idea was directly tested in this study showing a 10-fold decrease in RecA expression. The experimental set up in this system was different in that *lexA3* was overexpressed by placing a strong promoter in front of the *lexA* gene at its native locus instead of *lexA* being expressed off a plasmid.

There are a couple reasons why increasing the amount of *lexA3* led to increased UV sensitivity. The decreased amount of RecA may not be sufficient for proper DNA repair after UV exposure. LexA regulates many other genes including the genes important for nucleotide excision repair. Deletion of *uvrA* makes cells extremely sensitive to ultraviolet light (Seeberg et al., 1976). Overexpression of LexA3 may lead to such a depletion of NER proteins that the cell can no longer do UV repair properly.

Another possible reason for increased UV sensitivity is it has been shown *in vitro* that LexA competes with double-stranded DNA in a RecA filament. It was hypothesized that overexpressing LexA3 would make LexA outcompete the secondary DNA strand and make synapsis difficult for the RecA filament, thereby making the cells rec^- (Harmon

et al., 1996). By putting the cell in the position that it needs to repair DNA, one can select for suppressor mutants in either *lexA* or *recA* that disrupts the interaction between LexA and RecA. To do this, we combined *lexA2005* with a *dam-13* mutation.

In order to find *lexA* mutants that do not bind to the RecA filament, a genetic screen was designed. Work from Marinus has demonstrated that *lexA3* is synthetically lethal with a *dam* mutation (Marinus, 2000). Using this information, the *dam-13* mutation was P1 transduced into the *lexA3* overproducer strain and selected for by chloramphenicol selection. Isolates were screened for UV resistance of greater than 5J/m^2. The *recA* and *lexA* region of UV resistant isolates was PCR amplified and sequenced. Only mutations in *lexA* were discovered. A total of fifteen missense mutations and one frameshift mutation were isolated. Figure 1 shows the mutants mapped to LexA that were isolated. Each of the mutant strains had their SOS expression measured by using *sulAp-gfp* and LexA protein amount measured by Western blot. The *lexA* mutations were P1 transduced into a dam^+ background (SS6321) to compare SOS levels. (About 1000 cells analyzed) That information is given in Table A1.

The mutants appear to break into three categories: High SOS expression, medium SOS expression, and similar to wildtype SOS expression. The mutants in the high SOS expression category are *lexA2018* (Q8E) *lexA2007* (D129G), and *lexA2009* (T27A). All of these had SOS expression over half what would be considered half of fully SOS-induced. The medium SOS expression category has *lexA2019* (N171D), *lexA2013* (S103P), *lexA2006* (P25L), and *lexA2008* (R157C). These had less than half of fully-induced SOS expression but still higher than what is typically considered wildtype expression. The last category is similar to wildtype SOS expression. The mutants in this

category are *lexA2020* (V193A), *lexA2015* (L50V), *lexA2014* (V146A), *lexA2016* (N41S), *lexA2010* (P77S), and *lexA2017* (A42T). This group is somewhat surprising considering that many of these mutations have been implicated in binding DNA, but the SOS response expression measured by *sulAp-gfp* is not elevated beyond wildtype levels of SOS response expression.

The mutants can also be categorized by protein stability. While all of the mutants were less stable than *lexA2005*, some were more stable than others. The stable proteins are considered those that are less than a two-fold decrease whereas, the unstable proteins are considered those that are greater than a two-fold decrease. The stable mutant proteins are *lexA2018* (Q8E), *lexA2006* (P25L), *lexA2016* (N41S), and *lexA2017* (A42T). There is a loose correlation between protein amount and SOS expression with the exception of *lexA2018*. The unstable mutant proteins are *lexA2007* (D129G), *lexA2009* (T27A), *lexA2019* (N171D), *lexA2013* (S103P), *lexA2008* (R157C), *lexA2020* (V193A), *lexA2015* (L50V), *lexA2014* (V146A), *lexA2010* (P77S). A few mutants correlate with a high SOS response expression and low LexA protein amount such as *lexA2007* (D129G), *lexA2009* (T27A), and *lexA2019* (N171D). However, other mutants have a low LexA protein amount and low SOS response expression such as *lexA2020* (V193A), *lexA2015* (L50V) and *lexA2010* (P77S).

While the goal of this study was to find LexA or RecA mutants that no longer interacted with each other, what eventually became clear is that the LexA mutants that were isolated either disrupted DNA binding or destabilized the protein to a point where the SOS response would be induced.

Several mutants isolated in this study have previously been isolated from previous work exploring the residues important for DNA binding. The *lexA2006* (P25L) and *lexA2017* (A42T) have been isolated in a study Shnarr et al. 1990. Four other mutants, *lexA2009* (T27A), *lexA2016* (A42T), *lexA2015* (L50V), and *lexA2018* (Q8E), have not been isolated previously, but have been implicated in DNA binding from x-ray crystallographic studies Rice 2011). Both *lexA2015* (L50V) and *lexA2009* (T27A) appear to also affect protein stability as Western blot analysis on these mutants demonstrated that they were three-fold less stable than without the suppressor mutations. There is a drastic difference in SOS response expression between *lexA2016* (A42T) and *lexA2018* (Q8E) despite them both being implicated in DNA binding (Zhang et al., 2010). While there have been no *in vitro* studies done on these mutant LexA proteins, one might assume from the SOS response expression data that *lexA2016* (A42T) only affects DNA binding slightly, while *lexA2018* (Q8E) affects DNA binding more strongly. The amount of LexA2018 measured by Western blot may not be a strict reflection of protein stability. The promoter driving expression of *lexA3* is the *recA* promoter with a *recAo1401* and *recAo281* operator mutation (Uhlin et al., 1982; Wertman & Mount, 1985). Work in chapter two demonstrated that overexpressing *lexA3* with this promoter lead to a four-fold increase in the amount of *lexA3*. Strains with this amount of LexA3 have a ten-fold decrease in RecA protein amount. The *recAo281* operator supposedly eliminates operator activity and so in theory is not repressed by LexA. However, Western blot analysis demonstrated that the strains with both *recAo281* and *lexA3* overproducer have less RecA protein than the strains with *recAo281* and *lexA*$^{+}$. This suggests that if there is an increased amount LexA in the cell, operator activity can be partially restored. If *lexA2018*

(Q8E) more strongly affects LexA repressor activity compared to *lexA2016* (A42T), then it would follow that *lexA2018* (Q8E) would have more protein since transcription of *lexA* is not as inhibited as *lexA2016* (A42T). A caveat to this discussion is that it does not take into account the *recAo1401* mutation which together with the *recAo281* mutation may eliminate an operator activity regardless of the amount of *lexA3* in the cell.

LexA forms a dimer when it binds to DNA and mutations that disrupt dimerization can affect LexA's ability to bind operators as a repressor (Thliveris et al., 1991). This is seen with the commonly used *lexA3,51*. The *lexA51* allele codes for a frameshift that leads to a premature stop codon and a truncated protein (Mount, 1977). Since the C-terminus is necessary for dimerization, LexA is not able to repress the genes of the SOS response and the SOS response is constitutively expressed. Surprisingly, this study failed to identify any missense mutations that explicitly affected dimerization.

Electrostatic interactions play an important role in maintaining protein structure and stability. A number of mutations in the C-terminal domain of LexA were isolated that could possibly be in electrostatic interactions and therefore protein stability. Residues D129 and R197 seem to be important as mutations were isolated at their positions in the mutant screen as *lexA2007* (D129G), *lexA2012* (R197D), and *lexA2011* (R197S). All of these mutations removed the charged residue that was within electrostatic interaction with the other. In a similar vein, R157 may make electrostatic interactions with E170. In support of this idea, mutations were isolated at R157, *lexA2008* (R157C), and the residue neighboring E170, N171, *lexA2019* (N171D).

Tertiary structure and protein structure can be affected by the presence of proline which causes a "kink" in a polypeptide. Removal or addition of proline in a protein can

disrupt the local structure and may destabilize the protein. Two mutations, *lexA2010* (P77S) and *lexA2013* (S103P), either add or remove a proline. Both of these have a greater than 2-fold decrease in protein stability as measured by Western blot.

V146 is in the hydrophobic channel where the loop containing the scissile bond allosterically moves to facilitate the cleavage reaction. It is not presently obvious why the *lexA2014* (V146A) mutation allowed for suppression other than destabilizing the protein (2-fold decrease from *lexA2005*).

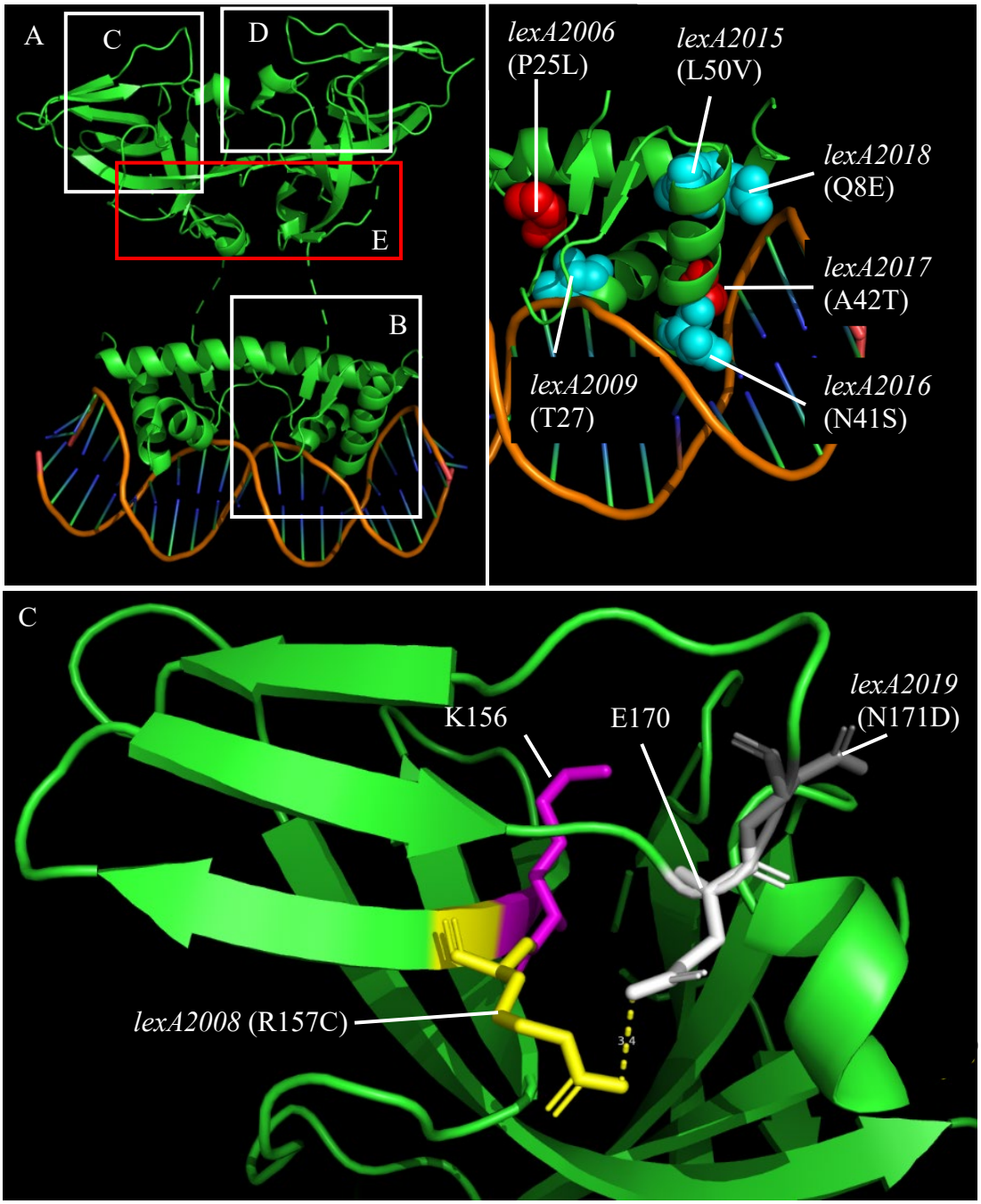
A surprising conclusion from this study is a general lack of correlation between SOS response expression and LexA protein amount. One would assume that a decreased amount of LexA would lead to higher SOS response expression. Additionally, one would assume that all the mutations in the DNA binding domain would lead to an elevated SOS response expression. However, only two of the mutations in the DNA-binding domain led to high levels of SOS response expression, *lexA2018* (Q8E) and *lexA2009* (T27A) and *lexA2009* was unstable (greater than a two-fold decrease). Another surprising result was a failure to recapitulate the elevated level of SOS response expression from the two previously discovered mutants in the DNA-binding domain, *lexA2006* (P25L) and *lexA2017* (A42T). This difference can be accounted for by the fact that all of the *lexA* mutants in this study are double mutants with *lexA3*, whereas all the mutants in Oertel-Buchheit et al. did not have *lexA3* and were therefore cleavable. Furthermore, unbound LexA is more susceptible to cleavage as binding DNA stabilizes the noncleavable form (Giese et al., 2008).

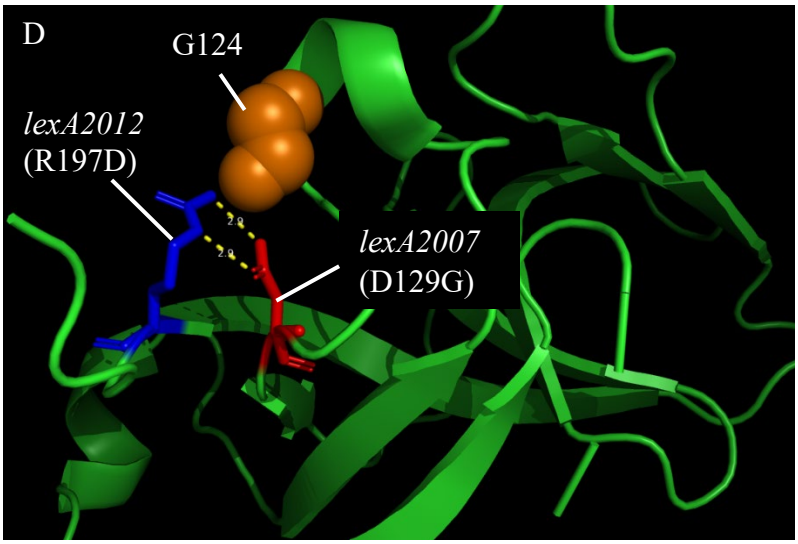
When designing a genetic screen, it is important to be cognizant of what exactly you are selecting for. From this genetic screen, it is apparent that the elevated expression of

the SOS response genes is what is important in a *dam* mutant. The way the cell was able to get around this problem was by preventing LexA from proper repressor activity. The fact that LexA was mutated and not just the operator sequence of a gene or two that was critical for the phenotype suggests that it is a series of genes in the SOS response that are important to be expressed at elevated levels in a *dam* mutant and not just one gene.

FIGURES

Figure A1: Mapped isolated mutants




D
G124
lexA2012
(R197D)
lexA2007
(D129G)

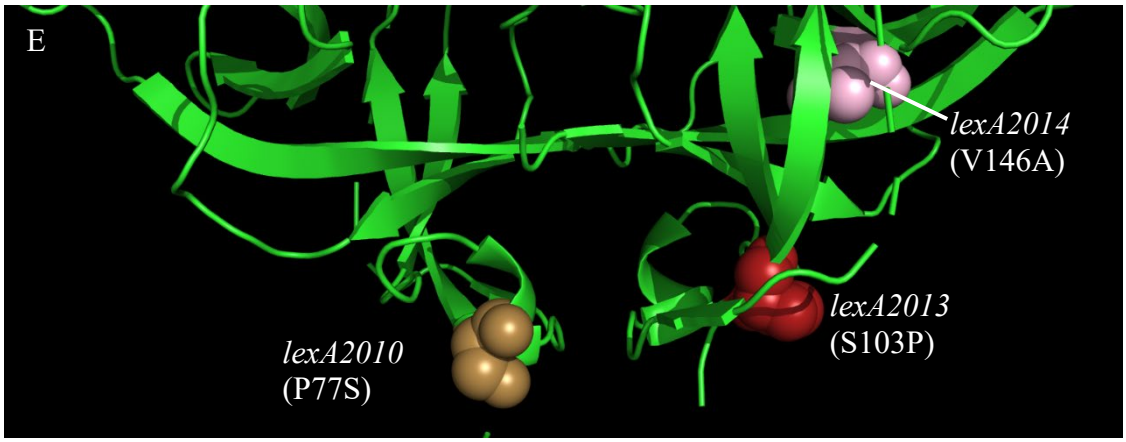

E
lexA2014
(V146A)
lexA2013
(S103P)
lexA2010
(P77S)

Figure A1: Suppressor mutants mapped to LexA crystal structure. (A): LexA bound to double-stranded DNA. LexA is binds to DNA as a dimer. The N-terminal domain is the DNA binding domain. The C-terminal domain contains the dimerization domain and the catalytic core. Each box is a zoomed in area for a category of mutants. (B): Mutations isolated in the DNA-binding domain. The red spheres (*lexA2006* (P25L) and *lexA2017* (A42T)) are mutations that have been isolated previously. The cyan spheres (*lexA2009* (T27A), *lexA2016* (N41S), *lexA2018* (Q8E), *lexA2015* (L50V)) are mutations that have been implicated in LexA-DNA interactions based on x-ray crystallographic data. (C): Residues near the catalytic domain that may play a role in electrostatic structural stability. K156 (magenta) is one of the catalytic residues for cleavage. Next to K156 is the yellow sticks, *lexA2008* (R156C), that may potentially form an electrostatic interaction with E170 (white sticks). Neighboring E170 is *lexA2019* (N171D) (grey sticks). (D): Residues near the dimerization domain that may play a role in electrostatic structural stability. G124 is important for dimerization. In red sticks is *lexA2007* (D129G) and in blue sticks is *lexA2012* (R197D) which may make electrostatic interactions with each other to stabilize the dimerization domain of the protein. (E): Mutations that affect protein stability. In the tan spheres is *lexA2010* (P77S) that is a removal of a proline and in the maroon spheres is *lexA2013* (S103P) that is an addition of a proline. In the pink spheres is *lexA2014* (V146A) which makes up a part of the hydrophobic channel that the loop with the labile peptide bond buries to bring the cleavage site to the catalytic residues. Aside from destabilizing the protein, there is no obvious effect on any protein functions.

Table A1: Characterization of *lexA2005,3* suppressor mutants

Mutation	Relative SOS expression	Relative Protein amount (n=2)
lexA^{+}	1.7±0.2	0.3
lexA2003 (Δ*lexA*)	56.9±2.0	0.0
lexA2018 (Q8E)	50.7±5.0	0.7
lexA2007 (D129G)	40.1±1.3	0.2
lexA2009 (T27A)	35.4±0.9	0.4
lexA2019 (N171D)	21.0±0.9	0.3
lexA2013 (S103P)	12.7±0.4	0.4
lexA2006 (P25L)	10.9±0.4	0.7
lexA2008 (R157C)	10.1±0.5	0.4
lexA2012 (R197D)	4.1±0.2	0.1
lexA2015 (L50V)	3.2±0.1	0.4
lexA2014 (V146A)	3.0±0.3	0.5
lexA2016 (N41S)	2.7±0.1	0.8
lexA2010 (P77S)	2.6±0.2	0.4
lexA2017 (A42T)	1.7±0.1	0.8
lexA2005 (*lexA3*op)	0.0±0.1	1.0

Table 1: Characterization of *lexA3* overproduction suppressors. Relative SOS expression was measured by expression from *sulAp-mCherry*. Fluorescent intensity was normalized to region on agarose pad without cells. A series of three fields of view were taken over three days for a total of nine pictures. Pictures were taken at 1000X final magnification. Images were quantified as described in Materials and Methods in chapter 2. For Western blots intensity of bands for LexA was normalized to a nonspecific band that showed consistent banding intensity regardless of *lexA* mutation. For the different mutants, the band intensity was normalized to *lexA3op* which was served as "1". Relative protein amount is an average of two experiments.

Table A2: Strains used in this study

Strain	*lexA* allele	Source or origin
SS7117	+	Laboratory stock
11776	*lexA3*	This study
SS12333	*lexA3*	This study
SS12345	*lexA3,2006*	This study
SS12362	*lexA3,2007*	This study
SS12363	*lexA3,2008*	This study
SS12377	*lexA3,2010*	This study
SS12397	*lexA3,2021*	This study
SS12398	*lexA3,2009*	This study
SS12831	*lexA3,2013*	This study
SS12832	*lexA3,2014*	This study
SS12833	*lexA3,2015*	This study
SS12834	*lexA3,2016*	This study
SS12835	*lexA3,2017*	This study
SS12836	*lexA3,2018*	This study
SS12837	*lexA3,2019*	This study
SS12838	*lexA3,2012*	This study

All strains have a JC13509 background. JC13509 is derived from SK362 strain and has the following genotype: F^- *lacMS286* Φ*80dIIlacBK1 sulB103 argE4 his-4 thi-1 xyl-5 mtl-1* Sm^R $T6^R$. The *lacMS286* Φ*80dIIlacBK1* codes for two partial non-overlapping deletions of the lac operon (Konrad, 1977; Zieg & Kushner, 1977). All strains are derivatives of SS7117 which have Δ*attB:: sulAp-mCherry*, the SOS reporter. Unless notated otherwise, all *lexA* mutants generated in this study have *lexA2005 cat* in addition to noted mutations(Renzette et al., 2005).

5.2 B. SUMMARY OF SRF MUTANTS IN LITERATURE

Below is a table summarizing the current collection of *srf* (*s*uppressor of *r*ec*F*) mutants in the literature. The mutants are arranged in ascending order by allele number. Amino acid changes are provided in the second column if they are known. References to where these mutants are described are given in the fourth column.

The *srf* mutations listed below were mapped onto the RecA protein crystal structure (Figure C1). The mutations do not localize to any particular locus on the protein, rather they span the entire molecule.

Allele number	AA change	SOS[a]	Reference
441[bc]	E38K, I298V	C	(Knight et al., 1984; Sassanfar & Roberts, 1991; Thoms & Wackernagel, 1988; Volkert & Hartke, 1984; T.-C. V. Wang et al., 1993; Witkin et al., 1982)
718	E38K, L126V	N	(McCall et al., 1987; T.-C. V. Wang et al., 1993)
720	G204S, T39I	N	(T.-C. V. Wang et al., 1993)
727	G204S, E18K	N	(T.-C. V. Wang et al., 1993)
730[c]	E38K	C	(Britt et al., 2010; Centore & Sandler, 2007; Gruenig et al., 2008; Lavery & Kowalczykowski, 1992; Long et al., 2009; Massoni et al., 2012; McCool et al., 2004; Renzette et al., 2007; Renzette & Sandler, 2008; T.-C. V. Wang et al., 1993; Witkin et al., 1982)
750	E38K, L126V, Unknown	N	(T.-C. V. Wang et al., 1993)
801	Q257P	?	(Thoms & Wackernagel, 1988; Volkert & Hartke, 1984; T. C. Wang & Smith, 1986; T. C. V. Wang et al., 1991)
802	Unknown	?	(Volkert & Hartke, 1984)
803[c]	V37M	N	(Lavery & Kowalczykowski, 1992; Long et al., 2009; M. V. Madiraju et al., 1988; M. V. V. S. Madiraju & Clark, 1990; Sandler & Clark, 1994)
1202[c]	Q184K	C	(Liu et al., 1993; W. B. Wang, Sassanfar, et al., 1988; W. B. Wang, Tessman, et al., 1988; W. B. Wang & Tessman, 1986)
1235	T39I	N	(W. B. Wang & Tessman, 1986) (Van Alstine, this work, unpublished data)
2020	T121I	?	(T. C. Wang & Smith, 1986; T. C. V. Wang et al., 1991)
4001[e]	Unknown	?	(Thoms & Wackernagel, 1988)
4011[e]	Unknown	?	(Thoms & Wackernagel, 1988)
4142[df]	F217Y	C	(Long et al., 2008, 2009, 2010; Skiba & Knight, 1994; Zutter et al., 2001)
4161[cf]	ΔC17	C	(Britt et al., 2010; Eggler et al., 2003; Lusetti, Shaw, et al., 2003; Lusetti, Wood, et al., 2003)
4187	(E38H)	C	Van Alstine et al. 2022
4186	(E38Q)	N	Van Alstine et al. 2022

4201	(E38N)	N	Van Alstine et al. 2022
4185	(E38S)	N	Van Alstine et al. 2022
4204	(Q184R)	C	Van Alstine et al. 2022
4205	(Q184H)	N	Van Alstine et al. 2022
4197[f]	(D36K)	C	Van Alstine et al. 2022

[a] A subset of *srf* mutations also confer a constitutive expression phenotype. The strains that exhibit constitutive SOS expression have a "C" in this column. Strains that exhibit normal SOS expression have an "N" in this column. Strains where the character of SOS expression was not determined have a "?" in this column.

[b] Strains with this mutation exhibit normal SOS expression at 30ºC and exhibit constitutive SOS expression at 42ºC.

[c] This mutation has also been examined *in vitro* and found to have an increased ability to compete for single stranded DNA compared to wildtype RecA.

[d] This mutation has also been examined *in vitro* and found to have an increased cooperativity compared to wildtype RecA

[e] This mutation's ability to suppress *recF* is reliant on *recJ*.

[f] Strains with these mutations exhibit normal SOS expression when expressed with the wildtype operator and exhibit constitutive SOS expression when combined with the *o1403* operator mutation, which increases the basal level of transcription 2 to 3-fold.

Summary of how these mutations were isolated and briefly characterized

While Françous Jacob was attempting to isolate temperature sensitive mutants of the lambda CI repressor, he and colleagues discovered a mutation that led to lethal filamentation and prophage induction that was suppressed at lower temperatures, but was expressed at elevated temperatures (GOLDTHWAIT & JACOB, 1964). This mutation, then initially called *tif-1* which stands for temperature induced filamentation, was later called *recA441* and was discovered to have two mutations. The first mutation, later called *recA730* (E38K), was responsible for the phenotype that we now know as the SOS response. The second mutation, later called *recA4162* (I298V), suppressed the constitutive SOS response phenotype at the lower temperatures. The *recA730* (E38K) mutation was discovered again independently by Ethel Tessman as *recA1211*.

Evelyn Witkin and colleagues, while studying *recA441*, were able to find another intragenic suppressor that suppressed the SOS constitutive phenotype, but this time at all temperatures. The double mutant was called *recA718*. The suppressor, later called *recA4164* (L126V), was found, along with *recA4162*, to suppress the SOS constitutive phenotype of *recAo1403 recA4142* (Long et al., 2009).

Volkert and colleagues directly selected for *recF* suppressors using a *recBC sbcBC recF* strain finding three mutants: *srf-801*(Q257P), *srf-802* (amino acid changed not determined), and *srf-803*(V37M). All these mapped to *recA* so therefore had their names changed to "*recA*" instead of "*srf*"(T. C. V. Wang, Madiraju, Templin, & Clark, 1991) *recA4001* and *recA4011* were discovered by Thoms and Wackernagel in a *recBC sbcB recF lexA51* strain. This strain grew very slowly so they were able to find suppressors that grew faster that spontaneously arose during growth. These were found to be reliant

on *recJ* for suppression, but have not been defined at a molecular level (Thoms & Wackernagel, 1988). *srf-2020* (T121I) was isolated by selecting UV radiation-resistant clones from UV-irradiated *ΔuvrB recF* cells (T. Wang & Smith, 1986). It was cloned along with *recA801* for preliminary characterization by (T. C. V. Wang, Madiraju, Templin, & Clark, 1991).

UmuCD' is the error-prone DNA polymerase V that has a role in SOS mutagenesis (Burckhardt et al., 1988). The PolV holoenzyme cannot form until the subunit UmuD interacts with a RecA filament and cleaves itself. The cleavage product, UmuD', is then able to complex with UmuC and start DNA synthesis. Cells that have SOS turned on constitutively are also expressing active PolV and therefore exhibit a greater spontaneous mutability than wildtype. Sweasy and colleagues found mutations in *recA* that exhibited a greater spontaneous mutability than *recA*$^{+}$ (Sweasy et al., 1990). *recA750* (E38K, L126V, third aa change not known) was selected as a temperature-resistant revertant of *recA718* (E38K, L126V) with *poIA12* mutation and does not grow on rich media at 37ºC. *recA720* (G204S, T39I) and *recA727* (G204S, E18K) were isolated as fully UV resistant and partially UV resistant suppressors of *recA430*. *recA430* is a mutation in loop 2 which prevents RecA from polymerizing on ssDNA as effectively as wildtype (Moreau & Roberts, 1984). A caveat with the Sweasy strains is these are *E. coli* B strains and they all have a *lon-11* mutation which is an IS in the promoter region of *lon*.(Sweasy, Witkin, Sinha, & Roegner-Maniscalco, 1990) Based on the assumption that SOS constitutive mutants of *recA* were mutants of RecA that bound to ssDNA tighter, it was tested by Wang and colleagues if a subset of these mutations (*recA718, recA720, recA730,* and *recA750*) along with *recA441* and *recA2020* were later tested to see if they would

suppress a *recF* mutation (T.-C. V. Wang, Chang, & Hung, 1993a). *recA730* (E38K), *recA720* (G204S, T39I), *recA750* (E38K, L126V, unknown), *recA441*(E38K, I298V) *and recA2020* (T121I) all were able to suppress *recF*.

Ethel Tessman in 1986 mutagenizing lambda phages containing the *recA* gene and grew them on UV irradiated wildtype *E. coli* strains and screening for dark blue plaques using a Mu d(Ap lac) transcriptional fusions of *dinD* promoter and *sulA* promoter found 5 novel RecA point mutants (*recA1202*(Q184K), *recA1212*(A179V), *recA1219*(E158K), *recA1222*(S25F), *recA1235*(T39I)) that are coprotease constitutive but still maintained wildtype level of recombination ability (Tessman & Peterson, 1985; W. Wang & Tessman, 1986). She also found 6 other point mutants that were SOS^C but did not maintain recombination proficiency. One of particular interest is *recA1235* (T39I) which is right next to E38. Tessman reported this as a SOS^C mutant that is rec^+; however, in our hands the mutation did not confer a constitutive SOS response. However, we did test to see if it was *srf* and confirm that it was (unpublished results). The SOS^C mutants in the Ethel Tessman study have not been as rigorously studied as *recA730* or *recA803* and suppressors for only one of the mutants has been published.

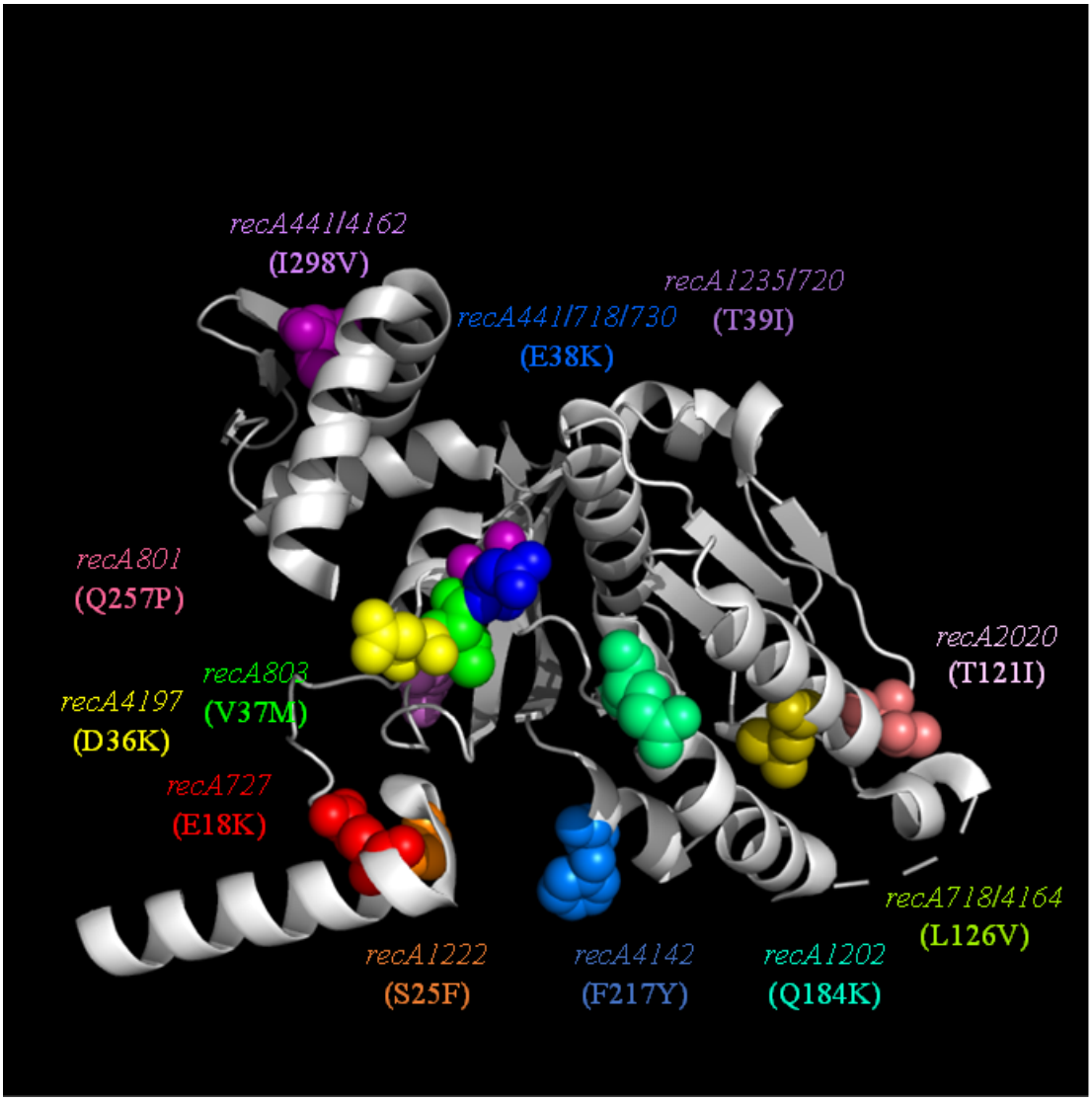


Figure B1: Location of color coded *recA srf* mutations on 2REB RecA crystal structure. Double mutants are listed first and then the name of it as a single mutant is given last. *recA441* and *recA718* are double mutants that share E38K. *recA720* and *recA727* are double mutants that share G204S (not shown).

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