EXPRESSION AND FUNCTION OF THE DNA DAMAGE PROTEIN UMUD OF ACINETOBACTER BAYLYI, AND ITS PREDICTED CLEAVAGE AND NUCLEOPHILIC ACTIVATOR RESIDUE MUTANTS IN ESCHERICHIA COLI

A Thesis
Presented to
the Faculty of the College of Science & Technology
Morehead State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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July, 2011
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As one part of the cellular response to DNA damage, an error prone polymerase (DNA polymerase V, composed of an UmuD'-UmuC protein complex), conducts SOS mutagenesis in *Escherichia coli*. However, the protein UmuD present in *Acinetobacter baylyi* strain ADP1 (UmuDAb) contains an extra N-terminal region. Furthermore, observations made in our lab have shown that ADP1 does not respond to DNA damage by SOS mutagenesis because of the absence of *umuC* gene. UmuDAb upregulates the transcription of *ddrR* (a DNA damage-responsive gene) in ADP1. We are thus investigating the function of this UmuDAb in the DNA damage response. Since UmuD of *E. coli* self-cleaves around 20 minutes after DNA damage to carry out SOS mutagenesis, and UmuDAb disappears ~20 minutes after mitomycin C (a DNA damaging agent) treatment, we hypothesize that UmuDAb also self-cleaves in response to DNA damage. We have created a mutation in the predicted cleavage site of UmuDAb. The mutated *umuDAb* gene was cloned into an expression vector and transformed into wild type, *umuD*, and *recA* strains of *E. coli*. Surprisingly, immunoblot analyses have shown that UmuDAb, mutated at the
predicted cleavage site of ADP1, still disappears after mitomycin C treatment when expressed in \textit{E. coli}. We have also created a mutation in a Lys-156 residue that, in \textit{E. coli}, activates nucleophilic attack mediated by serine at the predicted cleavage site. Immunoblot analyses have shown that UmuDAb that is mutated at the nucleophilic activator site does not disappear after mitomycin C treatment when expressed in \textit{E. coli}. Our observations conclude that lysine residue is essential for the cleavage of the protein like in \textit{E. coli}. Moreover, UmuDAb is expressed but does not disappear in \textit{recA} strains, which suggests that RecA is required for the self-cleavage of UmuDAb after DNA damage like in \textit{E. coli}. We thus speculate that UmuDAb cleaves in response to DNA damage and we are interested in studying the regulatory function of the cleaved UmuDAb in response to DNA damage. Future directions will involve more studies on the predicted cleavage site of ADP1 as well as the serine residue that mediates the cleavage of the protein.

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Chair
Acknowledgements

I would like to express my sincere thanks and deepest respect to Dr. Janelle Hare, the chairman of my thesis committee, for her continuous support. Her continued guidance, patience, research enthusiasm, and friendly attitude have provided me the inspiration to achieve the goal of conducting research and writing this thesis. I am very grateful to her for bearing with all the troubles and extending extra help toward my research project.

I would also like to extend my special thanks and appreciation to Dr. Geoffrey Gearner and Dr. Michael Fultz, who served as members of my thesis committee and for their continued guidance, suggestions, and support throughout the research project. I would also like to thank Dr. Douglas Dennis for providing the XL-10 supercompetent cells, and Dr. Leslie Gregg-Jolly and the Walker lab for providing the bacterial strains used in the study. A special thanks also goes to Alison Grice, Lynn Eisenhour and all the members of the Hare and the Dennis lab for creating a friendly environment in the lab and technical support.

Finally, I wish to thank my father Dilli Raj Adhikari, brothers Sanjeev and Saroj, sister-in-laws Samana and Sobita for their strong support and encouragements, and I would like to dedicate this thesis to my late mother Shova Adhikari.
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Introduction

Genomic DNA replication is necessary for the continuity of life, but as various endogenous or exogenous factors can damage DNA, cells must implement a variety of DNA damage repair mechanisms in order to sense and repair these damages. There are two main ways cells can sense and repair damages: error-free and error-prone repair mechanisms. Error-free repair mechanisms, as the name implies, repair the DNA damage without creating any mutations while error-prone mechanisms repair the DNA at the cost of increasing mutation loads in the DNA. Most research has been performed in *Escherichia coli* model system. Nevertheless, different types of organisms, prokaryotes and eukaryotes, have evolved various types of DNA damage repair systems and mechanisms. The focus of the research is to study the error-prone repair system, specifically SOS mutagenesis, a type of error-prone repair mechanism that has been extensively studied in *E. coli*.

This thesis will discuss different types of DNA damage and DNA damage repair systems. The SOS response system in *E. coli* and our developing information on a similar response system in *Acinetobacter baylyi* strain ADP1 which encompasses error-free and error-prone repair systems, will be discussed. Mainly, my thesis will test if there is cleavage in an SOS response protein, UmuDAb, of ADP1 after DNA damage. With the help of mutations that I have created at the predicted cleavage site and nucleophilic activator site of the protein, this thesis work will primarily test if the
SOS response protein self-cleaves in response to DNA damage. UmuD of *E. coli* self-cleaves around 20 minutes after DNA damage to carry out SOS mutagenesis. Interestingly, UmuDAb also disappears ~20 minutes after mitomycin C (a DNA damaging agent) treatment, and we hypothesize that UmuDAb also self-cleaves in response to DNA damage.

**DNA damage**

*Sources of DNA damage*

Sources of DNA damage can be either endogenous (within the cells) or exogenous (sources outside of the cells). Many naturally found factors as well as man-made artificial agents can induce DNA damage (Ames, 1979). Endogenous sources of DNA damage include errors that occur during DNA replication when proofreading function of DNA polymerases have failed to fix them. The cellular environment itself is thus a continuous source of DNA damage (Cassimeris *et al.*, 2010). A wide variety of chemical agents can alter specific bases within DNA. For example, reactive oxygen species such as hydroxyl radicals react with guanine to form 8-oxoguanine. 8-oxoguanine pairs with adenine instead of cytosine during DNA replication, which results in spontaneous transversion DNA mutations (Snyder *et al.*, 2007; Tropp, 2008). Deamination is another process that causes DNA mutation. For example, nitrous acid (HNO₂) can cause deamination by converting adenine to hypoxanthine, which can then base pair with cytosine rather than thymine to cause DNA mutation (Berge *et al.*, 2007).
Nucleotide bases are also subject to alkylation in addition to oxidation and deamination. Alkylating agents include mitomycin C (MMC), aflatoxin B₁, nitrogen mustard gas (bis[2-chloroethyl]methylamine) and ethylmethane sulfonate (EMS). MMC (See Figure 1 for structure) is a potent DNA crosslinker and crosslinking by MMC is specific for the duplex DNA sequence CpG•CpG (Tomasz, 1995). The 2-NH₂ group of the strand opposite to guanine is required for selective monoalkylation. The 2-NH₂ group can form a hydrogen bond with the C10' oxygen of the activated MMC, and this non-covalent bond increases the rate of formation of the covalent bond with the target guanine (Tomasz, 1995) (Figure 2). Thus, the double helix structure of DNA can be distorted and results in replication arrest and cell death if the crosslink is not repaired. Other compounds such as psoralens produced by the Chinese herb, Psoralea corylifolia can form inter-strand cross-links, which also prevent replication and gene expression because the cross-linked strands cannot be separated (Berg et al., 2007).
Base analogs resemble the normal bases in DNA and are sometimes converted into a deoxynucleotide triphosphate and become incorporated into DNA. The incorporation of a base analog such as 2'-aminopurine (2-AP), and 5'-bromouracil (5-BU) can be mutagenic, as the base analog pairs with the wrong base. 2-AP resembles adenine and pairs with thymine, while 5-BU is an analog of thymine and wrongly base pairs with adenine (Snyder et al., 2007).

One of the major exogenous sources of DNA damage is radiation. Radiation can be subdivided into two types: UV radiation and ionizing radiation (X-rays and gamma rays). The ultraviolet (UV) component of sunlight is a DNA-damaging agent (Cassimeris et al., 2010). UV light functions by covalently linking adjacent pyrimidine residues (thymine-dimers) along a DNA strand. The intrastrand cross-
linking causes a distortion of the double helix structure of DNA as pyrimidine dimers can fit into a double helix and completely block replication and gene expression (Berg et al., 2007). X-rays and gamma-rays cause a variety of DNA lesions and these lesions can be a result of direct damage to the DNA or indirect damage when the radiation produces reactive species such as reactive oxygen species (ROS) in the cells. Ionizing radiation is lethal to the cells and a double-strand break is the most common DNA damage caused by ionizing radiation (Cassimeris et al., 2010).

Types of DNA damage

Exposure of DNA to endogenous or exogenous DNA damaging sources causes mutations in DNA. The mutations result from changes in DNA sequence or deletions, insertions or rearrangement of DNA sequence. A base substitution is the simplest type of mutation, where a nucleotide pair in a DNA is replaced with a different nucleotide. Some base substitutions replace a pyrimidine base with another pyrimidine or one purine base with another purine base; these are called transition mutations. On the other hand, while a pyrimidine base is replaced with purine base or one purine base is replaced with pyrimidine base, the substitution is referred to as transversion mutations. The mutations in DNA sequence as a result of these base substitutions can change an amino acid sequence, truncate the protein, or shift the reading frame.

A missense mutation or non-synonymous mutation changes results in one amino acid being replaced with another and this replacement in a protein alters the
biological property of the protein. Diseases such as phenylketonuria and sickle-cell anemia are caused by missense mutations. A nonsense mutation is a base substitution that creates a new stop codon, making a polypeptide truncated. The premature chain termination renders the polypeptide non-functional. A silent or synonymous substitution changes the nucleotide sequence without changing the amino acid sequence. A silent substitution is so called because it is not detectable by changes in phenotype. In addition, a frameshift mutation caused by addition or deletion of a single base shifts the reading frames of the codons in mRNA. A frameshift mutation results in synthesis of nonfunctional protein as the frameshift alters the amino acids downstream from the site of mutation (Hartl et al., 2002). DNA damaging agents used in this study are UV radiation, which induces frameshift mutation in bacteria (Hartman et al., 1971; and Miller, 1985) and mitomycin C (MMC), which induces either a base substitution or a frameshift mutation (Minear et al., 1995).

**DNA damage and repair systems**

When a cell's DNA is damaged, the cell senses and respond to the DNA damage with activation of an error-free repair system or error-prone repair system (Figure 3). Most of the SOS genes are induced rapidly after DNA damage and are involved in an error-free DNA repair, such as base excision repair (BER), nucleotide excision repair (NER), and recombinational DNA repair (Friedberg et al., 2006).
Figure 3. DNA damage repair mechanism in prokaryotes.
However, if the levels of DNA damage are tremendous, then error-free repair systems cannot repair the damage and the replication is halted. Now, the mutagenic phase of SOS response is triggered (Walker, 1984; Echols and Goodman, 1990). After sensing DNA damage, a cell’s repair machinery attempts to fix the DNA damage and it may lead to the following scenarios: 1) the DNA is repaired perfectly, also known as error-free DNA repair; 2) the damage cannot be repaired or the repair is incomplete and the cell undergoes apoptosis; and 3) the cell carries out error-prone repair with increased levels of mutations in DNA.

**Error-free DNA repair**

Most of the repair pathways can sense the initial DNA lesion and function by removing the lesion (either a single base or larger area in the damaged region). The repair pathways then use the information on an undamaged complementary strand to synthesize nucleotides in the damaged area and thus restore the information content of the DNA.

In some cases, however, DNA damage can be simply reversed through the action of an enzyme such as photolyase. The system is called a direct repair system or photoreactivation repair system (Schul *et al.*, 2002). The photoreactivation is so called because this type of repair occurs only in the presence of visible light. The photolyase enzyme uses the energy of an absorbed photon to directly bind to a cyclic pyrimidine dimer (CPD), which is the damaged area, and cleaves the cyclobutane ring, restoring the original normal structure of the pyrimidine monomers (Yasui *et al.*, 8...
The damaged DNA can also be directly repaired in a second type of direct repair by the action of a group of enzymes known as alkyltransferases (Pegg and Byers, 1992). A wide variety of these enzymes with specificities for different types of lesions have been reported in prokaryotes (Cassimeris et al., 2010). One of the alkyltransferase enzymes, DNA glycosylase, can remove a variety of alkylated substrates. Alykltransferases restore the original structure of a base by transferring the alky group from a damaged base to themselves (Krokan et al., 1997). The irreversible attachment of the alkyl groups makes these enzymes inactive, therefore these alkyltransferases are also known as “suicide enzymes” (Cassimeris et al., 2010).

Sometimes wrong nucleotides are mistakenly inserted by DNA polymerase III into the newly synthesized strand of DNA during DNA replication. The incorrectly inserted nucleotides are then usually removed by an enzymatic DNA repair process known as DNA proofreading. DNA polymerase I acts as nuclease and recognizes and removes mismatched bases by hydrolysis at the 5' end of the mismatched nucleotide. Since DNA polymerase I has a 3'-5' exonuclease activity, the removal of a nucleotide at the 5' end results in a free 3' OH group in the preceding base; this allows for the insertion of a correct nucleotide (Liu, 2007).

Another type of DNA damage repair mechanism, the excision repair system can be further sub-divided into three types: base-excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). BER removes bases damaged by alkylation, deamination, and oxidation (Cassimeris et al., 2010). Classes of enzymes known as DNA glycosylases (similar to alkyltransferases) and AP endonucleases are
the key players in the BER system. DNA glycosylase removes the damaged base from DNA and attaches it into an active site of the enzyme, and cleaves the N-glycosyl bond of the deoxyribose. The next stage of the pathway is followed by cleavage of the phosphodiester backbone so that abasic [apurinic/apyrimidinic (AP)] sites left in the DNA can be removed and replaced. AP endonuclease recognizes the AP sites and cleaves the DNA backbone 5' to the AP site. As a result, there is a gap in the DNA with a 5'-deoxyribose phosphate and 3'-OH, which can then be repaired by DNA polymerase I and DNA ligase (Cassimeris et al., 2010).

On the other hand, the nucleotide excision repair (NER) removes a large ~12 base region containing a DNA lesion. The resulting gap is the filled in with nucleotides using the intact DNA strand as a template for DNA polymerase. A wide range of DNA lesions such as thymine dimers resulting from UV irradiation, aflatoxin-damaged bases, and DNA cross-links can be repaired by the NER pathway. A mutation in human nucleotide excision repair (NER) genes can cause a disease called xeroderma pigmentosum (XP), which leads to an 1,000 fold increase in the incidence of skin cancer (Cassimeris et al., 2010).

The mismatch repair is a type of excision repair, which recognizes and repairs the DNA containing the mismatch by its replacement with the correct sequence using the complementary strand as a template. The major genes involved in MMR are mutH, mutL, and mutS (Cox, 1997). The MutH protein first binds to a mismatch and the MutL protein is recruited. The MutH-MutL complex then activates MutH endonuclease, which nicks the DNA at the nearest unmethylated GATC site (Iyer et
The nick acts as an entry point for helicase II to unwind the DNA containing the mismatch. Exonucleases degrade the unwound DNA containing the mismatch and DNA polymerase III fills in the resulting gap using the intact DNA strand as a template (Duckett et al., 1996).

**Error-prone DNA repair**

When error-free mechanisms cannot repair DNA damage, then error-prone repair systems come into play. The “SOS response” was first described and coined by Miroslav Randman in 1974 (Radman, 1974). The SOS response comprises over 20 unlinked genes such as recA, lexA, *umuDC*, polB, recN, sulA, uvrA, uvrB, and uvrD (Friedberg et al., 1995) and are induced after a cell is exposed to a DNA-damaging agent such as UV irradiation or chemicals (Witkin, 1976; Walker, 1984). Most of the SOS genes are induced rapidly after DNA damage and are involved in error-free DNA repair, such as base excision repair (BER), nucleotide excision repair (NER), and recombinational DNA repair (Friedberg et al., 2006). The SOS response is an inducible system and encompasses error-free and error-prone functions. The error-prone SOS response is responsible for mutagenic events that occur when DNA damage cannot be repaired in an error-free manner (Walker, 1984; Echols and Goodman, 1990) and thus is called SOS mutagenesis.

When cells experience lethal DNA damage and error-free mechanisms cannot repair the damage but cells are under pressure to replicate, an alternate *umuDC*-dependent process that leads to translesion synthesis (TLS) is induced (Bridges and
Woodgate, 1985; Sutton et al., 2000; Goodman, 2002). TLS repairs UV-induced DNA damages and induces errors in the general vicinity of lesions. The error-prone response system is usually the last inducible system in effect in response to DNA damage when error-free repair systems cannot cope with the damage (Lodish et al., 2000). The *umuC* and *umuD* genes that act on SOS mutagenesis are turned on ~30 min after the cells have been exposed to UV (Sommer et al., 1998).

These mechanisms of DNA repair such as error-free and error-prone repair mechanisms are also found in eukaryotes (Hubscher et al., 2002; Gan et al., 2008). Mutations in any of these DNA-damage repair pathways may lead to cancer in humans.
The SOS mutagenesis system

SOS mutagenesis in E. coli

The DNA damage in *E. coli* by agents such as UV light, mitomycin C, or fungal metabolites like aflatoxin B₁ generates a signal that activates RecA protein to a form (RecA*) that can promote the proteolytic self-cleavage of the LexA repressor (Horii *et al.*, 1981). The result of the inactivation of LexA repressor by proteolytic cleavage is the increased expression of SOS genes such as *umuD*, *umuC*, *recA* (Little *et al.*, 1982; Walker *et al.*, 1984) and many other SOS response genes such as *polB*, *recN*, *sulA*, *uvrB*, and *uvrD* (Friedberg *et al.*, 1995). LexA is a repressor protein that negatively regulates the transcription of the SOS genes and other genes by binding to a 20 nucleotide “SOS box” (Lewis *et al.*, 1992) in the promoters of these genes and interferes with the RNA polymerase transcription of the SOS response genes (Mount *et al.*, 1972, and Brent *et al.*, 1981). With the extensive amount of research done on *E. coli*’s SOS mutagenesis response, we know that Umu proteins play a causal role in damage-induced mutagenesis in *E. coli* (Steinborn, 1978; Kato *et al.*, 1977). *E. coli* *umu* genes are found in an *umuDC* operon. The *umuD* gene is smaller in size and overlaps *umuC* gene by one base pair (Gonzalez *et al.*, 2002). The *umuD* and *umuC* genes encode for proteins of 15.0 and 47.7 kDa, respectively (Kitagawa *et al.*, 1985). RecA* also facilitates the cleavage of UmuD to its shortened form UmuD'. UmuD and UmuD' can bind to each other to form a heterodimer or combine with a copy of itself, forming a homodimer. The resultant heterodimer or homodimer can combine with UmuC, forming a variety of different complexes, including the error-prone DNA
polymerase V that carries out SOS mutagenesis. The Umu(D)$_2$C complex participates in regulating the *E. coli* cell division cycle after DNA damage by slowing down the cell cycle and allowing more time for error-free repair mechanisms to act (Opperman et al., 1999). The UmuDD'C complex seems to be an inactive form and may play a role in shutting off SOS mutagenesis by binding with the UmuD'. The (UmuD')$_2$C complex (the error-prone DNA polymerase V) (Figure 4) causes SOS mutagenesis (Tang et al., 1999). Thus, the SOS mutagenesis system requires $\text{umuD}^+\text{C}^+$ and $\text{recA}^+$ and allows the cells to replicate through unrepaired lesions in the template DNA that cannot be repaired in an error-free manner (Tang et al., 1999). The survival rate of the cells is increased while mutation rate is elevated as a result of the translesion synthesis (Friedberg et al., 1995; Sutton et al., 2000).

There are two DNA polymerases that carry out SOS mutagenesis: DNA pol IV and DNA pol V. DNA pol IV was first discovered in 1999 and reported to be encoded by the SOS gene *dinB* (Wagner et al., 1999). DNA pol IV lacks intrinsic 3'-5' exonuclease proofreading activity (Tang et al., 2000; Goodman, 2002). The fidelity of the DNA pol IV is achieved by its ability to differentiate between correct and incorrect base formation. In a normal cell, the level of DNA pol IV is approximately 250 molecules per cell and this can increase up to 10-fold upon DNA damage (Kim et al., 2001).

DNA pol V is a Y-family polymerase and is a major translesion synthesis (TLS) polymerase in *E. coli* (Goodman, 2002). Although DNA pol IV (*dinB*) is induced rapidly after DNA damage (Qiu et al., 1997 and Kim et al., 2001), DNA pol
V (UmuD'2C) is not detectable until 20-40 min post SOS induction (Sommer et al., 1998). The delayed expression is useful to the bacterium because it provides an opportunity for the cell to repair the damaged DNA before the cells undergo mutagenic translesion synthesis (Pham et al., 2002).

DNA pol V was also first identified in 1999 (Tang et al., 1999, and Reuven, et al., 1999) and is encoded by the *umuDC* locus. DNA pol V consists of a dimer of post-translationally cleaved UmuD' protein complexed with a monomer of UmuC (Woodgate, et al., 1989). The amount of DNA pol V in a normal cell is 15 molecules per cell while it can reach 200 molecules per cell upon DNA damage (Woodgate et al., 1991). DNA pol V also lacks 3'-5' exonuclease proofreading activity, therefore it is considered a low fidelity DNA polymerase. DNA pol V is essential for most in vivo damage-induced SOS mutagenesis in *E. coli* because of its ability to bypass lesions when the replication fork is stalled due to lethal DNA damage.

**SOS Mutagenesis in non-*E. coli* models**

SOS mutagenesis has also been studied in many different bacteria such as *Bacillus* (Love et al., 1984; Love et al., 1986), *Mycobacterium* (Movahedzadeh et al., 1997), and *Xanthomonas* (Yang et al., 2002). Studies have shown that the SOS mutagenesis systems found in these bacteria are similar, but not identical, to that of *E. coli*.

The SOS box and LexA repressor proteins are found to be conserved among these bacteria. The *recA* gene has been found in all sequenced eubacterial genomes.
RecA protein possesses the following wide range of functions in different bacterial species: 1) the homologous DNA pairing and strand exchange with a homologous duplex of DNA (Lusetti and Cox, 2002), 2) induction of SOS response (Walker et al., 2000), and 3) interaction with the mutagenic proteins to carry out translesion synthesis (Bridges and Woodgate, 1985; Goodman, 2002). Proteins homologous to RecA are found in both archaea and eukaryotes. In archaea, the RecA homolog is called RadA (Seitz et al., 1998; Yang et al., 2001; Wu et al., 2004), while two RecA homologs, Rad51 and Dmc1 proteins are found in eukaryotes (Bishop et al., 1992; Shinohara et al., 1992; Sung, 1994). In contrast, the number of $\textit{lexA}$ genes present in bacteria is variable, ranging from zero in \textit{Helicobacter pylori} (Campoy et al., 2002) to two in \textit{Xanthomonas axonopodis} pathovar citri (Yang et al., 2005). In addition, the SOS box sequences are also found to vary between different bacterial classes, such as TACTG(TA)$_3$CAGTA for \textit{E. coli} (Walker, 1984), TTAG(N$_6$)TACTA for \textit{Xylella fastidiosa} (Campoy et al., 2002), CCAACRNRYGTTCYC for \textit{B. subtilis} (Winterling et al., 1998), and GGTT(N$_2$)C(N$_4$)G(N$_3$)ACC for \textit{Geobacter sulfurreducens} (Jara et al., 2003). Interestingly, no sequence similar to known SOS boxes are found in ADP1 (Hare et al., 2006; Rauch et al., 1996).
Figure 4. SOS response and UmuD action in *E. coli*. A dimer of UmuD' modified post-translationally forms a complex with a monomer of UmuC protein. The complex is a DNA polymerase V, which can bypass lesions in a DNA [leading to translesion synthesis (TLS)]. This figure is copied from Ferentz *et al.*, 2001.
**Acinetobacter**

**General microbiology**

*Acinetobacter* are Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative, coccobacillary gammaproteobacteria with a G+C content of 39% to 47% (Rossau *et al.*, 1991). Members of the *Acinetobacter* genus are considered ubiquitous organisms in the environment; they can be abundantly recovered from virtually all samples such as soil and surface water (Baumann *et al.*, 1968). Members of the *Acinetobacter* genus have been historically classified by various researchers under numerous different taxonomic names such as *Bacterium anitratum, Herellea vaginicola, Mima polymorpha, Achromobacter, Alcaligenes, Micrococcus calcoaceticus, and Moraxella glucidolytica* and *Moraxella lwoifii* (Bergogne-Berezin *et al.*, 1996); at present the genus *Acinetobacter* is classified in Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Pseudomonadales*, and Family *Moraxellaceae*.

Members of *Acinetobacter* species are typically non-pathogenic; however, pathogenic strains of *A. baumannii* (Peleg *et al.*, 2008), *A. lwoifii* (Rathinavelu *et al.*, 2003), *A. ursingii*, *A. schindleri* (Nemec *et al.*, 2000), and *A. baylyi* (Chen *et al.*, 2008) have been reported.

Some of the bacterial species of *Acinetobacter*, such as *A. baumannii* are increasingly becoming a source of hospital-acquired infections (Peleg *et al.*, 2008). The resistance of *A. baumannii* to disinfectants and its ability to survive for a
prolonged period of time in a hospital environment helps the organism in nosocomial spread (Peleg et al., 2008). The ability of *A. baumannii* to upregulate or acquire resistance determinants makes it one of the most important species of clinical relevance. Strains of *A. baumannii* have been found to be resistant to most of the known clinical antibiotics (Peleg et al., 2007), which requires this species to be added to the list of organisms challenging the modern antibiotic era. *A. baumannii* are commonly associated with hospital acquired pneumonia and wound infections but currently the incidence of infections involving the nervous system, skin and soft tissue, and bone appears to be increasing (Peleg et al., 2008).

**General features of ADP1 for being a good model organism**

*Acinetobacter baylyi* strain ADP1 is a ubiquitous soil and aquatic bacterium and has a capacity for natural transformation during late log phase (Young et al., 2005). Because *Acinetobacter* spp. can use a variety of carbon sources, they can be grown on simple media in lab (Barbe et al., 2004). With an optimal temperature between 30°C and 37°C, ADP1 can even grow slowly in room temperature (Metzgar et al., 2004). Because of its relatively small genome size of 3.7 Mbp, which has recently been sequenced (Barbe et al., 2004), ADP1’s genes can be readily manipulated in the lab. Moreover, ADP1 is a good candidate as an industrial microorganism because they are harmless to humans as they do not carry any genes classically associated with pathogenesis or virulence (Barbe et al., 2004). Moreover, several antibiotics that are commonly used against *E. coli* are also effective against
ADP1, and many antibiotic resistance cassettes used in *E. coli* can also be transferred in ADP1 with ease (Kok *et al.*, 1995). Additionally, ADP1’s property of natural competence (Young *et al.*, 2005) and a natural tendency toward homology-directed recombination (de Vries *et al.*, 2002) makes it a comparatively superior candidate over *E. coli* for research involving genetic manipulation. ADP1 in their exponential growth phase are approximately 10-100 times as competent as calcium chloride treated competent *E. coli* (Metzgar *et al.*, 2004). All of these characteristic features of ADP1 point to the fact that ADP1 is an excellent bacterial model and surpasses the expectations of *E. coli*. Furthermore, as a non-enteric gammaproteobacteria, ADP1 is an excellent model to examine basic cellular processes other than *E. coli*.

**DNA damage response in Acinetobacter**

*Acinetobacter* *shares features with E. coli in response to DNA damage*

After DNA damage, at least two DNA damaging response genes are induced in ADP1: *ddrR* (DNA damage-responsive gene) and *recA* (Hare *et al.*, 2006; Rauch *et al.*, 1996). In its response to DNA damage, *ddrR* requires RecA for induction (Whitworth *et al.*, 2000). However, ADP1 shows some unique features in response to DNA damage. ADP1 does not respond to DNA damage with SOS mutagenesis (Berenstein, 1987). The *recA* gene is induced by DNA damage in ADP1 as in *E. coli*, but unlike in *E. coli*, the induction of *recA* does not require the RecA protein (Rauch *et al.*, 1996). Moreover, several *E. coli* DNA damage proteins such as DinB, σ^{38}, SulA, FtsEX, and LexA are not found in *Acinetobacter* spp. (Robinson *et al.*, 2010).
*A. baylyi* strain ADP1 MutS protein has been shown to recognize mismatches that arise during DNA replication and homologous recombination similar to *E. coli* (Young *et al.*, 2001). The MutS protein preferentially recognizes transition mismatches and 1-bp frameshifts. However, the protein does not recognize neither transversion mismatches nor large insertions or deletions (Young *et al.*, 2001).

**Absence of LexA and SOS box**

The ADP1 recA and ddrR promoters lack a known SOS box (Gregg-Jolly *et al.*, 1994; Hare *et al.*, 2006). ddrR is encoded next to the ‘extra-long’ UmuDAb in many *Acinetobacter* species, especially in *A. baumannii* (Personal communication with J. Bradley and J. Hare). Furthermore, there is absence of a LexA homolog in ADP1. This means that *umuDC* operon in ADP1 is constitutively expressed and is not regulated by DNA damage or recA (Hare *et al.*, 2006).

**ADP1 and other bacteria have “extra-long” UmuD**

The *umuDAb* gene found in *A. baylyi* has an extra N-terminus region unlike *E. coli* and other bacteria. The *umuDAb* gene of ADP1 is 612 bp long and encodes a gene product of 203 amino acids, while the *umuD* gene found in *E. coli* and other bacteria encodes 140 amino acids (Figure 5). The *umuD* found in ADP1 is thus about 1.5 times the size of *umuD* in *E. coli* and other bacteria (Hare *et al.*, 2006). This means that *umuDAb* gene of ADP1 has a capacity to encode a larger protein and this larger size may be what carries out a different regulatory response to DNA damage.
than $umuD$ of $E. \text{coli}$. Research has shown that the functioning of the $umuDAb$ encoded gene product is unlike other bacteria such as $E. \text{coli}$ (Hare et al., 2006).

Other bacteria such as $Synechococcus \text{elongatus}$, $Legionella \text{pneumophila}$ strain Lens, and $\text{Chromobacterium violaceum}$ have “extra-long” UmuD similar to ADP1 (Figure 8). The function of these “extra-long” UmuD homologs have not been studied in these bacteria but their sequence was identified in the sequenced genomes (Hare et al., 2006).

$umuC$ of ADP1 is mutated

The $umuC$ gene of ADP1 is interrupted and incomplete, and there is also an insertion sequence fragment in between the interrupted $umuC$ gene fragments (Figure 5; Hare et al., 2006). The $umuC$ fragments together contain 43% of the length of $E. \text{coli} \umuC$ gene (Hare et al., 2006). The 348-bp fragment of $umuC$ is positioned 5.9 kbp downstream of $umuDC$, which encodes 114 amino acids of UmuC with 28% identity to UmuC (Hare et al., 2006). The mutated $umuC$ gene encodes 39 amino acids with 85% identity to the amino-terminal end of UmuC. The $umuC$ fragments are interrupted by a putative transposase gene, which is 72 bp long and is homologous to ISEhe3, and is found in the opposite orientation (Hare et al., 2006). Moreover, ADP1 does not respond to DNA damage by SOS mutagenesis (Hare lab, data not shown), which correlates with the fact that ADP1 has a mutated $umuC$. This feature of ADP1 is unusual when compared to $E. \text{coli}$ model system. Thus, experiments performed in
ADP1 and its DNA damage response system might give us new insights into how UmuDAb acts, aside from its action with UmuC in DNA pol V.
Figure 5. Differences in ADP1 from *E. coli* model. The *umuDA*b is 1.5 fold longer than *umuD*. The *umuC* gene of ADP1 is interrupted and incomplete, and there is also a presence of insertion sequence fragment in between the interrupted *umuC* gene. A genetic locus, *ddrR* is present in ADP1, which has no homologs in *E. coli* or any other bacteria. This figure is adapted from Hare *et al.*, 2006.
**SOS proteins UmuD and LexA belong to a serine-protease family that self-cleaves**

UmuD and LexA both belong to a serine-protease family that uses a serine-lysine catalytic dyad mechanism that helps in the self-cleavage of the protein (Paetzel et al., 1997). The Ser-119 and Lys-156 residues of LexA are key residues needed for this cleavage (Little, 1991). First, the Ser-119 acts as a nucleophile and attacks the peptide bond in the cleavage site. Second, the uncharged Lys-156 acts as an activator by helping a proton to leave the serine hydroxyl group by donating a proton to the leaving amino group. Activated RecA (RecA*) facilitates this cleavage of LexA by lowering the pKₐ of the Lys-156 residue and thus indirectly helps in the cleavage of LexA (Figure 7). The peptide bond is now cleaved and a covalent ester intermediate is formed (Little, 1991).

The percent identity between MucA, a UmuD homolog and the putative UV protection protein of *Salmonella enteria* serovar Typhi (Horii et al., 1981) and LexA is ~28%, while UmuD is 31% homologous to amino acids in the COOH-terminal domain of LexA (Perry et al., 1985) (Figure 6). *E. coli* UmuD is 140 amino acids and shares carboxyl-terminal homology with LexA (Walker, 1996). The sequence homology between UmuD and LexA suggests that RecA* interacts with UmuD (Perry et al., 1985). Firstly, RecA* initiates the proteolytic cleavage of LexA. Secondly, the interaction of RecA* with UmuD results in the post-translation cleavage of UmuD, which precedes in a similar fashion as that of LexA repressor. RecA* is also thought to play a role in helping UmuD direct to its site of action.
(Perry et al., 1985). The post-translation cleavage of UmuD is essential as only the
dimer of UmuD' complexes with UmuC to form DNA pol V to carry out SOS
mutagenesis.
<table>
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<td>LexA</td>
<td><strong>PLVG-RVAAGEPLLAQIQHIEGHYQDVPSLFLK-PNALFLKVSGMNDKDIGMGLDLAYHTQDVVRNGQVYVARIDDEVT</strong>KRL</td>
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<tr>
<td>UmuD</td>
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<tr>
<td>MucA</td>
<td><strong>PFYIQRISAG FPSPAGYEQELNLHEYCVRHPGTSYLFLRVS <strong>GSS</strong>MED</strong>GRIHDD<strong>D</strong>VVL<strong>D</strong>RTLSA<strong>HGSIVVACIHEFTK</strong>RL</td>
</tr>
</tbody>
</table>

**Figure 6. Homology of UmuD and MucA proteins to the LexA protein.** Underlined amino acids indicate the identities between the LexA repressor and either the UmuD or MucA proteins. The Ala-Gly cleavage site of LexA is between amino acids 112 and 113 while the Cys-Gly putative cleavage site of UmuD is between amino acids 24 and 25. Adapted from Perry et al., 1985.
Figure 7. Mechanism of LexA repressor cleavage. Ser-119 and Lys-156 are essential for effective cleavage of the LexA repressor. RecA* is thought to play a role in the cleavage by lowering the pKₐ of Lys-156. UmuD cleavage after DNA damage is also assumed to proceed by a similar mechanism. This figure is adapted from Little, 1991.
**Does UmuDAb self cleave after DNA damage?**

Studies have shown as mentioned above, that after DNA damage UmuD gets expressed in *E. coli*, and then self-cleaves post-translationally to form truncated UmuD'. A dimer of UmuD' can then combine with UmuC protein to form DNA pol V, which can then carry out SOS mutagenesis. Similarly, in ADP1, at least two DNA damaging response genes are induced in ADP1: *ddrR* (DNA damage-responsive gene) and *recA* (Hare *et al.*, 2006). The induction of *ddrR* is dependent on the activation of UmuDAb. Therefore, the question we pose is: Does UmuDAb self-cleave to induce *ddrR*? To understand if UmuDAb indeed regulates *ddrR* expression after DNA damage, there is therefore a need to examine whether a self-cleavage of UmuDAb occurs after DNA damage.

**Goals for the thesis**

The possible cleavage of UmuDAb in *A. baylyi*’s response to DNA damage is poorly understood. However, UmuDAb contributes to DNA damage response by regulating *ddrR*. But we do not know if UmuDAb self-cleaves in response to DNA damage like *E. coli* UmuD or if such self-cleavage is required for its regulation of *ddrR*. Therefore, as a first step, we are interested in studying whether UmuDAb self-cleaves when induced to DNA damage or how UmuDAb behaves in response to DNA damage. At the same time, we are also interested in examining the effects of changing amino acid encoded by *umuDAb* on the potential cleavage of the gene.
product after inducing the cells to DNA damage. This will tell us if cleavage, if it is indeed happening, proceeds like in the *E. coli* model.

The cleavage site of *E. coli* UmuD is the bond between Cys-24 and Gly-25 (Perry *et al.*, 1985). Nohmi *et al* (1988) observed that by changing the Gly-25 residue of the putative Cys-Gly UmuD cleavage site to a glutamic acid or lysine residue significantly reduced the ability of UmuD to function in SOS mutagenesis. Changing the Cys-24 to a tyrosine residue caused a less severe reduction in the ability of UmuD to function in UV mutagenesis. Moreover, changing either Ser-60 or Lys-97 to an alanine residue drastically reduced the ability of UmuD to function in UV mutagenesis. Changing Ser-60 to a cysteine residue resulted in a less severe reduction in UmuD’s ability to function in UV mutagenesis than the Ser-60 to an alanine residue change (Nohmi *et al.*, 1988).

UmuDAb, the UmuD homolog of *A. baylyi* has many amino acids that involved in nucleophilic attack at the Cys$^{24}$-Gly$^{25}$ in *E. coli* and the lysine-$97$ that assists are essential in *E. coli* for Rec-A facilitated UmuD self-cleavage. Serine-$60$, which is in the nucleophile attack are well conserved in UmuDAb. The putative cleavage site in UmuDAb is Ala$^{83}$-Gly$^{84}$ (Hare *et al.*, 2006) unlike Cys$^{24}$-Gly$^{25}$ in UmuD of *E. coli*. However, studies have shown that the cysteine in UmuD when substituted for alanine, did not affect the biological function of the *umuD* gene product (Lee *et al.*, 1994). This observation leads us to an assumption that the UmuD and UmuDAb behave in an identical way. However, future studies will examine the
function and regulatory characteristic of the extra N-terminus amino acids of UmuDAb.

Multiple sequence alignment of UmuD and UmuD homologs suggests that alanine-83/glycine-84 is the cleavage site of UmuDAb since the serine and lysine residues needed for cleavage are conserved in *E. coli* UmuD and UmuD homologs in other bacteria (Figure 8). A helpful way to illustrate if the predicted cleavage site in UmuDAb is indeed where the protein cleaves would be to create different types of mutations in either the cleavage site or the residues that are essential for cleavage. The three goals of my project are to: 1) study expression and cleavage of normal UmuDAb after DNA damage, 2) mutate alanine in the putative Ala-Gly cleavage site to glutamic acid and study the expression of the mutated gene product in *E. coli* wild type and *recA* strains by Western analyses, and 3) mutate the lysine to alanine in the nucleophilic activator residue that helps in the cleavage of UmuD and study the expression of similar *E. coli* wild type and *recA* strains.

In this study, UmuDAb and its mutant versions will be expressed in *E. coli* because we understand how SOS mutagenesis occurs in *E. coli*. *E. coli* can uptake the plasmids with the *umuDAb* gene or any gene of interest easily. It is helpful for us to study about a protein in an *E. coli* model system where majority of pathways are understood rather than in ADP1 where less is known about different metabolic and physiologic pathways. The ease of obtaining different mutant strains of *E. coli* such as *umuD* and *recA* also make *E. coli* an excellent model system for us in order to examine the expression and function of UmuDAb.
Figure 8. Multiple alignment of UmuD homologs in different bacterial strains. The serine and lysine residues (second and third boxes) required for self-cleavage of UmuD in *E. coli* are well conserved in UmuDAb and other UmuD homologs. The cleavage site of *E. coli* UmuD is the bond between Cys-24 and Gly-25, while the multiple alignment suggests that the predicted cleavage site of UmuDAb is the bond between Ala-83 and Gly-84. This figure is adapted from Hare et al., 2006.
Methods and Materials

**Bacterial strains**

The bacterial strains used in this project are listed in Table 1: *Acinetobacter baylyi* strain ADP1, AB1157 (*Escherichia coli* K-12 wild type), AB2463 (*E. coli recA13*, isogenic to AB1157), 315 (*E. coli umuD*, isogenic to AB1157), DH5α (*E. coli recA*), and XL-10 Gold Ultracompetent cells.

All of the bacterial strains examined in this study were grown in nutrient agar (NA) or Luria-Bertani (LB) broth or tryptic soy broth (TSB). All of the bacterial strains were grown at 37°C.
<table>
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<th>Description</th>
<th>Relevant Characteristic(s)</th>
<th>Source</th>
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<td>Wild type, Gregg- Jolly lab communication with L. Grein-Jolly</td>
<td>Gregg-Jolly lab</td>
<td>Personal communication with L. Gregg-Jolly</td>
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<td>AB1157</td>
<td><em>Escherichia coli</em> K-12</td>
<td>F-, thr-1, araC14, leuB6(Am), Δ(gpt-promA)Δ2, lacY1, tsx-33, qsr'0, glnV44(Am), galK2(Oc), &amp; lambda', Rac-0, hisG4(Oc), rfbC1, mgl-51, rpoS396(Am), psL31(strR), kdgK51, xylA5, mtl-1, argE3(Oc), thi-1</td>
<td>Walker lab</td>
<td>Dewitt <em>et al.</em>, 1962</td>
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<td>AB2463</td>
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<td>Unpublished, personal communication with P. Buening of the Walker lab</td>
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<td>Stratagene</td>
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Plasmids used

The plasmids used in this project are listed in Table 2. The pJH1 plasmid contains 2.2 kbp ADP1 chromosomal DNA encompassing the $umuDC$ operon and the $ddrR$ locus cloned into pUC19, which confers ampicillin resistance (Hare et al., 2006). The pJH1 plasmid presumably contains a promoter 5' upstream of $umuD$. Construction of plasmids pIX-1 and pIX-2 was based upon pJH1.

QIAGEN's EasyXpress Protein Synthesis Kit was used to construct the plasmids pIX-1 and pIX-2 that carry the $umuDAb$ gene from pJH1 and which were used in the Kit's two-step PCR process for generating PCR products for in vitro translation. The first round PCR uses sense and antisense primers that bind to the $umuDAb$ open reading frame (ORF) and adds extra 'attachment' base pairs for round 2 PCR. In round 2 PCR, the adapter primers provided by the kit that encode an N-terminal histidine tag or affinity tag attach to the 'extra' bp from round 1 PCR. The adapter primers add a T7 promoter, ribosomal binding site, and T7 transcription terminator. The overall two-step PCR process results in PCR products that contain multiple cloning sites well-suited for cloning into the EasyExpress pIX3.0 vector. The PCR products, once cloned into pIX3.0 (Qiagen), can produce larger amounts of protein in large scale in vitro reactions. Another benefit of this cloned expression construct is that the expression construct can be used to transform *E. coli* cells for conventional in vivo expression. The pIX-1 and pIX-2 plasmids were constructed by G. Howington and S. Wheeler in 2009. The pIX-2 plasmid contains the 612 bp ORF.
of umuDAb plus a ribosomal binding site, and T7 terminator cloned into QIAGEN’s EasyExpress pIX3.0 vector and thus encodes a N-terminus histidine-tagged UmuDAb. The pIX-1 plasmid contains the 612 ORF of umuDAb cloned after T7 promoter in the pIX3.0 vector, but encodes no histidine-tag in either the N- or C-terminus of UmuDAb.

Both plasmids pIX-2GtoE and pIX-2KtoA are derived from pIX-2. The plasmid pIX-2GtoE constitutively expresses UmuDAb that has a mutation in its predicted cleavage site (Gly-84➔Glu). Similarly, the plasmid pIX-2KtoA constitutively expresses UmuDAb that has a mutation in its nucleophilic activator site (Lys-156➔Ala).
Table 2. Plasmids used in this study

<table>
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<th>Plasmids</th>
<th>Description</th>
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</tr>
</thead>
<tbody>
<tr>
<td>pJH1</td>
<td>~2.2 kbp <em>Acinetobacter baylyi</em> strain ADP1 chromosomal DNA encompassing the <em>umuDC</em> operon and <em>ddrR</em> locus cloned into the ampicillin resistant pUC19 cloning vector</td>
<td>Hare <em>et al.</em>, 2006</td>
</tr>
<tr>
<td>pIX-1</td>
<td>612 bp of pIX3.0 containing umuDAb plus a T7 promoter, ribosomal binding site, and T7 terminator, no affinity tag</td>
<td>This study</td>
</tr>
<tr>
<td>pIX-2</td>
<td>612 bp of pIX3.0 containing umuDAb plus a T7 promoter, ribosomal binding site, and T7 terminator, N-terminus histidine-tagged</td>
<td>This study</td>
</tr>
<tr>
<td>pIX-2GtoE</td>
<td>612 bp of pIX3.0 containing umuDAb plus a T7 promoter, ribosomal binding site, and T7 terminator with predicted cleavage mutation, N-terminus histidine-tagged, derived from pIX-2</td>
<td>This study</td>
</tr>
<tr>
<td>pIX-2KtoA</td>
<td>612 bp pIX3.0 containing umuDAb plus a T7 promoter, ribosomal binding site, and T7 terminator with nucleophilic activator mutation, N-terminus histidine-tagged, derived from pIX-2</td>
<td>This study</td>
</tr>
</tbody>
</table>
Making mutations in UmuDAb

In previous experiments performed by Nohtni et al. (1987), a mutation from glycine to glutamic acid at the second amino acid residue of the C²⁴-G²⁵ cleavage site of E. coli UmuD reduced SOS mutagenesis drastically. Similarly, a mutation of the nucleophilic activator residue lysine to alanine had a severe effect on SOS mutagenesis. So, mutations were constructed at these locations in UmuDAb, using the QuickChange™ Site-Directed Mutagenesis System (Stratagene, CA), a common and widely applicable PCR-based protocol for creating site-directed mutations.

The plasmids pIX-2 or pJH1 were PCR amplified with the sense and antisense primers and other contents as shown in Table 4. The designed primers are complementary to the opposite strands of the plasmids. These primers are extended by PfuTurbo DNA Polymerase during the temperature cycling of the procedure. The amplified PCR products were restriction digested with DpnI to get rid of original non-mutagenized parental DNA and were transformed into XL-10 Gold Ultracompetent cells (Stratagene). The mutagenized plasmids, after purification from XL-10 cells, were transformed into E. coli wild type (AB1157) and umuD strains (315) and selected on 100 µg/mL ampicillin media plates.

Primer Design

The primers for the site-directed mutagenesis were designed using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, catalog no. 200518) instruction manual, which was used to follow the parameters of the sense and
antisense primers. An automated tool at the Agilent website was used to design the primers (http://www.genomics.agilent.com). The umuDAb sequence (ACIAD2729) (Barbe et al., 2004, and www.genoscope.cns.fr/agc/mage/acinetoscope), was used as a template for designing primers. When designing a primer for mutagenesis purposes, these characteristics must be strictly followed: 1) Both of the forward and reverse primers must be contain the desired mutation and anneal to the sequences on opposite strands of the plasmid; 2) Primers should be between the lengths of 25 & 45 bases; 3) Primers should have a melting temperature (Tm) of ≥ 78°C, 4) The desired mutation should be in the middle of the primer with ~ 10-15 bases of the correct sequence on both sides; 5) The primers should have a minimum GC content of 40% and preferably terminate in one or more G or C bases; 6) Primers must be purified by polyacrylamide gel electrophoresis (PAGE) or high-performance liquid chromatography (HPLC) (QuickChange™ Site-Directed Mutagenesis Kit).

After their design, primers were constructed and purchased from Integrated DNA Technologies. The forward and reverse primers for the mutated glycine to glutamic acid residue in the cleavage site were g181a_t182gS and g181a_t182gAS respectively. Similarly, forward and reverse primers K97ASense and K97AntiSense were constructed and purchased for the mutated lysine 156 to alanine nucleophilic activator residue mutation. The mutation primers used in this study to create site-directed mutagenesis are listed in Table 3.
<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Purpose</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>g181a_t182gS</td>
<td>Forward primer for mutation of glycine to glutamic acid residue at the</td>
<td>5'-CAGAACGTGTTGCTGCTGAGTTGCCATCACCTGCACAG-3'</td>
</tr>
<tr>
<td></td>
<td>predicted cleavage site</td>
<td></td>
</tr>
<tr>
<td>g181a_t182gAS</td>
<td>Reverse primer for mutation of glycine to glutamic acid residue at the</td>
<td>5'-CTGTGCAAGGTGATGGCAACTCAGCAGCAACACGTTTCTG3'</td>
</tr>
<tr>
<td></td>
<td>predicted cleavage site</td>
<td></td>
</tr>
<tr>
<td>K97ASense</td>
<td>Forward primer for mutation of lysine to alanine residue at the</td>
<td>5'-GTTGACAATGAGTTTACGGTGACCCGTCGTGATCGATCATC-3'</td>
</tr>
<tr>
<td></td>
<td>nucleophilic activator site</td>
<td></td>
</tr>
<tr>
<td>K97AntiSense</td>
<td>Reverse primer for mutation of lysine to alanine residue at the</td>
<td>5'-GATGATCGATCATCAGACGGGCTACCCTAAACTCATGCTA-3'</td>
</tr>
<tr>
<td></td>
<td>nucleophilic activator site</td>
<td></td>
</tr>
</tbody>
</table>
PCR Amplification

The purpose of PCR amplification was to produce large amounts of mutated DNA products from a small amount of the original, wild type DNA encoding $\textit{umuD}$.

The sample reaction was prepared as follows:

Sample Reaction Preparation

The following contents were placed in a thin walled 0.2 mL PCR tube and amplified in a Veriti™ 96 Well Thermal Cycler (Applied Biosystems, CA).

<table>
<thead>
<tr>
<th>Amount (µL)</th>
<th>Ingredient</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10X reaction buffer</td>
<td></td>
</tr>
<tr>
<td>3.78</td>
<td>dsDNA template, 10ng/µL</td>
<td>Either pIX-2 or pJH1</td>
</tr>
<tr>
<td>10</td>
<td>Oligonucleotide #1, final concentration of 125ng/10 µL</td>
<td>Forward primer</td>
</tr>
<tr>
<td>10</td>
<td>Oligonucleotide #2, final concentration of 125ng/10 µL</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>1</td>
<td>dNTP mix, 25mM</td>
<td></td>
</tr>
<tr>
<td>20.22</td>
<td>ddH2O</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$\textit{PfuTurbo}$ DNA polymerase (25 U/µL)</td>
<td></td>
</tr>
</tbody>
</table>

After the PCR tubes were placed in the PCR thermal cycler, an amplification reaction was run with the parameters as shown in Table 5.
Table 5. Cycling parameters for the QuickChangeTM site-directed mutagenesis method

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68°C</td>
<td>5.5 minutes</td>
</tr>
</tbody>
</table>

Following temperature cycling, the reaction was placed on ice for 2 minutes to cool the reaction to ≥ 37°C.

10 µL of PCR product was mixed with loading dye and run in a 0.7% gel. Lambda DNA cut with HindIII was used as a molecular weight marker. The remaining PCR product was digested with DpnI.

**DpnI digestion of the amplification products**

The purpose of DpnI digestion is to allow the mutagenized plasmid to be transformed but not the original parental plasmid. The parental methylated DNA is digested by DpnI and the PCR products remain intact because the newly synthesized DNA is non-methylated. This step ensures us that only the mutagenized plasmid is transformed. 1 µL of the DpnI restriction enzyme (10 U/µL) was added directly to each of the amplification reactions. Each reaction was gently and thoroughly mixed.
by pipetting the solution up and down (6-8 times). The reaction mixtures were spun down in a microcentrifuge for 1 minute and were incubated at 37°C for 1 hour.

**Transformation of XL-10 Gold Ultracompetent cells**

The XL-10 Gold Ultracompetent cells (QuickChange® XL Site-Directed Mutagenesis Kit, Stratagene, cat no. 200517-4, lot no. 006047569) are designed for efficient transformation of large DNA molecules. The XL-10 Gold strains are also designed to allow cloning of methylated DNA and aids in producing higher quality of miniprep DNA. The XL-10 Gold Ultracompetent cells stored at -80°C were gently thawed on ice. 50 µL of the Gold Ultracompetent cells were aliquoted to a prechilled 14-mL BD Falcon polypropylene round-bottom tube (Becton Dickinson, catalog no. 352059) for 30 minutes. 1 µL of the \textit{DpnI} treated DNA was added to the cells in the Falcon tube on ice for few minutes. The transformation reaction was heat shocked at 42°C for 45 seconds. The reaction tubes were placed on ice for 2 minutes. 0.5 mL of SOC media preheated at 42°C SOC media was added to the transformation tubes and the tubes were incubated at 37°C for 1 hour in a shaking incubator at 225 rpm. After the 1 hour incubation, 250 µL of the transformation reaction was plated on two nutrient agar (NA) plates containing ampicillin (100 µg/mL), and 48 µL X-Gal. The plates were then incubated at 37°C for >16 hours.

The nutrient agar (NA) plates were checked for the growth of white colonies. The white colonies were restreaked in nutrient agar plates containing ampicillin (100 µg/mL) and incubated overnight at 37°C.
**Plasmid Purification**

The purpose of this step was to purify the pIX-2 plasmids containing the *umuD* cleavage and nucleophilic activator residue mutations so that they could be then transformed into *E. coli* wild type (AB1157) and *umuD* (315) competent cells where the mutated gene would be expressed. The QIAprep® Spin Miniprep Kit (QIAGEN Sciences, catalog no. 27106) was used to purify the plasmids from the overnight cultures of the transformed XL-10 Gold Ultracompetent cells. Several microcentrifuge tubes were filled with 1 mL of double distilled H₂O (ddH₂O). Applicator tips were used to acquire colonies from the overnight plate cultures and colonies were transferred into the microcentrifuge tubes containing sterile water and resuspended. The tubes were centrifuged for 2 minutes at 14,000 rpm. The supernatant was discarded. The bacterial pellets were resuspended in 250 µL Buffer P1 (stored at 4°C) containing 100 µg/mL of RNAse A. 250 µL of Buffer P2 was added and gently and thoroughly mixed by inverting the tubes 4-6 times. 350 µL of Buffer N3 was added and immediately mixed thoroughly by inverting the tubes 4-6 times. The solution became cloudy at this point. The tubes were centrifuged for 10 minutes at 13,000 rpm. A white compact pellet was seen in the tubes after centrifugation. The supernatants from the tubes were applied to QIAprep spin columns by pipetting and centrifuged for 1 minute. The flow-through was discarded. 0.75 mL of the Buffer PB was added to the QIAprep spin columns and centrifuged for 1 minute. The flow-through was discarded and centrifuged for an additional minute to get rid of the excess wash buffer in the columns. The QIAprep columns were placed
in new microcentrifuge tubes and 30-50 µL of EB (Elution Buffer) was added to the center of the QIAprep column. The tubes were let stand for 1 minute and centrifuged for 1 minute at 13,000 rpm. The concentration of the plasmids was measured using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) and the plasmids were stored at 4°C.

**Confirmation of the mutations**

To confirm the presence of the mutations in pIX-2, samples of the plasmids containing the predicted cleavage and nucleophilic activator residue mutations were either digested with *DdeI* restriction enzyme or sent to Alpha Biolaboratory in Burlingame, California for DNA sequencing. *DdeI* could confirm the Gly to Glu predicted cleavage mutation because a restriction site was created as a result of the mutation, which resulted in additional DNA fragment when the samples were run in agarose gel.

**Transformation of competent wild type and *umuD* E. coli**

Transforming the AB1157 and 315 strains with the purified plasmids containing the mutated cleavage or nucleophilic activator residues in *umuD* enables us to study the expression of the mutated gene products in wild type and *umuD* E. coli strains. 200 µL of each frozen CaCl₂ competent AB1157 & 315 cells (made competent in Hare lab) were transferred to a pre-chilled 14-mL BD Falcon polypropylene round-bottom tube. 1 µL of the purified and stored plasmid from the purification step was added to the two Falcon tubes on ice for 30 minutes. The tubes
were then transferred to a 42°C water bath for 90 seconds. The tubes were immediately transferred to an ice bath and the reactions were allowed to chill for 2 minutes. 800 µL of preheated SOC medium was added to the tubes. The tubes were incubated in a 37°C incubating shaker for 45 minutes at 225 rpm to allow the bacterial cells to recover and express ampicillin resistance. After incubation, the tubes were centrifuged for a minute at 15000 xg and all of the liquid was plated on a nutrient agar (NA) plate containing 100 µg/mL of ampicillin per tube.

**Confirmation of plasmid presence in cells**

Since the plasmids pIX3.0 and pJH1 contain an ampicillin resistance (*ampR*) gene as a selectable marker, only the competent cells that contain the plasmid with the *ampR* gene are able to survive in ampicillin-containing medium. *ampR* functions by encoding for a protein that makes the cells transformed with the plasmids resistant to antibiotic ampicillin.

**Storage of bacterial cultures**

The purpose of this step was to store the transformed bacterial cultures appropriately so that these cultures can be used in the future. Overnight cultures of bacterial strains were grown in tubes containing 3 mL of Luria-Bertani (LB) broth and 3 µL (100 mg/mL) of ampicillin. The following day the culture tubes were vortexed and 750 µL of the liquid cultures were transferred to appropriately labeled microcentrifuge tubes. 250 µL of sterile 60% glycerol was mixed into the
microcentrifuge tubes containing the liquid cultures. The tubes were vortexed briefly and stored at -80°C.

**Collection of *E. coli* cell lysates and quantitation of protein**

*Lysate preparation*

Crude, total protein cellular lysates were prepared from overnight cultures grown in 3 mL of LB broth added with ampicillin (100 µg/mL) in a 37°C shaking incubator. 3 mL of the overnight cultures were 1:10 diluted in 25 mL of LB plus ampicillin (100 µg/mL) in an Erlenmeyer flask and grown at 37°C with shaking at 200 rpm for an additional 3 hours. After 3 hours, 12.5 mL of the cultures was transferred to a new each flask and 12.5 µL of mitomycin C (+MMC) (2 µg/mL) was added to the new flask. MMC is a potent DNA cross-linker, and induces DNA damage in our experimental setup. Both cultures were grown for additional necessary time (minutes to hours). Samples 1.5 mL were then collected in microcentrifuge tubes from either the “+MMC” or “-MMC” at the following increments: 0 minutes, 15 minutes, 30 minutes, 45 minutes, and 60 minutes. Each sample was spun for 3 minutes at 15,000 rpm. The supernatant was decanted and each pellet was resuspended in a solution of 950 µL Laemmli buffer (63 mM Tris HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, pH 6.8) and 50 µL of 2-mercaptoethanol (Sigma Chemical Co., lot no. 129 F04061). The samples were stored in the -20°C freezer and boiled for 5 minutes and placed in ice for 5 minutes before use.
Protein Quantitation Assay

To ensure that constant amounts of proteins from the lysates were loaded in each gel, we performed the RC DC Protein Assay (Bio-Rad). 5 µL of DC Reagent S was added to each 250 µL of DC Reagent A (Bio-Rad, catalog no. 500-0113) resulting in a mixture called Reagent A'. Five dilutions of a protein standard, bovine plasma gamma globulin (Bio-Rad, catalog no. 500-0005, purchased in solution as part of the Bio-Rad protein quantitation assay kit), were prepared by dilution in Lemmalei buffer. The resultant concentration of the protein ranged from 0.2 mg/mL to 1.5 mg/mL. These diluted proteins were used to prepare a standard curve each time the assay was performed. 25 µL of standards or lysate sample was placed in a clean microfuge tube containing 125 µL of Reagent A'. 125 µL of RC Reagent I (Bio-Rad, catalog no. 500-0117) was added to each tube. The tubes were vortexed briefly and incubated for 1 minute at room temperature. 125 µL of RC Reagent II (Bio-Rad, catalog no. 500-0118) was added to each tube, vortexed briefly, and centrifuged at 15,000 g for 3-5 minutes. The supernatants were discarded and the liquid was allowed to drain completely from the tubes. 127 µL of Reagent A' was added to each tube and vortexed. The tubes were incubated at room temperature for 5 minutes or until the precipitate was completely dissolved. The tubes were vortexed, 1 mL of DC Reagent B (Bio-Rad, catalog no. 500-0114) was added to each tube and vortexed immediately again. The tubes were incubated at room temperature for 15 minutes. The absorbances were recorded at 750 nm with the relative blue color of intensity correlating with increasing protein. A standard based on the readings from the BSA
standards was created and the amount of protein in each lysate sample was determined.

**Western Immuno-blot and analysis of proteins**

Either a Mini-PROTEAN® TGX™ gel (4-20% Tris-HCl, Bio-Rad) or a Criterion™ Precast gel (10-20% Tris-HCl, 1.0 mm, BIO-RAD) was run in order to separate various proteins by size. Frozen lysates were boiled for 5 minutes and put on ice for 5 minutes each time a PAGE was performed. The amount of protein samples ranging from 1.75 µg to 12 µg (determined from RC DC Assay) were loaded in the gel. The amount of protein corresponds to the micrograms (µg) of total protein per lane. The gel was run for 1 hour at 125 volts for a Mini-PROTEAN® gel or 3 hours at 125 volts for the Criterion™ gel at 4°C. The gel running buffer was composed of: 25 mM Tris base, 192 mM glycine, and 10% SDS.

After running the gel for an appropriate time, the proteins in the gel were transferred to an Immun-Blot PVDF membrane (Immun-Blot™ PVDF Membrane, Bio-Rad, lot no. D103267 for 10 x 15 cm or Immun-Blot™ PVDF Membrane, Bio-Rad, lot no. BR0028003 for 7 x 8.4 cm) using the Trans-Blot apparatus (Bio-Rad, Criterion™ Blotter). The apparatus was run for 1 hour at 100 volts at 4°C with transfer buffer (25 mM Tris base, 192 mM glycine, 10% methanol (Fisher Scientific, lot no. 107631)).

After the protein was transferred to the membrane, the membrane was air dried, pre-wetted in 100% methanol and incubated overnight in 0.05% Tween-20
in Tris Buffered Saline (TBS) (25 mM Tris, 137 mM NaCl, 2.7 mM) containing 5% milk (Blotting grade blocker non-fat dry milk, Bio-Rad, catalog no. 170-6404) on a Belly Dancer (Stovall Life Sciences Inc.) at speed 2 at 4°C. The following day, the membrane was incubated in 0.05% TBS Tween-20 containing 2.5% milk and diluted primary antibody.

Primary antibodies used were either an anti-peptide antibody directed at a 15 amino acid peptide located 79 amino acids upstream of the predicted cleavage site (Figure 9) or polyclonal antibody. The polyclonal anti-UmuD antibody was prepared by GenScript Corp., New Jersey, USA. Purified UmuDAb proteins were constructed by GensScript from the umuD sequence and the proteins were injected in rabbits. The serum containing the anti-UmuD polyclonal antibody were then isolated from the rabbits. Anti-peptide 2 (peptide specific) or polyclonal anti-UmuD antibodies were used at a dilution of 1:10,000, and secondary antibody (Goat Anti-Rabbit HRP Conjugate, Bio-Rad, catalog no. 170-5046) was used at dilution of 1:32,000. All antibody incubations were carried out separately for 1 hour each. The membrane was washed five times with 0.01% TBS-Tween 20 for 10 minutes in between the primary and secondary antibody incubation and after secondary antibody incubation. Precision StrepTactin-HRP Conjugate (Bio-Rad, catalog no. 161-0380) at a dilution of 1:10,000 was added with the secondary antibody. The secondary antibody is linked to an enzyme horseradish peroxidase (HRP) and binds to the primary antibody. The HRP cleaves a chemiluminescent substrate, resulting in generation of luminescence proportional to the quantity of protein present in the membrane. Chemiluminescent
substrate (SuperSignal® West Pico Chemiluminescent Substrate, Thermo Scientific, lot no. LC 141629) was prepared for the membrane and was gently pipetted onto with the PVDF membrane. After incubating in the dark with the substrate for 10 minutes, the film was sandwiched between transparency papers. The membrane sandwich was placed into an X-ray film cassette and the orientation was marked. The membrane was exposed to X-ray film in a medical film processor (Konica Minolta, SRX-101A, Z & Z Medical, Inc.) for different time exposures and the film was developed with developer (Med-Dent Developer, White Mountain Imaging, catalog no. 2501-FG) and fixer (Med-Dent Developer, White Mountain Imaging, catalog no. 2502-FG).

**Figure 9. Sequences recognized by anti-peptide antibody.** Primary anti-peptide antibody recognizes the 15 amino acids (shown in box) upstream of the predicted cleavage site. The dotted box represents the predicted cleavage site of UmuDAb.
Figure 10. Steps involved in a Western blotting technique. Adapted from Lodish, 2008.
Results

The main objective of my research was to determine if the UmuDAb protein was being expressed in wild type and \textit{umuD} mutants of \textit{E. coli}, and was self-cleaving over time after the cells were treated with a DNA damaging agent. In addition, we also wanted to determine if the mutations in the predicted cleavage or nucleophilic attack residues blocked or inhibited the disappearance (cleavage) of the protein over time. Therefore, Western blot analysis was performed and observations made were compared with \textit{E. coli} model for similarities and contrasts.

The \textit{umuD} found in ADP1 is about 1.5 times the size of \textit{umuD} in \textit{E. coli} and other bacteria and research has shown that the functioning of the \textit{umuDAb} encoded gene product is unlike other bacteria such as \textit{E. coli} (Hare \textit{et al.}, 2006). Western blot analyses performed in Hare lab have shown the size of the protein to be approximately 23 kDa (Experiments in Hare lab and this study). The pJH1 plasmid containing the native promoter expresses the \(\sim\)23 kDa protein. The pIX-2 plasmid containing the constitutive promoter also expresses the protein of \(\sim\)23 kDa.

Table 6 shows features of UmuD participation in \textit{E. coli}. My objectives of the thesis are to address similar processes or features in ADP1. Different approaches have been taken to investigate these features in ADP1. In \textit{E. coli}, UmuD self-cleaves in a reaction mediated by *RecA after DNA damage (Shinagawa \textit{et al.}, 1988; Burckhardt \textit{et al.}, 1988) and to investigate if UmuDAb self-cleaves in response to DNA damage, \textit{umuDAb} will be expressed in \textit{umuD} strains of \textit{E. coli}. In order to examine RecA
involvement in the disappearance (cleavage) of UmuDAb, UmuDAb will be expressed in DH5α and AB2463 cells that lack recA gene and these cells will be treated with MMC. The apparent cleavage time scale of UmuDAb will be calculated as the half-life after the cells expressing UmuDAb are treated with MMC. To examine the predicted cleavage site of UmuDAb, the glycine residue at the predicted cleavage site of the protein at A^{83}-G^{84} site will be mutated to glutamic acid. Similarly, the conserved lysine residue that acts in *E. coli* as a nucleophilic activator will be mutated to alanine to observe its effects in the cleavage of the protein.

Table 6. Features of the *E. coli* involvement of UmuD in the SOS mutagenesis of DNA damage response

<table>
<thead>
<tr>
<th>Processes/Features of UmuD cleavage</th>
<th><em>E. coli</em></th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleavage time scale</td>
<td>~20 min after DNA damage</td>
<td>Opperman <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Cleaved where?</td>
<td>C^{24}-G^{25}</td>
<td>Nohmi <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>Amino acids required for cleavage</td>
<td>Ser, &amp; Lys</td>
<td>Nohmi <em>et al.</em>, 1988</td>
</tr>
<tr>
<td>SOS mutagenesis phenotype required</td>
<td>C^{24}-G^{25} Cleavage, Ser, &amp; Lys</td>
<td>Nohmi <em>et al.</em>, 1988</td>
</tr>
</tbody>
</table>
UmuDAb protein is expressed in *E. coli*

*Wild type cells express UmuDAb, which disappears over time after MMC treatment*

Western analysis showed that UmuDAb of ADP1 is expressed in the wild type *E. coli* strain AB1157 and the size of the protein is approximately 23 kDa as predicted by the open reading frame (Hare *et al.*, 2006) (Figure 8). The expression of the protein disappeared over time in cells treated with 2 µg/mL of MMC, whereas the levels of UmuDAb were consistent over time in non-MMC treated cells. The apparent “disappearance” of UmuDAb (Figure 11, lanes 2 and 3) could be as a result of cleavage of the protein at the predicted cleavage site in response to MMC-induced DNA damage because when anti-peptide antibody is used, the larger ~16 kDa expected cleavage product cannot be expected to be seen or recognized on the Western blot. This is because primary anti-peptide antibody recognizes the 15 amino acids (shown in box) upstream of the predicted cleavage site (Figure 9).

A cross-reacting protein of approximately 19 kDa is seen in the Western blot (Figure 11, lanes 2, 3, 4, 5, 6, and 7; and Figure 12 A and B, lanes 3-18 for both). Furthermore, AB1157 cells alone can express the cross-reacting protein (Data not shown, personal communication with M. Whitaker of Hare lab). The 19 kDa protein is neither UmuD nor UmuDAb because the molecular weights of UmuD and UmuDAb are 15.0 (Kitagawa *et al.*, 1985) and 23.0 kDa respectively. The control cells that contain the vector alone with no *umuDAb* do not express UmuDAb but the cross-reacting proteins are still seen (Figure 11, lanes 8 and 9).
Figure 11. Expression and disappearance or no disappearance of UmuDAb in wild type, *umuD*, and *recA* strains of *E. coli*. The cells were treated with MMC for 1 hour. “+” indicates cells treated with 2 µg/mL of MMC, “-” refers to no MMC treatment. Primary antibody used was anti-peptide 2. Lanes 8 and 9 contain proteins from wild type with vectors pUC19 and pIX3.0, alone respectively.
**E. coli UmuD is not required for UmuDAb disappearance after DNA damage**

Western blot analysis indicated that UmuDAb was also expressed in *E. coli* cells that completely lack their *umuD* gene. The size of the expressed protein was approximately 23 kDa. UmuDAb disappeared over time in MMC untreated cells in a time-dependent manner whereas the amounts of the protein were consistent over time in non-MMC treated cells (Figure 12 A and B). Because the *umuD* *E. coli* completely lack the *umuD* gene that encodes its own UmuD (Personal communication with P. Buening), and UmuDAb disappeared over time in MMC treated cells, we conclude that *E. coli* UmuD is not required for or involved in UmuDAb disappearance.

**UmuDAb disappears in both wild type and umuD strains of E. coli**

In order to examine the phenomenon of disappearance of UmuDAb more closely with respect to its timing, more short-term protein lysates were collected after MMC treatment. Figure 12 A and B suggest that UmuDAb is expressed, but disappears after MMC-induced DNA damage over similar amounts of time in both wild type and *umuD* strains of *E. coli*. UmuDAb was expressed and the protein disappears over time with MMC treatment. This suggests that MMC acts as a DNA damaging agent and UmuD is self cleaving, which is consistent with *E. coli* model. Other experiments in the Hare lab (Personal communication with S. Wheeler and K. Lambert of Hare lab) also have shown that UV-C exposure causes disappearance (apparent cleavage) of UmuDAb.
Figure 12 A. Expression of UmuDAb in wild type *E. coli*. UmuDAb is expressed from pIX-2 and disappears over time in wild type with MMC treatment. "+" indicates cells treated with 2 µg/mL of MMC, "-" refers to no MMC treatment. Primary antibody used was anti-peptide 2 antibody.
Figure 12 B. Expression of UmuDAb in *umuD* strains of *E. coli*. UmuDAb is expressed from pIX-2 and disappears over time in *umuD* mutants with MMC treatment. "+" indicates cells treated with 2 µg/mL of MMC, "-" refers to no MMC treatment. Primary antibody used was peptide-specific anti-peptide 2 antibody.
Timing of UmuDAb disappearance resembles that of *E. coli*

In order to compare the disappearance of UmuDAb in wild type and *umuD* strains of *E. coli* in a more quantitative manner such as to see if the protein cleave with same timing, ImageJ Software (National Institutes of Health) was used to determine the percent of UmuDAb remaining after DNA damage. The percent remaining was calculated at different intervals of times after addition of MMC to the cultures: 0 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 25 minutes, 30 minutes, 45 minutes, and 60 minutes. The decay of UmuDAb for all the experiments was calculated to be ~20 minutes, which is similar to ~ 20 minutes (Opperman *et al.*, 1999) for *E. coli*. The timing of disappearance of the protein was determined as a half-life time from a percent remaining versus time (min) graph for the protein as shown in Figure 13.
Figure 13. **Half-life of UmuDAb protein.** Percentage of UmuDAb remaining over time manner in both MMC treated and untreated samples of wild type and *umuD* strains of *E. coli* expressing UmuDAb. The horizontal and vertical dotted lines represent the half life for the disappearance of UmuDAb in wild type and *umuD E. coli*. 
**RecA is required for the cleavage of UmuDAb**

In *E. coli*, RecA is activated by DNA damage to subsequently bind to UmuD and facilitate its self-cleavage (Shinagawa *et al*., 1988; Burckhardt *et al*., 1988). To assess the role of RecA in the disappearance of UmuDAb, UmuDAb disappearance was examined in the DH5α *recA* strain of *E. coli* that was used to produce pIX-2. The wild type and *recA* strains of *E. coli* were grown for >16 hours, then 1:10 diluted and grown for additional 3 hours. The cells were then treated with MMC for 1 hour. In wild type and *umuD* strains, UmuDAb disappears after a 1 hour treatment with MMC (Figure 14). In DH5α cells that are mutated in their *recA* gene, however, the level of UmuDAb expression was consistent over time and did not disappear. This suggests that RecA is required for the disappearance of UmuDab after DNA damage, and is consistent with the idea that the “disappearance” is cleavage.

However, the DH5α strain of *E. coli* contains many additional mutations in its genotype, relative to AB1157, in addition to *recA*, therefore it was necessary that we use an isogenic strain of wild type AB1157 that has a mutation in its *recA* gene only. AB2463 is an isogenic strain of wild type AB1157 with a mutation in its *recA* gene that encodes for RecA (Horii *et al*., 1979). To further assess the role of RecA in the disappearance of UmuDAb after DNA damage, AB2463 cells containing various plasmids such as pJH1, pIX-2 and pIX-1 were grown as described in Materials and Methods, and treated with MMC for 1 hour. The *recA*+ strain AB1157 was also treated with MMC as a control. As shown in Figure 15, UmuDAb does not disappear.
in recA strains after 1 hour MMC treatment. However, UmuDAb disappears in the recA+ strains, offering further evidence that RecA is required for the disappearance of UmuDAb after DNA damage.
Figure 14. Expression of UmuDAb in recA⁻ and recA⁺ strains of E. coli. Western blot analyses showing the expression and disappearance or no disappearance of UmuDAb in DH5α recA⁻ and recA⁺ strains after cells were treated with MMC for 1 hour. “+” indicates cells treated with 2 µg/mL of MMC, “-” refers to no MMC treatment. Compare lanes 2 and 3; lanes 8 and 9; and lanes 10 and 11.
Figure 15. Disappearance of UmuDAb in recA+ strains of E. coli. Western blot analyses showing the expression and disappearance or no disappearance of UmuDAb in AB2463 recA− and AB1157 recA+ strains after cells were treated with MMC for 1 hour. "+" indicates cells treated with 2 µg/mL of MMC, "−" refers to no MMC treatment. The vector used was pIX3.0.
UmuDAb mutant in predicted cleavage site still disappears in time-dependent manner after MMC treatment

To test whether the observed disappearance of UmuDAb was really a result of the cleavage in the predicted cleavage site of the protein, I constructed a mutation in the predicted cleavage site of the protein at A\textsuperscript{83}-G\textsuperscript{84} site. The glycine at the predicted cleavage site was mutated to glutamic acid because by changing the Gly-25 residue of the putative Cys-Gly cleavage site to a glutamic or lysine residue in \textit{E. coli} UmuD significantly reduced the ability of UmuD to function in SOS mutagenesis (Nohmi \textit{et al.}, 1988).

Proteins lysates were prepared from the wild type and \textit{umuD} \textit{E. coli} as described in the Materials and Methods and examined with Western blot analyses with a polyclonal antibody directed against the entire UmuDAb protein.

Figure 16 shows the effect of the predicted cleavage site Gly-84 to Glu-84 mutation on the disappearance of UmuDAb. Wild type that contained the plasmids with the predicted Gly-84 to Glu-84 cleavage mutations in \textit{umuDAb} express the protein, which similar to the unmutated UmuDAb, almost completely disappears at 60 minutes with MMC treatment (Figure 16, lanes 6 and 7). Similar results were seen in the \textit{umuD} strain background: the mutated UmuDAb protein still disappeared after 60 minutes of MMC treatment (Figure 16, lane 9).

This first immunoblot, shown in Figure 16 was performed on sample lysates collected at 0 minutes and 60 minutes only. To obtain better observations on the disappearance of an UmuDAb, immunoblot was re-run with lysates collected on
different dates at 0 minutes, 30 minutes, 45 minutes, and 60 minutes. Moreover, the primary antibody used this time was polyclonal antibody that recognizes the entire UmuDAb unlike peptide-specific antibody that is sequence specific directed at a 15 amino acid peptide located 79 amino acids upstream of the predicted cleavage site (Figure 9). This is desirable because the polyclonal antibody is directed against the whole UmuDAb protein and therefore also recognizes the cleaved UmuD' product.

Figure 17 shows the effect of the predicted cleavage site Gly-84 to Glu-84 mutation on the disappearance of UmuDAb. The levels of UmuDAb in either the wild type or umuD strains were consistent in cells that were not treated with MMC (Figure 17, lanes 2, 4, and 6 or lanes 13 and 15). The un-mutated UmuDAb expressed by the wild type and umuD strains disappeared over time with MMC treatment (Figure 17, lanes 2, 3, and 5 or lanes 13, and 14). Wild type cells that contain the plasmids with the predicted Gly-84 to Glu-84 cleavage mutations in umuDAb express the protein, which completely disappears at 60 min with MMC treatment (Figure 17, lanes 7, 8, and 10). Similar results were seen in the umuD strain background: the UmuDAb cleavage mutants still disappeared after 60 min of MMC treatment.

In contrast to the results seen in Figure 16, with the use of polyclonal antibodies, we see a ~14 kDa cleavage product appearing in only MMC treated cells, which may correspond to a UmuD' form (Figure 17, lanes 3, 5, 8, 10, and 16). The larger molecular weight, ~16 kDa of cleavage product for cleavage mutants suggests that UmuDAbG84E might be using an alternate cleavage site. Figure 18 shows the percentage of UmuDAb remaining in a time-dependent manner in both MMC treated
and untreated samples of wild types and cleavage mutants, as determined by the ImageJ program.
Figure 16. Expression of UmuDAb by wild type and *umuD* predicted cleavage mutants. “+” indicates cells treated with 2 µg/mL of MMC, “-” refers to no MMC treatment. The primary antibody used was peptide-specific anti-peptide 2 antibody.
Figure 17. Expression of UmuDAb by wild type and umuD predicted cleavage mutants. "+" indicates cells treated with 2 µg/mL of MMC, "-" refers to no MMC treatment. The primary antibody used was polyclonal antibody that recognizes entire UmuDAb.
Figure 18. The percentage of UmuDAb remaining in a time-dependent manner in both MMC treated and untreated samples of wild types and cleavage mutants. The solid lines represent the UmuDAb in samples untreated with MMC while the dotted lines represent the UmuDAb in samples treated with MMC.
UmuDAb mutant in nucleophilic activator residue fails to undergo cleavage after treatment with MMC

Because the UmuDAb expressed by the cleavage mutants disappeared surprisingly after MMC-induced DNA damage (Figure 16 and 17), we speculate that the protein cleaves in a site rather than the predicted cleavage site or the protein uses alternate mechanism for the cleavage. Therefore, I constructed a mutation in the lysine residue (Lys-156) of the UmuDAb that acts as a nucleophilic activator for the cleavage mediated by serine. The lysine at the nucleophilic activator residue was mutated to alanine because by changing the Lys-97 residue to alanine in *E. coli* UmuD drastically reduced the ability of UmuD to function in SOS mutagenesis (Nohmi *et al.*, 1988).

Proteins lysates were prepared from the wild type and *umuD* *E. coli* as described in the Materials and Methods and examined with Western blot analyses with a polyclonal antibody directed against the entire UmuDAb protein.

In Figure 19 A and B, we see that UmuDAb is expressed by wild type and *umuD* strains of *E. coli* and disappears in a time-dependent manner in MMC treated wild type and *umuD* mutants, disappearing completely at 60 min. This disappearance is dependent on MMC treatment, as UmuD does not disappear in MMC untreated cells.

Figure 19 A and B show that UmuDAb that is mutated in its nucleophilic activator residue (Lys-156 to Ala-156), is also expressed by wild type and *umuD*
mutants of *E. coli*. However, UmuDAb containing the nucleophilic activator residue mutation does not disappear over time after MMC treatment in either in wild type or *umuD* mutants of *E. coli*. The levels of expression of UmuDAb is consistent over time in both MMC-treated and untreated samples of wild type and *umuD* mutants. This suggests that the “disappearance” is actually cleavage, mediated by a reaction like that of other peptidases such as LexA and UmuD, which depend on the Lys-Ser enzymatic dyad (Figure 7).

Figure 20 represents a graph that shows the percentage of UmuDAb remaining in a time-dependent manner in both MMC treated and untreated samples of wild type and nucleophilic activator residue mutants whereas Figure 21 represents a graph that shows the percentage of UmuDAb remaining in a time-dependent manner in both MMC treated and untreated samples of *umuD* mutants and nucleophilic activator residue mutants. Figure 20 shows that the levels of UmuDAbKtoA is consistent over time in the wild type nucleophilic activator residue mutants treated with MMC while the UmuDAb has completely disappeared in wild type cells after MMC treatment. Similarly, Figure 21 shows that the levels of UmuDAbKtoA is consistent over time in *umuD* nucleophilic activator residue mutants after MMC treatment while the protein has completely disappeared in *umuD* cells that express UmuDAb after MMC treatment.
Figure 19 A. Expression of UmuDAb by wild type nucleophilic activator residue mutants. "+" indicates cells treated with 2 µg/mL of MMC, "-" refers to no MMC treatment. The primary antibody used was polyclonal antibody that recognizes the entire UmuDAb.
Figure 19 B. Expression of UmuDAb by *umuD* nucleophilic activator residue mutants. "+" indicates cells treated with 2 µg/mL of MMC, "-" refers to no MMC treatment. The primary antibody used was polyclonal antibody that recognizes the entire UmuDAb.
Figure 20. Graph showing the percentage of UmuDAb remaining in a time-dependent manner in both MMC treated and untreated samples of wild types and nucleophilic activator residue mutants. The solid lines represent the UmuDAb in samples untreated with MMC while the dotted lines represent the UmuD in samples treated with MMC.
Figure 21. Graph showing the percent of UmuDAb remaining in a time-dependent manner in both MMC treated and untreated samples of *umuD* mutants and nucleophilic activator residue mutants. The solid lines represent the percent of UmuDAb in samples untreated with MMC while the dotted lines represent the percent of UmuDAb in samples treated with MMC.
What we know now of *A. baylyi* model system

Table 7 contains the summary of results after numerous Western blot analyses were performed to better understand the role and action of UmuDAb action after DNA damage response. Most of the processes or features of UmuDAb action after DNA damage have been addressed while some of the features still need to be determined, which will be the basis of future experiments.

**Table 7. Processes/features observed in *E. coli* model system and that were addressed in this study.**

<table>
<thead>
<tr>
<th>Processes/Features</th>
<th><em>E. coli</em> UmuD</th>
<th><em>A. baylyi</em> UmuDAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RecA involvement</td>
<td>Requires RecA</td>
<td>Requires RecA</td>
</tr>
<tr>
<td>Cleavage Time Scale</td>
<td>~20 min</td>
<td>~20 min</td>
</tr>
<tr>
<td>Cleaved where?</td>
<td>C^{24}-G^{25}</td>
<td>Not the predicted A^{83}-G^{84}</td>
</tr>
<tr>
<td>Amino acids required for cleavage</td>
<td>Ser, &amp; Lys</td>
<td>Lys is required Ser – no data</td>
</tr>
<tr>
<td>Phenotype required for SOS mutagenesis</td>
<td>C^{24}-G^{25} Cleavage via Ser &amp; Lys</td>
<td>No data</td>
</tr>
<tr>
<td>UmuD involvement</td>
<td>UmuD self cleavage</td>
<td><em>E. coli</em> UmuD is not required, suggests UmuDAb cleavage</td>
</tr>
</tbody>
</table>
Various endogenous or exogenous factors can damage DNA, and in order to sense and repair these damages, cells have developed a variety of DNA damage repair mechanisms. As already mentioned above, there are two main ways by which cells can sense and repair damages: error-free repair and error-prone repair mechanisms. A cell’s repair machinery is always on lookout attempting to fix these DNA damages and a cell may have the following three fates as a result of this attempt to deal with DNA damages: 1) the damaged DNA is repaired perfectly by error-free DNA repair; 2) the cell may undergo programmed cell death or apoptosis if the damage cannot be fixed or if the repair is partial; and 3) the cell may carry out error-prone repair with elevated levels of mutation loads in DNA.

When the levels of DNA damage is so extensive that error-free DNA repair, such as BER, NER, and recombinational DNA repair (Friedberg et al., 2006) cannot repair the damage, DNA replication is stalled. This triggers the mutagenic phase of the SOS response (Walker, 1984; Echols and Goodman, 1990), also known as SOS mutagenesis that leads to translesion synthesis (TLS) (Bridges and Woodgate, 1985; Goodman, 2002).

After DNA damage, single-stranded DNA generated by a halt in replication converts a key regulatory protein RecA to its activated form (RecA*), which facilitates the autocleavage of LexA repressor and induces several SOS genes such as *umuD, umuC, polB, recN, sulA, uvrB, and uvrD* to be expressed (Friedberg et al.,
1995). LexA represses the expression of \textit{umuD} and \textit{umuC} genes by binding to a “SOS box” (Mount et al., 1972). RecA* also facilitates the self-cleavage of UmuD to its cleaved form UmuD', which can complex with UmuC to form the error-prone DNA polymerase V (Tang et al., 1999) that carries out SOS mutagenesis. UmuD$_2$C complex acts as checkpoint inhibitor of cell division by slowing down the cell cycle and allowing more time for error-free mechanism to act (Opperman et al., 1999). UmuD protein may play a role in shutting off SOS response by binding with UmuD' and forming a heterodimer UmuDD'C complex (Battista et al., 1990).

The \textit{umuDAb} gene found in ADP1 has an extra N-terminus region unlike \textit{E. coli}. However, bacteria such as \textit{Synechococcus elongatus}, \textit{Legionella pneumophila} strain Lens and \textit{Chromobacterium violaceum} have “extra-long” UmuD similar to ADP1 (Figure 8). The \textit{umuDAb} gene of ADP1 is 612 bp long and encodes a protein of 203 amino acids, while \textit{umuD} gene found in \textit{E. coli} encodes 140 amino acids (Figure 5). The \textit{umuDAb} is about 1.5 times the size of \textit{umuD} in \textit{E. coli} (Hare et al., 2006). In \textit{E. coli}, UmuD self-cleaves in a reaction mediated by *RecA after DNA damage (Shinagawa et al., 1988; Burckhardt et al., 1988) to carry out SOS mutagenesis. In this research, we were interested in investigating if UmuDAb also self-cleaves similar to \textit{E. coli} UmuD in response to DNA damage.

Multiple sequence alignment of UmuDAb and UmuD homologs in \textit{E. coli} and other bacteria show that many amino acids required for RecA mediated UmuD self-cleavage in \textit{E. coli} are conserved in UmuDAb (Figure 8). For example, the cysteine-24/glycine-25 cleavage site, serine-60 that carries out the nucleophilic attack at the
UmuD cleavage site, and lysine-97 that acts as a nucleophilic activator are all conserved in UmuDAb. However, the multiple sequence alignment suggests that alanine-83/glycine-84 is the putative cleavage site in UmuDAb. We have thus created different types of mutations in either the predicted cleavage site or the residues that are required for effective cleavage in UmuDAb. The Gly-84 at the predicted cleavage site was mutated to Glu-84, while the Lys-156 that acts as a nucleophilic activator in UmuDAb cleavage was mutated to Ala-156. The mutated umuDAb genes were then transformed into E. coli wild type (AB1157) and umuD (315) competent cells so that the mutated gene would be expressed and the functions of UmuDAb could be studied.

Western blot analyses in our lab have shown that UmuDAb is expressed in both wild type and umuD strains of E. coli. The size of the protein is ~23 kDa as predicted by the open reading frame (Hare et al., 2006). The expression of the protein disappeared over time in both wild type and umuD strains of E. coli after MMC treatment (Figure 12 A and B). We have proposed that the apparent “disappearance” of UmuDAb was as a result of cleavage of the protein at the predicted alanine-83/glycine-84 cleavage site in response to MMC-induced DNA damage.

UmuD of E. coli self-cleaves post-transcriptionally in a reaction mediated by RecA to form UmuD'. A homodimer of UmuD' then complexes with a monomer of UmuC (Woodgate et al., 1989) to form a (UmuD')_2C complex, an error-prone DNA polymerase V (Tang et al., 1999), which can conduct SOS mutagenesis in E. coli. Similarly, Western blot analyses have suggested that E. coli UmuD is not required for UmuDAb disappearance in response to MMC-induced DNA damage in umuD (315)
strains of *E. coli*. 315 strains of *E. coli* completely lack the *umuD* gene that encodes its own UmuD (Personal communication with P. Buening). Since the UmuDAB is expressed in *umuD* strains and disappears over time after MMC treatment (Figure 12B, we conclude that UmuDAB is self-cleaving in response to DNA damage or at least *E. coli* UmuD is not required for UmuDAB cleavage.

The timing of disappearance of UmuDAB was calculated to be ~20 min (Figure 13). The disappearance timing was similar to that of *E. coli* UmuD, where after UV irradiation, uncleaved UmuD accumulates over UmuD' for 20 min. After 20 minutes, UmuD' have been shown to accumulate while uncleaved UmuD slowly disappears (Opperman *et al.*, 1999). Experiments in the Hare lab (Personal communication with S. Wheeler and K. Lambert) also have suggested that UV-C exposure also causes apparent cleavage of UmuDAB in a way similar to the disappearance of UmuDAB in response to MMC-induced DNA damage. However, the timing of the disappearance of UmuDAB in response to UV-C-induced DNA damage has yet to be determined.

RecA is activated by DNA damage in *E. coli* in response to DNA damage, which then binds to UmuD and mediates its self-cleavage (Shinagawa *et al.*, 1988; Burckhardt *et al.*, 1988). Similarly, our data clearly indicate that RecA is required for the cleavage of UmuDAB (Figure 14 and 15). UmuDAB is expressed by cells containing the native and constitutive promoter (Figure 14). UmuDAB disappears in recA+ (Figure 14, lanes 4 and 5; lanes 6 and 7) after MMC treatment of the cells for 1 hour. However, in recA− strains of *E. coli* (Figure 14, lanes 2 and 3; lanes 8 and 9;
lanes 10 and 11), the levels of UmuDAb is consistent after MMC treatment for 1 hour. Moreover, Western blot performed on AB2463, an isogenic strain of wild type AB1157 that has recA mutation only, clearly suggested that RecA is required for the cleavage of UmuDAb (Figure 15). UmuDAb does not disappear in recA− strains of E. coli after MMC treatment (Figure 15, lanes 6 and 7; lanes 9 and 10; lanes 12 and 13), while UmuDAb disappears in recA+ strains of E. coli. This suggests that RecA is required for cleavage of UmuDAb after MMC-induced DNA damage similar to the cleavage of UmuD in E. coli after DNA damage.

The experiments of Nohmi et al., 1988 suggested that when the Gly-25 at the predicted cleavage site of UmuD in E. coli to Glu-25, UmuD’s ability to function in SOS mutagenesis was significantly reduced since the cleavage of the protein was tremendously blocked. In contrast, the mutation of Gly-84 residue in the predicted cleavage site of UmuDAb to Glu-84 did not reduce or block the cleavage of UmuDAb (Figure 16 and 17). The protein mutated at the predicted cleavage site still disappeared after wild type and umuD strains of E. coli after MMC treatment. Therefore, we speculate that the mutation does not affect the protein in its cleavage. Interestingly, a protein of ~16 kDa protein (Figure 17, lanes 8, 10, and 16) is also recognized by the polyclonal antibody that suggests that the protein may have found an alternate site for the cleavage.

Nohmi et al., 1988 observed that when the nucleophilic activator residue, Lys-97 was mutated to Ala-97, there was a substantial reduction in the amount of UV mutagenesis. The lysine residue is essential for the effective cleavage of UmuD in a
reaction mediated by a nucleophilic serine residue. Similarly, our data indicate that UmuDAb protein that has a mutation in its Lys-156 residue fails to undergo cleavage after MMC-induced DNA damage (Figure 19 A and B). This strongly suggests that lysine is a key amino acid essential in the effective cleavage of UmuDAb protein similar to that of *E. coli* UmuD.

Table 7 summarizes the studies that were done as part of my research project. The data supports the hypothesis that UmuDAb self-cleaves in response to DNA damage as *E. coli* UmuD. Moreover, RecA is also required for the cleavage of UmuDAb as in *E. coli*. Furthermore, the data strongly suggest that Lys-156 is essential for the effective cleavage of UmuDAb in a mechanism similar to that of Lys-97 in *E. coli* UmuD.

Future directions may involve studies with creating mutations in the alanine residue of the predicted cleavage site and serine residue that carries out the nucleophilic attack in the cleavage site. The mutated UmuDAb will be expressed in wild type and *umuD* strains of *E. coli* and the effect of these mutations on the cleavage of the protein will be studied.
References Cited


Horii, T., T. Ogawa, and H. Ogawa. 1981. Nucleotide sequence of the *lexA* gene of *E. coli*. *Cell* **23:**689-697


