Discovery of a DNA Damage Response in *Acinetobacter baumannii* and Analysis of Translesion Synthesis DNA Polymerases of Both *A. baumannii* and *Escherichia coli*

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DEDICATION

This dissertation is dedicated to my family and fiancé for their endless support and love.
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ABSTRACT OF DISSERTATION

Acinetobacter baumannii is a dangerous opportunistic pathogen that has quickly emerged as a source of nosocomial infections for immunocompromised patients. It is able to survive desiccation and disinfection in the hospital setting where it can rapidly acquire resistances to multiple antibiotics. Escherichia coli gains antibiotic resistances through the induction of error-prone translesion synthesis (TLS) DNA polymerases, part of a global response to DNA damage. These DNA polymerases, mainly DNA Pol IV (DinB) and DNA Pol V (UmuD’2C), permit cells to replicate their DNA past potentially lethal fork-stalling lesions, albeit at a mutagenic cost.

We hypothesized that A. baumannii gains antibiotic resistances through a yet undetermined response akin to the E. coli paradigm. Surprisingly, we find that A. baumannii isolates have acquired multiple genes encoding putative DNA Pol V components. In the A. baumannii 17978 isolate, classic DNA damage response genes and TLS DNA polymerases are induced, and antibiotic resistant mutants are dramatically increased upon DNA damage and desiccation both in a RecA-dependent manner. However, the mechanism regulating the A. baumannii DNA damage response is likely different than E. coli based on nuances in gene induction. These data strongly support the discovery of an A. baumannii DNA damage-inducible response that directly contributes to antibiotic resistance acquisition. Our findings also imply that the number of DNA Pol V genes in each strain may directly influence mutation frequencies. We therefore analyzed the function of the multiple A. baumannii 17978 umuD, umuC and single dinB gene products in E. coli, to determine their activities. Although the A. baumannii DNA Pol V components appear to be inactive in E. coli, DinB is mostly functional, suggesting that the regulation and requirements of TLS differ between these bacterial species.
Lastly, we examined the poorly understood carboxy-terminal domain of *E. coli* UmuC, the catalytic subunit of DNA Pol V, to determine its structural role in regulatory protein-protein interactions. Using a carboxy-terminal fragment of UmuC, we find that expression causes diverse changes in DNA damage-induced mutagenesis and cell viability, depending on the type of damage or stress. These effects are independent of HtpG, the Hsp90 chaperone homologue that we hypothesized to play a role in UmuC stability. C-terminal fragment solubility is dependent on DNA damaging conditions, indicating the involvement of other damage-induced interacting factors necessary for stability. Since UmuC orthologues are conserved in bacteria, these results provide insights into the regulation of mutagenesis and the evolution of antibiotic resistances in most bacteria, including *A. baumannii*. 
TABLE OF CONTENTS

Dedication .............................................................................................................................. ii
Acknowledgements ................................................................................................................ iii
Abstract of Dissertation ........................................................................................................ iv
Table of Contents ................................................................................................................... vi
List of Figures ......................................................................................................................... vii
List of Tables ........................................................................................................................ ix
Introduction ........................................................................................................................... 1

**Chapter 1:** Antibiotic resistance acquired through a DNA damage-inducible response in *Acinetobacter baumannii* ........................................ 6

**Chapter 2:** Functional analysis of multiple, putative *Acinetobacter baumannii* 17978 DNA polymerase V gene products in *Escherichia coli* .......... 49

**Chapter 3:** Examining the role of the carboxy-terminal domain of *Escherichia coli* DNA polymerase V subunit, UmuC ................................. 72

Concluding Remarks ........................................................................................................... 106

References ............................................................................................................................ 109
LIST OF FIGURES

CHAPTER 1

Figure 1.1  The *A. baumannii* 17978 predicted *umuC* and *umuD* genes are organized differently than *E. coli* ................................................................. 36

Figure 1.2  Representative, evolutionarily conserved DNA damage response genes are expressed in *A. baumannii* 17978 .................. 37

Figure 1.3  The predicted *A. baumannii* TLS DNA polymerases and other DNA damage response genes are induced by DNA damage and regulated by RecA................................................................. 38

Figure 1.4  Intracellular concentrations of *A. baumannii* 17978 DNA damage-inducible proteins increase upon UV irradiation .............. 40

Figure 1.5  Mutation frequency is elevated upon treatment with DNA damaging agents or upon desiccation in a *recA*-dependent manner ........................................................................................................ 41

Figure 1.6  *A. baumannii* DinB shares sequence similarity to *E. coli* DinB ...... 42

Figure 1.7  Predicted UmuC proteins from *A. baumannii* 17978 are similar to *E. coli* UmuC ........................................................................................................ 43

Figure 1.8  Plasmid-borne *A. baumannii* *dinB* complements certain phenotypes of *dinB*-deficient *E. coli* ..................................................... 44

Figure 1.9  There is no effect of Ab-*dinB* on the frequency of MMS-induced rifampicin mutants ........................................................................... 45

CHAPTER 2

Figure 2.1  All plasmid-borne *A. baumannii* genes are expressed upon UV-irradiation in *E. coli* Δ*umuDC* ................................................................. 65

Figure 2.2  *A. baumannii* 17978 *umuDC* s do not rescue *E. coli* Δ*umuDC* from UV-sensitivity ..................................................................................... 66

Figure 2.3  *A. baumannii* 17978 *umuDC* s do not confer UV-induced mutagenesis in *E. coli* Δ*umuDC* ................................................................. 67
A. baumannii 17978 umuD s do not complement E. coli ΔumuD for UV-induced mutagenesis..........................................................68

A. baumannii 17978 umuDC s do not affect MMS-induced mutation frequencies in an alkylation damage-sensitive strain of E. coli..........................................................69

CHAPTER 3

Model of hypothesis and method..........................................................96

Schematic of UmuC carboxy terminus construct .........................97

Complementation of the mutator strain, umuC122::Tn5, with UmuC carboxy terminus results in decreased mutagenesis and increased hydroxyurea resistance ..........................................................98

Cell viability of umuDC+ strains bearing pC-terminus varies depending on the treatment..........................................................99

The C-terminus construct increases the frequency of mutagenesis upon treatment with MMS in a manner requiring dinB and umuDC but independent of htpG..........................................................100

UmuC C-terminus increases mutagenesis upon treatment with ciprofloxacin and decreases mutagenesis upon UV irradiation........101

SOS induction is required to detect soluble UmuC C-terminus protein ..........................................................102

Solubility of UmuC C-terminus protein is not dependent on htpG, active HtpG, or umuDC ..........................................................103
LIST OF TABLES

CHAPTER 1

Table 1.1 Oligonucleotides used in this study ..........................................................46
Table 1.2 Comparison of number of putative TLS DNA polymerase genes from select isolates of A. baumannii ..............................................................47
Table 1.3 Mutation signatures of desiccation-induced A. baumannii 17978 Rif^R mutants ..................................................48

CHAPTER 2

Table 2.1 Strains and plasmids ..............................................................................70
Table 2.2 Oligonucleotides used in this study .......................................................71

CHAPTER 3

Table 3.1 Strains and plasmids ..............................................................................104
Table 3.2 Oligonucleotides used in this study .......................................................105
INTRODUCTION

All cells, both prokaryotic and eukaryotic, must deal with damage to their DNA from exogenous and endogenous sources such as ultraviolet (UV) light, ionizing radiation, chemicals, and cellular metabolism. DNA damage will ultimately kill cells that are unable to deal with it, thus they have evolved mechanisms to cope with this constant challenge (1). Bacteria in particular have evolved sophisticated and interlinked DNA damage and stress responses to repair or tolerate potentially lethal DNA lesions, survive environmental stress, and ultimately promote genetic variability (2, 3). In *Escherichia coli*, the model prokaryotic organism, one such system is called the SOS response (1, 4, 5).

The SOS response induces around 200 genes (6) involved in high-fidelity DNA repair and homologous recombination (1, 7), low-fidelity DNA damage tolerance and mutagenesis (5, 8-10), persistence (11, 12), and virulence (13, 14). Enzymes part of high-fidelity repair processes such as homologous recombination, nucleotide excision repair, and base excision repair are called upon first to repair DNA lesions (1). When damage becomes too great, translesion synthesis (TLS) DNA polymerases are activated to permit bypass of replication fork-stalling lesions, i.e. the damage is tolerated and high-fidelity processes will repair the lesions in subsequent rounds of replication (9). These enzymes are known to cause mutations by replicating DNA in an error-prone manner, giving rise to profound consequences such as antibiotic resistance acquisition in pathogenic bacteria and the formation of eukaryotic cancer cells (4, 8, 9, 15, 16).

The *E. coli* SOS gene network is negatively regulated by the LexA global repressor, which binds to an operator region upstream of each SOS gene called the LexA box (or SOS box) (17, 18). When replication forks become stalled by DNA lesions or other replication stress, the
single stranded DNA that builds up is coated by RecA, forming RecA/ssDNA nucleoprotein filament (RecA*). RecA* promotes the autocleavage of LexA, thus SOS genes are derepressed and transcription commences (1). High-fidelity repair enzymes have LexA boxes with low binding specificities, allowing these genes to be quickly activated upon DNA damage. The polB and dinB genes, encoding TLS DNA polymerases (Pols) II and IV (DinB), respectively, also have weak LexA boxes because their gene products are able to bypass certain DNA lesions in a mostly error-free manner. In contrast, the umuDC operon, encoding low-fidelity DNA Pol V (UmuD’2C), is tightly bound by LexA, which ensures that it is one of the last enzymes to be induced (1, 9, 18). DNA Pol V bypasses a variety of DNA lesions including those produced by UV-light, but is highly error-prone and responsible for the majority of SOS-induced mutagenesis (5, 19, 20). Because of this feature, it is highly regulated and used only as a last resort when other high-fidelity mechanisms are exhausted (1, 9).

Orthologues of the Y-family of DNA polymerases, including *E. coli* DinB and UmuC, Rev1, and Rad30 are found in all domains of life (21). DinB’s function remained elusive for many years even though DinB orthologues are the most ubiquitous of the Y-family polymerases (9, 19) and it is the most abundant DNA polymerase in SOS-induced *E. coli* cells (22). Recently, DinB was shown to bypass certain N²-dG lesions with high fidelity (23) and be involved in non-TLS functions such as replication check-points (24), error-prone homologous recombination (25, 26), and transcription coupled repair (27). The human DinB orthologue, Pol kappa, plays a role in nucleotide excision repair in addition to TLS (28). Moreover, the human orthologue of *E. coli* UmuC and *S. cerevisiae* Rad30, Pol eta, has been found to be impaired in those with xeroderma pigmentosum variant (XP-V) syndrome, a disease that promotes both extreme sensitivity to sunlight and skin cancer (9).
The Y-family DNA polymerases derive their error-prone replication feature from the lack of a 3’ to 5’ exonuclease proofreading subunit (4, 19) and an enzyme active site that is more structurally open in comparison to high-fidelity replicative DNA polymerases (e.g. E. coli DNA Pol III; (29-33)). This open active site allows for the accommodation of distorted Watson-Crick base pairing and damaged bases with bulky adducts, albeit while sacrificing stringent geometric checking of incoming nucleotides (9, 29, 30). The chances of incorporating the wrong nucleotide are therefore greater for Y-family Pols. The misincorporation of nucleotides, called single nucleotide polymorphisms (SNPs), during cellular replication has vast implications in life; it is not only part of the molecular basis of evolution in all organisms (34, 35), but is specifically one way that bacteria are able to gain resistances to antibiotics (8, 15, 36, 37).

The rise of pathogenic bacteria that are multi-drug resistant (MDR) or in some cases, pan-drug resistant, has steadily risen over the years and far surpassed the rate in which new antibiotics are being discovered (38, 39). The emergence of the “ESKAPE” group of MDR pathogens is presently of greatest concern because they cause the majority of clinical infections and “escape” the effects of antibacterial drugs. This group includes: Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species (38). A. baumannii, in particular, has become notorious for infecting US military personnel that have been seriously injured in battle and transported to military hospitals. For this reason, it has gained the nickname, “Iraqibacter,” but also infects immunocompromised patients in hospitals around the world (40-44). Acinetobacter species appear to be ubiquitous in nature, while A. baumannii does not seem to be typically found in the environment (44). A. baumannii causes hospital- and community-acquired pneumonia, bacteremia, meningitis, urinary tract infections, and wound and burn infections (from military
combat). It is so dangerous in the intensive care hospital setting because of its ability to form a biofilm, withstand both desiccation and disinfection, and survive on equipment and surfaces for very long periods of time (40-42, 44, 45).

*A. baumannii* and other pathogenic bacteria can gain antibiotic resistances through horizontal gene transfer, plasmids, and the modification of genes that encode porins, transmembrane efflux pumps, lipopolysaccharides, ribosomes, and other antibiotic targets such as DNA gyrase and topoisomerase IV (40-42, 44). The genetic basis of these modifications is changes in the genome that result in either altered expression of genes or in changes in the primary amino acid sequence of these proteins. Since TLS DNA polymerases generate SNPs in DNA sequences, their induction during times of stress is a way in which bacteria are able to “speed up” the process of evolution and select for increased fitness (36, 46).

In this work, we wanted to uncover a yet to be determined DNA damage response in *A. baumannii* to gain a better understanding of its role in antibiotic resistance acquisition. Although the *E. coli* SOS response is considered the paradigm and *A. baumannii* appears to lack LexA (47), bacterial DNA damage responses are broad and LexA-independent regulated systems exist (3). In chapters one and two, our aim was to: (i) discover whether or not a regulated DNA damage response exists in *A. baumannii*; (ii) if so, examine the mechanisms of its regulation; (iii) assess the overall effect of DNA damaging conditions on *A. baumannii*’s ability to evolve antibiotic resistance; and (iv) determine the specific roles TLS DNA polymerases play in this response. We also focused in on the regulation of *E. coli* DNA Pol V to gain insights into the regulation of this highly mutagenic DNA polymerase. Chapter three focuses on the carboxy-terminal domain of UmuC, the catalytic subunit of DNA Pol V. This domain is a poorly understood region of the enzyme but is thought to be involved in regulatory protein-protein
interactions. Taken together, this work provides crucial insights into how an important opportunistic pathogen responds to DNA damage and gains antibiotic resistances. Furthermore, we have enhanced our knowledge of the mechanisms of regulation of DNA damage responses and of mutagenic TLS in both *A. baumannii* and *E. coli*. 
CHAPTER 1

Antibiotic resistance acquired through a DNA damage-inducible response in

*Acinetobacter baumannii*

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ABSTRACT

*Acinetobacter baumannii* is an emerging nosocomial, opportunistic pathogen that survives desiccation and quickly acquires resistance to multiple antibiotics. *Escherichia coli* gains antibiotic resistances by expressing genes involved in a global response to DNA damage. Therefore, we asked whether *A. baumannii* does the same through a yet undetermined DNA damage response akin to the *E. coli* paradigm. We find that recA, and all of the multiple error-prone DNA Polymerase V genes, those organized as *umuDC* operons and unlinked, are induced upon DNA damage in a RecA-mediated fashion. Consequently, we found that the frequency of rifampicin resistant (Rif<sup>R</sup>) mutants is dramatically increased upon UV treatment, alkylation damage and desiccation also in a RecA-mediated manner. However, in the *recA* insertion knockout strain, in which we can measure *recA* transcript, we find *recA* is induced by DNA damage, while *uvrA* and one of the unlinked *umuC* genes are somewhat derepressed in the absence of DNA damage. Thus, the mechanism regulating the *A. baumannii* DNA damage response is likely different than *E. coli*. Notably, it appears that the number of DNA Pol V genes may directly contribute to desiccation-induced mutagenesis. Sequences of the *rpoB* gene from desiccation-induced Rif<sup>R</sup> mutants show a signature consistent with *E. coli* DNA Polymerase V-generated base pair substitutions, and match that of sequenced *A. baumannii* clinical Rif<sup>R</sup> isolates. These data strongly support an *A. baumannii* DNA damage-inducible response that directly contributes to antibiotic resistance acquisition, particularly in hospitals where *A. baumannii* desiccates and tenaciously survives on equipment and surfaces.
INTRODUCTION

Acinetobacter baumannii is a gram-negative coccobacillus that has quickly become a major nosocomial pathogen in hospitals worldwide, particularly infecting critically ill and immunocompromised patients in intensive care units (40, 42). With its tenacious resistance to desiccation and disinfectants (40), it is able to live on hospital equipment, including plastics, fabrics, and dry surfaces for long periods of time (48-52). Due to A. baumannii’s ability to readily gain multiple antibiotic resistances (42, 53), there is now a high incidence of multi-drug resistant strains in many hospitals, which are sometimes resistant to every antibiotic available to clinicians (54-58). Therefore, there is an increasing need to understand the underlying mechanisms that permit A. baumannii to readily evolve in the hospital environment. Though horizontal gene transfer and homologous recombination are important for A. baumannii to gain antibiotic resistance (42, 59), it is unclear how A. baumannii regulates, if at all, systems that govern recombination and mutagenesis.

A well understood mechanism by which Escherichia coli and possibly other bacteria can become resistant to antibiotics is through the elevated expression of gene products that increase mutagenesis (10, 36). The E. coli SOS response, a well-characterized global transcriptional response triggered by DNA damage, replication stress, or antibiotics (1, 60), ultimately helps cells survive poor environmental conditions. The SOS response induces over 40 genes (18) involved in DNA repair (1); mutagenesis (1, 5, 8, 10); homologous recombination (7); virulence (13); and tolerance and persistence to fluoroquinolones (11).

In E. coli, DNA damage (1) or other effectors such as nucleotide starvation (61) trigger DNA replication fork arrest, which in turn signals induction of the SOS gene response. RecA initiates the response by coating single stranded DNA that accumulates at stalled replication
forks, forming a nucleoprotein filament. Also known as RecA*, this filament promotes autocleavage of LexA, the global transcriptional repressor of the SOS gene network, through an endowed co-protease activity. It is LexA proteolysis which ultimately permits the expression of SOS-regulated genes (1). RecA is also necessary for homologous recombination (62) and participates in the DNA damage tolerance pathway by forming complexes with translesion synthesis (TLS) DNA polymerases DinB (or DNA Pol IV; (63)) and DNA Pol V (63-65). *A. baumannii* encodes a predicted *recA* gene that when knocked out sensitizes it to DNA damage and a number of different stressors (66). Moreover, *recA* and *ddrR* (encoding a protein of unknown function) are induced upon UV irradiation in *Acinetobacter baylyi* ADP1 (67, 68), a non-pathogenic strain of *Acinetobacter*, suggesting a key role for RecA in mechanisms involved in stress survival. Nevertheless, efforts to identify a global DNA damage response in *Acinetobacter* have not been pursued. The lack of a LexA homologue in this genus has undoubtedly hindered efforts to identify such a response (47).

Damaged DNA must be either repaired or tolerated for a cell to survive. UvrA is one of the first gene products in which elevated expression can be detected upon DNA damage in the *E. coli* DNA damage response (18). This enzyme is part of the nucleotide excision repair (NER) pathway that detects DNA-distorting lesions, e.g. those produced by UV irradiation (69), and recruits the NER components to repair them. The *E. coli* DNA damage response also induces error-prone Y-family TLS DNA polymerases, Pol V and DinB, as well as B-family DNA Pol II, to perform DNA synthesis past replication stalling lesions that have been left behind on the template DNA. These lesions stall DNA replication because they cannot be used as template by replicative DNA polymerases. Y-family DNA polymerases have a relatively open active site compared to replicative DNA polymerases, permitting the accommodation of damaged bases. In
addition, they lack an exonuclease activity, which enables other DNA polymerases to proofread DNA synthesis. Because of these features, Y-family DNA polymerases are generally more error-prone on undamaged DNA than replicative, high-fidelity DNA polymerases (4, 5, 19, 30, 70). This low fidelity DNA synthesis increases mutagenesis and can lead to acquisition of antibiotic resistance through the modification of certain gene products (10, 36). The mutation signatures of DNA Pol V and DinB are base-pair substitutions and -1 frameshifts, respectively (20, 63, 71). Notably, sequenced clinical A. baumannii strains from different locations worldwide have multiple mutations that result in quinolone resistance (72-74), possibly the result of base-pair substitutions made by mutagenic Y-family DNA polymerases.

Y-family DNA polymerases are evolutionarily conserved from bacteria to humans (21). DNA Pol V (UmuD’-C) is composed of the catalytic enzyme, UmuC, and a homodimer of the accessory protein UmuD’. UmuD’ is the product of the co-protease activity of RecA* on UmuD; it is a 24 residue amino-terminal truncation of full-length UmuD. The error-prone DNA Pol V is known to bypass UV-induced DNA lesions and it is responsible for most UV-induced mutagenesis; because of this, umuD and umuC are highly regulated in E. coli to minimize the intracellular concentration of active DNA Pol V (1). A. baumannii is capable of UV-induced mutagenesis and it has also been observed that it carries multiple umuD and umuC genes (75, 76). It has been assumed that these genes are responsible for the mutagenesis. However, since there are multiple umuD and umuC genes, it is not yet known whether one or all of them are expressed upon DNA damage.

Therefore, we sought to assess whether a common response to DNA damage exists in A. baumannii by determining whether E. coli canonical DNA damage genes (e.g. recA, uvrA), as well as the multiple error-prone DNA polymerase genes, are induced upon DNA damage. We
also investigated induced mutagenesis, an output of the DNA damage response, and assessed the impact of having multiple \textit{umuD} and \textit{umuC} genes. In this report we present evidence that supports the existence of an \textit{A. baumannii} inducible DNA damage response in which RecA plays a major regulatory role. We demonstrate that this response increases mutagenesis and is one of the mechanisms used by \textit{A. baumannii} to acquire antibiotic resistances upon clinically relevant DNA damaging conditions.
MATERIALS AND METHODS

Strains and growth conditions. A. baumannii ATCC 17978 (77) and ATCC 19606 (78) were purchased from The American Type Culture Collection (ATCC). The isogenic A. baumannii 17978 recA deficient mutant (recA::Km) was the generous gift of the Bou Lab (Universitario A Coruña, Spain). All GenBank accession numbers, including those of strains used for in silico analyses, are shown in Table 1.2. A. baumannii and E. coli cultures were routinely grown at 37º C in Luria Broth (LB) or on LB agar. MICs were determined using a standard liquid broth dilution method (79). For all strains, 100 µg mL\(^{-1}\) of rifampicin (Rif, Calbiochem), and 30 µg mL\(^{-1}\) of Kanamycin (Km, Sigma) were used.

Homology searches and sequence alignments. A. baumannii protein sequences were obtained from the NCBI protein-protein BLAST search engine (80) using E. coli protein sequences as query. Genomic sequences that were not annotated were hand-curated accordingly. Genomic organization of ATCC 17978 umuDC operons were determined by finding the predicted open reading frames of the genes of interest in the available genome sequence. Protein sequences were aligned using the multiple sequence alignment tool of CLC Main Workbench (CLC Bio). Gene locus tags for these A. baumannii 17978 genes are as follows: umuD(A1S_0636) and umuC(A1S_0637), umuD(A1S_1174) and umuC(A1S_1173), umuD(A1S_1389), umuC(A1S_2008), umuC(A1S_2015), and dinB(A1S_0186).

Construction of A. baumannii 17978 dinB::Km. The dinB::Km insertion knockout was created using a method developed by Aranda et al. (66) with some modifications. An amplicon of approximately 3000 bp was constructed by splicing by overlap extension PCR (81). This fragment contains a kanamycin resistance gene insertion at bp 414-612 (resulting in a 198 bp deletion) of the A. baumannii 17978 dinB gene (see Table 1.1 for oligonucleotide sequences).
The Km gene was amplified by PCR from pUA66 (82) using Kan-F and Kan-R oligonucleotides (Table 1.1). \textit{dinB}-int-R and \textit{dinB}-nest-F (Table 1.1) were used to amplify the 5’ end of the \textit{dinB} gene and approximately 550 bp upstream of \textit{dinB}. \textit{dinB}-int-F and \textit{dinB}-nest-R (Table 1.1) were used to amplify the 3’ end of the \textit{dinB} gene and approximately 500 bp downstream of \textit{dinB}. Finally, using \textit{dinB}-nest-F and \textit{dinB}-nest-R (Table 1.1), the three pieces were joined together by PCR. All PCRs were carried out using Gotaq Green Master Mix (Promega). This 3000 bp product was ligated into pGEM Easy T-Vector (Promega) using T4 DNA ligase (Promega) and the resulting \textit{dinB}::Km plasmid was introduced into \textit{A. baumannii} 17978 cells by electroporation at 1.8 mV for 5 ms following standard \textit{E. coli} protocols (83). \textit{A. baumannii} \textit{dinB}::Km colonies were confirmed by sequencing (Tufts Core Facility) using chromosomal flanking oligonucleotides \textit{dinB}-up-F with \textit{dinB}-down-R; \textit{dinB}-up-F with Kan-R; and \textit{dinB}-down-R with Kan-F (Table 1.1). Kanamycin was used at 35 ug ml$^{-1}$ for selection in \textit{A. baumannii} and plasmid maintenance in \textit{E. coli}.

\textbf{UV, MMS, and ciprofloxacin treatment.} Saturated cultures of \textit{A. baumannii} 17978 (~10$^9$ cells; parental) and \textit{A. baumannii} 17978 \textit{dinB}::Km were diluted 1:1000 in LB broth and grown for 2.5 hours. They were then sub-cultured for 2 hours, 3 consecutive times by diluting cultures each time 1:50 to ensure cells were in exponential phase. \textit{A. baumannii} 17978 \textit{recA}::Km cultures were grown similarly, with the exception of the final growth cycle being 4 hours. For UV treatment, 10 mL saturated cultures were spun down, resuspended in equal volume of SMO (100 mM NaCl, 20 mM Tris-HCl pH 7.5), and 2 mL samples were placed evenly in a sterile glass petri dish. Samples were irradiated in the dark under a UV germicidal lamp with 270 J m$^{-2}$ for parental and \textit{dinB}::Km, or 5 J m$^{-2}$ for \textit{recA}::Km, resulting in approximately 2-20% survival. Parallel samples of the parental strain were also irradiated with 100 J m$^{-2}$. 

13
For MMS and ciprofloxacin treatments, cultures were grown to exponential phase as described for UV treatment. 25 mM methyl methanesulfonate (MMS; Sigma; 1X MIC) or 6 µg ciprofloxacin mL\(^{-1}\) (Sigma; 10X MIC) were used to treat the parental and \(\text{dinB}\::\text{Km}\) cultures for 1 hour. In addition, parental strain cultures were treated for 2 and 3 hours with ciprofloxacin. For \(A. \text{baumannii}\ 17978\ \text{recA}\::\text{Km}\) cultures, 0.8 mM MMS (1X MIC) or 1 µg ciprofloxacin mL\(^{-1}\) (10X MIC) were used. After treatment, which resulted in 10-fold killing for all strains used after 1 hour, cells were spun down and washed in SMO two times.

**Semi-quantitative RT-PCR.** UV-treated samples were incubated for 1 hour prior to RNA extraction to allow for gene expression. Total RNA was obtained by following the RNA Protect and RNeasy protocols (Qiagen). Absence of DNA was verified by carrying out a PCR with Go-taq 2X Master Mix (Promega) and the same oligonucleotide sets as described below for RT-PCR (Table 1.1) at the highest concentration of total RNA used for RT-PCR (100 ng). Total RNA concentration was measured by a spectrophotometer nanodrop at \(A_{260}\) (Nanodrop 2000, Thermo Scientific). Equal amounts of total RNA (100 ng) from treated and untreated samples were 10-fold serially diluted and used as template for the SuperScript III One-Step RT-PCR System with Platinum Taq (Life Technologies) kit. The concentrations of the serially diluted total RNA were measured, within the nanodrop’s limit of detection of 1 ng µL\(^{-1}\), and were determined to be within approximately 10% of the predicted concentration. PCR conditions were followed per manufacturer’s recommendations. Oligonucleotides (Table 1.1) were designed to be specific for amplifying either the unique junctions between \(\text{umuD}\) and \(\text{umuC}\) in the \(\text{umuDC}\) operons, or to the unlinked \(\text{umuC, umuD, dinB, uvrA(A1S}_3295), \text{recA(A1S}_962\) and 16s rRNA (A1S_r01) open reading frames. cDNA was separated by electrophoresis in 1% agarose (SeaKem) gels. Gel images were analyzed using ImageJ 1.46r software (Wayne Rasband, NIH,
USA). The software provides a measurement of the thickness and intensity of the separated electrophoresis bands. The area of each band was determined to learn the specific mRNA concentration present at each dilution from treated and untreated samples, which is in turn divided by the total RNA dilution factor. Changes in relative expression were thus calculated.

**Spontaneous and induced-mutagenesis.** For all mutagenesis assays, bacterial cultures were started with $\leq 100$ cells to reduce the probability of preexisting mutants in the starting inoculum. For UV-induced mutagenesis, samples were treated as described for UV-treatment ($270 \text{ J m}^{-2}$ for parental), with the exception that cultures were grown 1 time at a 1:50 dilution from the starting saturated culture. After treatment, samples were immediately diluted 1:10 in LB medium-containing flasks wrapped in tin foil and grown to saturation. Then, the appropriate cell dilutions were deposited on LB plates with and without rifampicin to assess, respectively, the number of rifampicin resistant mutants ($\text{Rif}^R$) and the total number of CFUs. Colonies were counted after 24 hours of incubation. Mutation frequency was calculated by dividing the number of $\text{Rif}^R$ mutants by the total number of CFUs. Spontaneous $\text{Rif}^R$ mutants from untreated saturated cultures were determined as described. Statistical significance was calculated using a student t-test.

For MMS-induced mutagenesis, cultures were grown and treated as described for MMS-treatment, with the exception that cultures were grown one time at a 1:50 dilution directly from the saturated cultures. After the 1-hour treatment, washed cultures were diluted 1:3 in LB medium and grown to saturation. $\text{Rif}^R$ mutation frequency was determined as described above.

The protocol used for desiccation-induced mutagenesis is a modification of the one used by Aranda et al. (66). 0.5 mL of saturated cultures were deposited onto sterile 0.45 $\mu$m, black gridded 47 mm filters (Millipore) by filtration. Filters were dried inside a closed, sterile petri
dish at 37°C for 24 hours (recA\(^+\) strains) or 6 hours (recA::Km). 3-5 fold killing was observed for \(A.\) baumannii 17978, dinB::Km, and \(A.\) baumannii 19606 strains and 15-fold killing for recA::Km. In addition, an exponential phase culture of \(A.\) baumannii 17978 was desiccated as described above for 24 hours, which resulted in 15-fold killing.

**Sequencing of Rif\(^R\) mutants.** Colony PCR was performed according to the Go-Taq 2X Master Mix (Promega) protocol on 32 individual desiccation-induced Rif\(^R\) mutants from 6 independent \(A.\) baumannii 17978 recA\(^+\) experiments and also on 10 individual dinB::Km Rif\(^R\) mutants from 5 independent \(A.\) baumannii 17978 dinB::Km experiments. Oligonucleotides rpoB-1441F and rpoB-2095R (Table 1.1) amplify a 654 bp region of rpoB (locus A1S_0287) where Rif\(^R\)-inducing base pair substitutions are frequently located (37). Sequencing (Tufts Core Facility) was carried out using the same oligonucleotide set. The data obtained were analyzed using CLC Main Workbench (CLC Bio).

**Immunoblotting.** Cells were spun down and lysed with Bugbuster (Novagen) after UV treatment. Total protein concentration was determined for each sample with Bradford reagent (Biorad) following the manufacturer’s protocol. Equal amounts of total protein per sample, mixed 1:1 with Laemml Sample Buffer (2x; Sigma), were separated by SDS-PAGE on a 4-12% Bis-Tris gel (Life Technologies) with 1x MOPS buffer (Life Technologies). After electrophoresis, proteins were transferred to a PVDF membrane (Immobilon-P; Millipore) and incubation with primary and secondary antibodies was carried out according to published procedures (83). Bound antibodies were detected with Luminata Crescendo Western HRP Substrate (Millipore) followed by autoradiography or imaging on a Typhoon 8600 (GE Healthcare) using ImageQuant 5.2 software (Molecular Dynamics). Gel images were analyzed using ImageJ 1.46r software (Wayne Rasband, NIH, USA; see prior methods section). Relative
fold-change in expression was determined by dividing the obtained intensities by the intensity of 
the untreated sample.

Polyclonal rabbit anti-DinB antibody, the generous gift of Dr. Takehiko Nohmi (84), was 
affinity purified (85) and diluted 1:100. Polyclonal rabbit anti-UvrA antibody (Covance) was 
generated using purified UvrA protein (the generous gift of Dr. Ben Van Houten) and used at a 
1:10,000 dilution. Rabbit polyclonal anti-RecA (Abcam, Cambridge, MA) was used at a 
1:10,000 dilution while the mouse monoclonal anti-RpoB (Abcam, Cambridge, MA) was used at 
a 1:5,000 dilution.

**E. coli strains, plasmids, and growth conditions.** *Escherichia coli* strain P90C (86) 
ΔdinB::Km (lab stock) derivative was used as wild-type. MG1655 ΔalkA tag dinB (87) is the base excision repair-deficient strain (gracious gift of Ivan Matic, Université Paris Descartes). Plasmids used in this study include: pVector (pWKS30, (88)), pEc-\( \text{dinB}^{\text{native}} \) (pYG768, (22)), pEc-\( \text{dinB}^{\text{lac}} \) (pYG782, (22)); pAb-\( \text{dinB}^{\text{native}} \) and pAb-\( \text{dinB}^{\text{lac}} \) were constructed for this work. *E. coli* strains were routinely grown in Luria broth (LB) and supplemented with 200 µg/mL ampicillin (Ap; Sigma) for plasmid maintenance.

**Construction of pAb-\( \text{dinB}^{\text{lac}} \).** *Acinetobacter baumannii* dinB (gene locus A1S_0186) from strain ATCC 17978 was amplified by PCR using the oligonucleotides 5’-ATG CGC AAA ATC ATT CAT ATC G-3’ and 5’-TTA CCA TAA GGA CAA CTG AAA GTC G-3’ with Platinum *Taq* DNA polymerase High Fidelity (Life Technologies). The amplification product was purified and ligated into pGEM cloning vector (Promega). The PstI and SacII Ab-\( \text{dinB} \) fragment was subcloned into the low copy number plasmid pWKS30 under the *lac* promoter. The resulting pAb-\( \text{dinB}^{\text{lac}} \) plasmid was sequenced with M13 forward and reverse oligonucleotides (Tufts Core Facility).
Construction of pEc-dinB<sub>native</sub>. Site-directed mutagenesis was performed on plasmid pYG768 (contains <i>E. coli</i> <i>dinB</i> under its native promoter; (22)) using the Gene-Tailor kit (Life Technologies), according to manufacturer’s instructions. Using oligonucleotides 5’-ACC AGT GTT GAG AGG TGA GCT AGC AAT GCG TAA AAT CAT TC-3’ and 5’-GCT CA CCT CTC AAC ACT GGT AAA GTA TAC AGT GAT TTC AGG-3’, a Nhel restriction site was inserted between the starting <i>E. coli</i> <i>dinB</i> methionine codon and the native promoter region. Resulting plasmid was confirmed by sequencing (Tufts Core Facility) using oligonucleotides 5’-GGG ATA ATT GGC GGT GCT GAT CAC and 5’-CCG GCG CAT TGAG ATT ATG GTG C-3’. The Nhel restriction site was added so that the <i>A. baumannii</i> <i>dinB</i> gene could be inserted into the plasmid directly downstream of the <i>E. coli</i> <i>dinB</i> promoter.

Construction of pAb-dinB<sub>native</sub>. <i>A. baumannii</i> <i>dinB</i> was amplified by PCR with oligonucleotides that introduced restriction site Nhel on the 5’ end and HindIII on the 3’ end of the gene (5’ GGG GGC TAG CAA TGC GCA AAA TCA TTC ATA TCG-3’, 5’-CTG CAA GCT TTT ACC ATA AGG ACA ACT GAA AGT CG-3’). The amplification product was cloned into the Nhel and HindIII sites of pEc-dinB<sub>native</sub>, resulting in Ab-dinB directly downstream of the native <i>E. coli</i> <i>dinB</i> promoter. The newly constructed plasmid was sequenced (Tufts Core Facility) with 5’-CCG GCG CAT TGA GAT TAT GGT GC-3’, 5’-TAA TAC GAC TCA CTA TAG GG-3’, 5’-CTC ATG GAC ATG GCA GAG CG-3’, and 5’-GCA ACT GAA TGC CCG AGG TG-3’.

<i>E. coli</i> Survival Assays and DNA damage treatments. For survival assays, three independent <i>E. coli</i> cultures were grown to saturation. Cultures were serially diluted in SMO and 10 µL spots were deposited on LB-Ap agar with methyl methanesulfonate (MMS; Acros Organics), ethyl methanesulfonate (EMS; Acros Organics), 4-nitroquinolone-1-oxide (4-NQO;
Sigma), or nitrofurazone (NFZ; Sigma) at the concentrations specified in figure legends. NFZ and 4-NQO plates were incubated in the dark for 20 hours, and MMS plates were incubated for 20-40 hours depending on the strain and concentration. Percent survival was determined by calculating the fraction of colony forming units (CFUs) grown with the DNA-damaging agent per total number of CFUs grown on LB.
RESULTS

Most *A. baumannii* genomes encode multiple error-prone DNA polymerases genes organized either as operons or as unlinked genes. We wanted to know if *A. baumannii* regulates the error-prone translesion synthesis (TLS) DNA polymerases in response to DNA damage or environmental stress, because this would account for a yet undetermined mechanism of genomic evolution and antibiotic resistance acquisition in this organism.

To gain insights into the expression, genetic context and relevance of these predicted TLS DNA polymerase genes in *A. baumannii* 17978, we searched the sequenced genomes of 10 independent *A. baumannii* isolates (Table 1.2) for genes whose products show similarity with the *E. coli* TLS DNA polymerases UmuC, DinB, and DNA Pol II and the accessory protein, UmuD. This was done using the standard protein-protein BLAST search engine made available by NCBI (80); genomic sequences that were not annotated were hand-curated accordingly. Interestingly, we found no *polB* genes (encoding TLS DNA Polymerase II) in these genomes (Table 1.2). As in *E. coli*, *A. baumannii* isolates have only one putative *dinB* gene. DinB homologues from *A. baumannii* share sequence similarity with *E. coli* DinB with E values less than or equal to 2x10^-69, and were found to have nearly 100% sequence conservation between *A. baumannii* isolates (Fig. 1.6). Not surprisingly, we discovered that *A. baumannii* DinB is also recognized by *E. coli* polyclonal antibody (see below and Fig. 1.4).

Because *E. coli* DNA Pol V (composed of UmuD’2C) is extensively regulated to minimize unnecessary mutagenesis (1), it is very surprising that the majority of *A. baumannii* genomes encode multiple, putative *umuC* and *umuD* homologues (Table 1.2). There is even one isolate, *A. baumannii* ATCC 17978, with four putative *umuC* and three *umuD* homologues. We found that isolates have acquired different combinations of the number of *umuC* and *umuD* genes
(Table 1.2), both on the chromosome and on plasmids (e.g. strain ACICU; Table 1.2). The total intracellular concentration of active DNA Pol V will depend on the expression of these multiple umuC and umuD genes. However, even if an isolate has acquired numerous umuC genes, A. baumannii DNA Pol V activity likely depends on enough supporting umuD gene products (89). Because A. baumannii 17978 has more copies of both umuC and umuD genes, it may have the potential for more DNA damage-induced mutagenesis (or DNA Pol V-induced) than the other isolates listed (Table 1.2).

Conserved catalytic residues of the active site (90) were used to validate A. baumannii 17978 umuC gene products’ homology to E. coli UmuC (Fig. 1.7). Each of the putative UmuC homologues, annotated in Genbank as either RumB, DNA-directed DNA polymerase, or DNA repair protein, share sequence similarity with E. coli UmuC throughout the protein sequences with E values less than or equal to 7x10^-82 (Fig. 1.7). Similar E values were found for all putative A. baumannii umuC genes listed in Table 1.2. UmuD protein sequences of all A. baumannii isolates share sequence similarity with E. coli UmuD with E values less than or equal to 7x10^-18, in agreement with previous reports (76, 91).

In A. baumannii 17978, we found that the four umuC genes are uniquely organized, and different than E. coli. Figure 1.1 diagrams the arrangements of the two umuDC operons, the two unlinked umuC, and the one unlinked umuD gene of A. baumannii 17978. There are interesting differences between A. baumannii and E. coli even within the umuDC operons: for instance, in E. coli, umuD and umuC genes overlap by one nucleotide (Fig. 1.1, top; (1)). In contrast, we found that the open reading frame (ORF) of umuC(A1S_0637) overlaps the ORF of umuD(A1S_0636) by 20 nucleotides, and the ORF of umuC(A1S_1173) does not overlap the umuD(A1S_1174) ORF at all. Instead, the umuC(A1S_1173) ORF starts 3 nucleotides after the
stop codon of \textit{umuD} (Fig. 1.1). In \textit{E. coli}, the -1 frameshift within the ORF of the \textit{umuDC} operon is part of the regulation of expression of the \textit{umuD} and \textit{umuC} gene products, resulting in significantly less translation of \textit{umuC} than \textit{umuD}, and thus a low intracellular concentration of DNA Pol V molecules (1). Therefore, it is likely that these gene arrangements in \textit{A. baumannii} would influence the synthesis of their gene products as well.

**Predicted TLS DNA polymerase and other DNA damage response genes are expressed in \textit{A. baumannii} 17978.** We wanted to ascertain whether the predicted multiple \textit{umuC}, \textit{umuD} and the single \textit{dinB} genes are expressed in \textit{A. baumannii} 17978, since this isolate has acquired the most TLS DNA polymerases of those sequenced (Table 1.2). To also examine the role of RecA, if any, in gene expression, we obtained an isogenic \textit{A. baumannii} 17978 strain with a kanamycin gene cassette inserted within \textit{recA}, rendering its gene product functionally inactive (\textit{recA::Km}; (66)). We hypothesized that RecA would play a key role in the induction of the aforementioned genes as well as other DNA damage response genes in \textit{A. baumannii}, despite lacking a discernable LexA. We measured mRNA transcript levels by semi-quantitative RT-PCR to determine basal level gene induction (Fig. 1.2). Total RNA was purified from untreated \textit{A. baumannii} cells; then the same amount of starting RNA template was used for subsequent RT-PCRs. The relative mRNA expression levels were thus obtained using gel electrophoresis image analysis (refer to Materials and Methods). Each gene’s basal level of expression was calculated as a percentage of 16S rRNA expression, a standard housekeeping gene, in both the \textit{recA}\textsuperscript{+} and \textit{recA::Km} strains. This analysis permits the assessment of any differences in the relative basal level of expression between the examined genes. It should be noted here that we are able to measure \textit{recA} expression in the \textit{recA::Km} strain, because of the kanamycin cassette insertion.
The \textit{recA} oligonucleotides are specific to the 5' end of the gene (first 260 bp), a region that remains intact on the chromosome of the \textit{recA::Km} strain.

We found that the \textit{A. baumannii umuDC} operons, the unlinked \textit{umuD} and \textit{umuCs, dinB, uvrA} and \textit{recA} are expressed because we detected their respective transcripts (Fig. 1.2). Notably, \textit{umuD}(1389) and \textit{recA} had the highest relative basal level of expression in the \textit{recA}+ strain (Fig. 1.2). The \textit{umuDC}(0636-0637) operon, unlinked \textit{umuC}(2008), \textit{uvrA} and \textit{dinB} had the second highest level of relative expression in the \textit{recA}+ strain. Lastly, the \textit{umuDC}(1174-1173) operon and unlinked \textit{umuC}(2015) had lowest relative basal level expression in the \textit{recA}+ suggesting that these genes may be the most tightly regulated of those analyzed in \textit{A. baumannii} 17978. In the \textit{recA::Km}, we found a similar gene expression profile, however one surprising difference is evident: \textit{umuC}(2015) and \textit{uvrA} have marked higher relative basal level expression in \textit{recA::Km} compared to the \textit{recA}+ (Fig. 1.2). This suggests a role for RecA in the regulation of these genes; possibly an involvement in repression.

\textit{A. baumannii TLS DNA polymerases are upregulated as part of a RecA-mediated DNA damage response.} \textit{Escherichia coli} and other bacteria manage genomic instability in response to DNA damage or environmental stress by regulating a globally induced response, the SOS gene network (1, 5). The lack of an identifiable LexA homologue has made it difficult to characterize a similar damage response in \textit{Acinetobacter} (47, 76). In the classic \textit{E. coli} DNA damage response, the orchestrated upregulation of stress-response proteins is controlled at the level of transcription (13, 18, 92). We assessed whether we could detect changes in gene expression after treatment with three different DNA damaging agents: MMS, ciprofloxacin and UV. These agents are known to induce the DNA damage regulatory system in \textit{E. coli} through varying mechanisms. MMS is a cytotoxic DNA alkylating agent that produces replication fork-
stalling 3-methyladenine (3-meA) lesions (87). Ciprofloxacin is an antibiotic that is a strong inducer of the SOS response in *E. coli* (11, 93); it causes replication stress because it traps the gyrase-DNA complex and blocks DNA replication, potentiating DNA double-strand breaks (94). UV irradiation is also classically used as a strong inducer of the SOS response (95) because it produces fork-stalling DNA lesions such as thymine-thymine dimers (1). Like *E. coli*, *A. baumannii* is sensitive to killing by UV, and the *recA::Km* strain is extremely sensitive as predicted ((66); data not shown). *A. baumannii* 17978 *recA*+ or *recA::Km* strains were each treated with MMS at their respective MIC and ciprofloxacin was used at a clinically relevant concentration of 10X the MIC. To compare between strains with dramatically different sensitivities to DNA damaging agents, we used doses of drugs or UV treatments in which they had the same viability. Otherwise, cells would either die (e.g. if a UV dose typically used for a *recA*+ is used for a *recA::Km*) or the treatment would not elicit a response (e.g. if a UV dose typically used for a *recA::Km* strain is used for a *recA*+).

We determined first whether there is induction of *A. baumannii* DNA Pol V genes upon treatment with DNA damaging agents. In the *recA*+ strain the levels of expression for all *umuDC* operons and unlinked *umuD* and *umuC* loci increased upon all three treatments (Fig. 1.3A; black bars). The gene expression profiles differ for each treatment, but the *umuDC* (1174-1173) operon has the highest fold increase in expression in each case. We also saw drastic differences in induction between treatments. For instance, *umuC* (2015) was only modestly upregulated upon MMS-treatment (~1.5-fold), but upon ciprofloxacin- and UV-treatment, its expression increased ~10- and ~4-fold, respectively (Fig. 1.3A). *umuD* (1389) gene expression was induced ~10-fold for MMS- and ciprofloxacin-treatment and only 2-fold upon UV-treatment (Fig. 1.3A).
Conversely, it is apparent that in recA::Km induced levels were either greatly reduced when compared to recA+ or not induced at all (Fig. 1.3A; white bars). Remarkably, we found some genes are induced even in the absence of recA, as exemplified by the umuDC(0636-0637) operon during ciprofloxacin- and UV-treatment and the umuDC(1174-1173) operon during UV-treatment (Fig. 1.3A).

We next examined the induction of two DNA damage response genes recA and uvrA, and the other Y-family DNA polymerase, DinB (or DNA Pol IV). Like the DNA Pol V genes (Fig. 1.3A), there was induction of expression of recA and uvrA in the recA+ strain (Fig. 1.3B; black bars). The induction of recA upon ciprofloxacin treatment was quite dramatic (34-fold; Fig. 1.3B), suggesting that RecA is likely an important part of this response, and that ciprofloxacin is a strong inducer of the A. baumannii DNA damage response, as it is for E. coli (11, 93).

Because we were able to measure recA expression in the A. baumannii recA::Km strain, we were able to see that its expression in recA::Km was almost equal to that of recA+ during MMS treatment (Fig. 1.3B). Upon ciprofloxacin treatment, recA was expressed comparatively higher than other genes in the recA::Km strain (Fig. 1.3B). It was also expressed approximately one-third as much as was seen in recA+. In contrast, recA was significantly induced (~30-fold) upon UV-treatment in recA::Km compared to recA+ (~5-fold; Fig. 1.3B), suggesting deregulation of recA in the absence of RecA.

No significant changes in expression were observed for dinB in either recA+ or recA::Km, which is similar to the 16s rRNA control (Fig. 1.3B). In a time course with ciprofloxacin- or UV-treatment, we found no detectable differences in the levels of induction of many genes, including dinB, in comparison to the results shown in Fig. 1.3 (recA+; data not shown).
In response to persistent DNA damage or replication stress, transcript upregulation should coincide with an increase in protein levels (1, 18). We tested for a change in abundance of three DNA damage-inducible proteins in response to UV induced-damage. We selected RecA, UvrA, and DinB, because each of these is encoded by a single gene in *A. baumannii* 17978. DinB was of particular interest since we were unable to see a detectable increase in transcript. We predicted that antibodies raised against the *E. coli* proteins would recognize the respective *A. baumannii* homologues, given the similarity in their predicted primary sequences.

Increasing levels of all three proteins in response to increasing doses of UV irradiation were observed in *A. baumannii* 17978 (Fig. 1.4). The relative increase in RecA protein expression at 160 J m\(^{-2}\) compared to untreated was 40-fold; UvrA, 2.5-fold; and DinB, 3-fold (Fig. 1.4). No change was observed in the housekeeping protein, RpoB, the RNA polymerase β subunit (Fig. 1.4). Although the use of different antibodies precludes comparison of the amplitude of induction of the three proteins, the simultaneous increase in abundance of all three in response to DNA damage is consistent with a DNA damage regulatory program in *A. baumannii*. While a change in the expression of *dinB* at the level of transcription was undetectable (Fig. 1.3B), the observable increase in protein over time strongly suggests that DinB is induced upon DNA damage.

Taken together, these data provide evidence for a bona fide DNA damage-inducible response in *A. baumannii* with TLS as a key component. The induction of a host of genes, including the multiple DNA polymerase V components, was shown using a DNA alkylating agent, UV irradiation and treatment with an antibiotic frequently used by clinicians at clinically relevant concentrations. High-level induction of these genes is dependent on RecA, but the data also suggests that the role of RecA in *A. baumannii* gene regulation is different than the *E. coli*
paradigm. These results are also consistent with the hypothesis that *A. baumannii* may induce this DNA damage response as a possible mechanism for genomic evolution upon multiple stressors. We therefore sought to gain evidence for the role of the DNA damage response in *A. baumannii* induced-mutagenesis.

*A. baumannii* recA-dependent DNA damage response contributes to induced mutagenesis. We set forth to test whether this response is responsible for DNA damage-induced mutagenesis by using an established rifampicin resistance (Rif$^R$) assay (96). Rifampicin is an antibiotic frequently coupled with colistin and used by clinicians to treat multidrug-resistant *A. baumannii* infections (37). Rifampicin targets the β subunit of the bacterial RNA polymerase holoenzyme, RpoB. Only base pair substitutions, i.e. not frameshifts, in the *rpoB* gene lead to select residue changes in the target site of RpoB, decreasing the effectiveness of rifampicin binding (96). These base pair substitutions can be the result of error-prone DNA polymerase such as DNA Pol V (20). *A. baumannii* clinical Rif$^R$ isolates have been shown to have mutations in *rpoB* (37), validating this assay for use in *A. baumannii*. Both parental *A. baumannii* 17978 and the *recA*::Km isogenic strains were tested for induced mutagenesis by selecting for Rif$^R$ mutants after exposure to UV and to the alkylating agent MMS. We also constructed an *A. baumannii* 17978 *dinB*::Km insertion knockout strain to assess the impact of DinB on induced mutagenesis. TLS DNA polymerase gene products are necessary in *E. coli* for both survival and induced mutagenesis in cells that have accumulated UV- and MMS-induced DNA lesions (1, 87, 93), and we know (see previous sections) that these genes are induced by treatment with these reagents in *A. baumannii*.

As shown in Figure 1.5A, in the parental *recA*$^+$ strain there was a dramatic increase (~30-fold for UV; ~400-fold for MMS) in the frequency of DNA damage-induced Rif$^R$ mutation
frequency (UV, grey bars; MMS, black bars) when compared to spontaneous Rif$^R$ mutation frequency (white bars). No significant increase in MMS- or UV-induced Rif$^R$ mutation frequency was observed for recA::Km (Fig. 1.5A). Interestingly, a significantly lower spontaneous mutation frequency (3.5-fold; P<0.01) was found for the dinB::Km strain when compared to parental (Fig. 1.5A), and it is also not statistically different than that of recA::Km (P>0.05). UV- and MMS-induced mutation frequencies for dinB::Km were the same as parental; however, the fold-increase of induced compared to spontaneous mutation frequencies was larger than dinB$^+$ (70-fold for UV, 1400-fold for MMS).

Together these data demonstrate that rifampicin resistance can be acquired through the recA-dependent DNA damage response in A. baumannii, likely resulting from DNA base pair substitutions in the rpoB gene (37). The dinB::Km data also suggests a role for A. baumannii DinB in generating spontaneous mutations, and emphasizes that the multiple DNA Pol Vs likely have a greater role in induced-mutagenesis.

**Desiccation-induced mutagenesis is recA-dependent.** From these data, we became intrigued by the possibility that A. baumannii may be able to mutate in the hospital setting as the result of environmental processes likely to produce DNA damage. It is known that A. baumannii is able to survive on hospital equipment for long periods of time and has considerable desiccation tolerance (48, 49). Desiccation and desiccation-rehydration cause various DNA lesions including alkylation, oxidation, cross-linking, base removal, and strand breaks (97); and it has been reported that A. baumannii 17978 recA::Km cells are sensitive to desiccation stress (66). It is likely that A. baumannii cells on the surfaces of hospital equipment incur these types of desiccation-induced DNA lesions. We hypothesized that these DNA lesions would result in elevated mutagenesis when cells are rehydrated. We simulated desiccation-induced DNA-
damage by drying *A. baumannii* cells on filters for a period of time resulting in standardized killing (see materials and methods). As expected, and in agreement with previous findings (66), *recA::Km* cultures were more sensitive to drying than the parental (data not shown). Cells were rehydrated and grown in rich liquid medium to assess the frequency of Rif\(^R\) mutants. As seen in Figure 1.5B, the mutation frequency post-desiccation (grey bars) compared to pre-desiccation (white bars; spontaneously arising mutations only) was significantly increased (~50-fold) in the *A. baumannii* 17978 *recA*\(^+\) strain. No significant increase in mutation frequency was observed in the *A. baumannii* 17978 strain lacking *recA* post-desiccation (P=0.2). Post-desiccation mutation frequency of *A. baumannii* 17978 *dinB::Km* matches the frequency of *A. baumannii* 17978 (data not shown), and we can again infer that there is a lessened role for *A. baumannii* DinB in induced-mutagenesis and a greater role for DNA Pol Vs. Together these results correlate with the results of DNA alkylation-induced mutagenesis (MMS; Fig. 1.5A), since it is probable that cells incur DNA alkylation lesions from desiccation (97).

Because *A. baumannii* 17978 has 4 predicted *umuC* and 3 predicted *umuD* genes (Fig. 1.1 and Table 1.2), we expected that a strain with fewer TLS genes would result in fewer Rif\(^R\) mutants upon desiccation rehydration. As a proof of concept, we used the strain *A. baumannii* 19606, which possesses 2 predicted *umuC* loci (HMPREF0010_03135 and HMPREF0010_00311) that are the same as those present in *A. baumannii* 17978 (A1S_1173 and A1S_2008, respectively). The 2 predicted *A. baumannii* 19606 *umuD* loci, HMPREF0010_00986 and HMPREF0010_03136, are also present in the *A. baumannii* 17978 genome as A1S_1389 and A1S_1174, respectively. Moreover, we have shown that these common loci were induced upon DNA damage (Fig. 1.3). We compared the frequency of Rif\(^R\) mutants after desiccation between these two strains. Like *A. baumannii* 17978, we found that
there were significantly more Rif\(^R\) mutants for \textit{A. baumannii} 19606 post-desiccation compared to pre-desiccation (~7-fold; \(P<0.01\); Fig. 1.5B). Remarkably, this increase is significantly less (~7-fold) than the increase observed for \textit{A. baumannii} 17978 (Fig. 1.5B) even though both strains are comparably sensitive to desiccation. Therefore, these data suggest a correlation between the number of genes encoding error-prone DNA Pol V and the number of desiccation-induced Rif\(^R\) mutants.

We then tested the hypothesis that the \textit{A. baumannii} 17978 rec\(^A\) desiccation-induced Rif\(^R\) mutants were the result of \textit{rpoB} base pair substitutions. The \textit{rpoB} gene from 32 individual colonies was sequenced and it was found that all isolates had indeed acquired mutations in this gene (Table 1.3). Sequence analysis revealed single base pair substitutions that result in amino acid substitutions. Our data coincides with published clinical Rif\(^R\) isolates containing amino acid substitutions for aspartic acid at position 525, histidine at position 535, serine at position 540, leucine at position 542 and isoleucine at position 581 (37). At these positions, we found the recognized D525Y, H535L and S540Y substitutions (37) as well as a number of novel substitutions that are indicated in Table 1.3. We also found new substitutions of the glutamic acid in position 522 for lysine, leucine or arginine.

In addition, the \textit{rpoB} sequence from 10 \textit{A. baumannii dinB::Km} desiccation-induced Rif\(^R\) mutants was sequenced. Many of the same mutations as those in \textit{dinB}\(^+\) were found, including amino acid substitutions at positions 522, 525, 535, 540, 542, 566, and 581 (Table 1.3). Two mutations, D525V and R566C, were also found to be unique to \textit{dinB::Km}. The majority of \textit{dinB::Km} mutations are transversions (7 out of 10), as are the majority of \textit{dinB}\(^+\) mutations (21 out of 32). Analysis of the total \textit{dinB}\(^+\) and \textit{dinB::Km} sequences combined reveals the majority (67%; 28 out of 42) of base pair substitutions to be transversions (Table 1.3), a signature of DNA
Pol V in *E. coli*, and all but one listed substitution (A to G transition) are also known to be DNA Pol V generated (45).

**DISCUSSION**

*A. baumannii* is desiccation resistant, which permits long-term survival and transmission in hospital environments. It also quickly becomes multidrug resistant, and has thus become a major worldwide health concern (40, 42, 48, 49, 53). It is clear that homologous recombination, horizontal gene transfer and plasmids play a role in antibiotic resistance acquisition (40, 42, 59), though the underlying regulatory mechanisms, if any, have remained unknown. A global response to DNA damage or harsh environmental conditions has been shown to play a key function in antibiotic and virulence acquisition in other organisms (13, 14), but it has been unclear whether such a response exists in *A. baumannii*. In this study, we present evidence for a bona fide *A. baumannii* global DNA damage-inducible response, and identify this response as one important mechanism of antibiotic resistance acquisition.

It has been unclear why *A. baumannii* isolates have acquired, most likely through horizontal gene transfer (76), multiple *umuDC* operons and unlinked *umuCs* or *umuDs* (Fig. 1.1 and Table 1.2). This is in stark contrast to *E. coli*, which highly regulates a single *umuDC* operon to minimize the intracellular concentration of active DNA Pol V (1). We found that these multiple DNA Pol V gene components are all expressed at different levels in *A. baumannii* 17978 (Fig. 1.2) and induced upon DNA damage (Fig. 1.3A). Different DNA damaging agents caused distinct expression of the multiple *umuD* and *umuC* genes (Fig. 1.3A), consistent with an idea in which the multiple DNA Pol Vs may have different lesion-bypass abilities (and mutation signatures; Table 1.3). Thus, these possibly provide *A. baumannii* 17978 with multiple
alternatives to cope with DNA damage. The unlinked *umuD*(1389) is ubiquitously present in all the *A. baumannii* genomes analyzed (Table 1.2). Its role in the *A. baumannii* DNA damage response is likely similar to its role in *E. coli*. Indeed, this *umuD* gene product is most closely similar to the *A. baylyi* *umuD* gene product shown to be cleaved in *E. coli* in response to DNA damage (91), suggesting its role in the DNA Pol V complex might be similar to that of *E. coli* UmuD’.

We found that DinB is also induced by DNA damage based on a detectable increase in protein levels upon UV treatment (Fig. 1.4), but we were unable to detect increased *dinB* transcript (Fig. 1.3B) even over a time course of treatment (data not shown). We do not yet understand the reason for this discrepancy. We tested whether *A. baumannii* DinB (Ab-DinB) activity is conserved and we found that *E. coli dinB::Km* is complemented by Ab-*dinB* on a low copy number plasmid (Fig. 1.8). Like *E.coli* DinB, which accurately bypasses N2-furfuryl-dG lesions generated by nitrofurazone and other N2-dG lesions generated by 4-nitroquinolone-1-oxide (23, 98), complementation with plasmid-borne Ab-*dinB* rescues cells from nitrofurazone- and 4-NQO-induced death (Fig. 1.8). In contrast and to our surprise, Ab-*dinB* does not complement *E. coli dinB::Km* upon treatment with alkylating agents (Figs. 1.8 & 1.9). In addition, *A. baumannii dinB::Km* cells are neither sensitive to alkylating agents (data not shown) nor are they more or less mutagenic upon treatment than *dinB*+ (Fig. 1.5A). These results suggest that Ab-DinB has different lesion bypass activities than *E.coli* DinB, and also provides more support for our hypothesis that mutagenesis and TLS are dominated by the DNA Pol Vs in *A. baumannii* 17978, especially considering there is no DNA Pol II in the *A. baumannii* sequences analyzed (Table 1.2).
Here we provide evidence for RecA regulating the induction of \textit{A. baumannii} DNA damage response genes (Fig. 1.3). RecA is essential to mount a DNA damage response in \textit{E. coli} (1) and is necessary for \textit{A. baumannii} to survive DNA damage and general stress (66). Interestingly, we also observed RecA-independent induction of some genes, and RecA may also have an autoregulatory role (Fig. 1.3B). The precise mechanistic role of RecA in the regulation of the \textit{A. baumannii} DNA damage response remains unknown, as does the yet unidentified LexA-like transcriptional repressor. DNA damage responses vary from bacteria to bacteria (13, 68, 99, 100) so it is possible that (i) a protein unidentifiable by primary and secondary structure has evolved a similar function as LexA or (ii) there is no LexA-like repressor, and the regulation in \textit{A. baumannii} is different to that of \textit{E. coli}’s. While both of these options are currently being investigated, this study suggests the latter is most likely. In agreement with this idea, we find no DinI homologue in \textit{A. baumannii}, a protein that turns off the SOS response in \textit{E. coli} by inhibiting LexA cleavage promoted by RecA nucleoprotein filament (5). We also failed to complement a \textit{lexA}(Def) strain of \textit{E. coli} with plasmids containing \textit{A. baumannii} genes encoding LexA-like candidates, including \textit{umuD}(1389) (A. MacGuire and V.G. Godoy, unpublished data). \textit{umuD}(1389) may still have a regulatory role in \textit{Acinetobacter} spp., as it does in \textit{A. baylyi}, a notion put forth by Hare et al. (91).

\textit{A. baumannii} is notorious for readily incorporating foreign DNA such as transposons, IS elements, and antibiotic resistance encoding islands into its genome (40, 42, 75). Therefore it has the ability to acquire antibiotic resistances possibly from a wide range of bacteria. In combination with error-prone DNA polymerases, both inherent and acquired through these means, \textit{A. baumannii} could evolve new resistances when faced with environmental stress by generating base pair substitutions in a variety of cellular targets (58, 73, 74). Our finding that \textit{A.}
*baumannii* mutates upon desiccation-rehydration (Fig. 1.5B), is not only novel, but it has
obvious implications in the clinical setting: improper disinfection of *A. baumannii* from surfaces
could lead to desiccation-induced mutagenesis. Importantly, current methods of disinfection are
lacking in their ability to kill *A. baumannii* or hinder further antibiotic resistance acquisitions.
Use of UV-light as a sterilizing agent in hospitals (101-104) may even promote mutagenesis
(Fig. 1.5A) if not done properly. Incorporation of a RecA inhibitor (105, 106), for example, into
new disinfectants may be a viable option in the near future as novel inhibitors continue to be
discovered and patented (107, 108). This would impede the DNA damage response, suppressing
both induced mutagenesis and homologous recombination in the hospital, and thus limiting
evolution of antibiotic resistance (15, 109, 110). Another intriguing use for a bacterial RecA
inhibitor includes combining it with antibiotic treatment as a combination therapy, thereby
increasing bacterial susceptibility and the therapeutic effects of the antibiotic (110).

In summary, we have uncovered a mechanism that may aid *A. baumannii* in genomic
evolution and acquisition of antibiotic resistance. This global DNA damage response has
hallmark features of those that are well understood; however, it is clear that the system in place is
by no means conventional. Elucidation of the more intricate details of this system will further
efforts to combat this deadly opportunistic pathogen.
ACKNOWLEDGEMENTS

This work was supported by the 1RO1GM088230-01A1 award from NIGMS to V.G. Godoy. We would like to thank Marin Vulic for critical reading of the manuscript, the Bou lab for generously sending us the *A. baumannii* recA::Km strain, Ivan Matic for the *E. coli* ΔalkA tag dinB strain, and Dr. Ben Van Houten for the UvrA protein. We would also like to thank Ashley MacGuire for providing her unpublished data and other members of the Godoy lab for helpful discussions.
Figure 1.1. The *A. baumannii* 17978 predicted *umuC* and *umuD* genes are organized differently than E. coli. (A) There is one *umuDC* operon in the *E. coli* (Ec) chromosome in which the *umuD* open reading frame (ORF) is expressed approximately 10-fold better than *umuC* due to a -1 frameshift between the two ORFs (17). This frameshift in the gene is depicted as overlapping arrows. (B) *A. baumannii* 17978 (Ab) has two putative *umuDC* operons, an organization similar to the one in *E. coli*, but within the *umuDC*(0636-0637) operon there is an overlap between the *umuD* and *umuC* genes of 20 nucleotides (depicted by overlapping arrows). In the *umuDC*(1174-1173) operon, we find no overlap between the two predicted genes. There are also two unlinked predicted *umuC* genes and one unlinked predicted *umuD* gene. For easier identification, locus tags (“A1S_” not included before number) are included as part of each *A. baumannii* gene name. Arrows represent predicted ORFs and white boxes represent promoter (P) or putative promoter (P*) regions.
Representative, evolutionarily conserved DNA damage response genes are expressed in \textit{A. baumannii} 17978. The predicted genes encoding DNA damage response genes are all expressed in the recA$^+$ strain though at different levels. Relative expression of each gene is shown as a percent of 16s rRNA expression, a standard housekeeping gene. In the recA::Km strain, most genes analyzed have no detectable change in relative basal level gene expression. Some genes showed modest detectable decreases and modest to moderate increases in expression, which suggests a role for RecA in gene regulation. Semi-quantitative RT-PCR was performed on total RNA purified from untreated cultures of \textit{A. baumannii} 17978. See Materials and Methods section for details of this experimental procedure. Gene specific RT-PCR primers were used to amplify approximately 300bp of either the unique junctions between the umuD and umuC genes organized as operons, or unique sequences of the unlinked genes. Locus tags from the \textit{A. baumannii} ATCC 17978 genome (“A1S” not included before number) are included as part of the umuD and umuC names. Data from a representative experiment is shown.
Figure 1.3. The predicted *A. baumannii* TLS DNA polymerases and other DNA damage response genes are induced by DNA damage and regulated by RecA. (A) Expression of putative DNA Polymerase V genes. All *umuD* and *umuC* loci are upregulated upon MMS,
ciprofloxacin, or UV light treatment in the recA+ strain. In the recA::Km strain, most genes have no change in expression, which is denoted as a fold change of 1. We also observed increased expression for some of the genes though lower than in recA+. (B) Expression of other DNA damage response genes. The three DNA damaging conditions examined resulted in upregulation of recA and uvrA in the recA+ strain. uvrA is regulated by RecA, as shown by its high expression in the recA+ strain. Notably, a large increase in recA expression is seen in the UV-treated recA::Km strain. There is no increase in expression of dinB or the 16S rRNA control in either strain. The recA+ and recA::Km strains were treated with 25 mM or 1.5 mM, respectively, of MMS for 1 hour; 6 µg mL−1 or 1 µg mL−1, respectively, of ciprofloxacin for 1 hour; and 270 J m−2 or 5 J m−2, respectively, of UV light. Semi-quantitative RT-PCR was performed on total RNA purified from treated and untreated cultures as described in Figure 1.2 legend and Materials and Methods. Locus tags from the A. baumannii ATCC 17978 genome (“A1S_” not included before number) are included as part of the gene names. Data from a representative experiment is shown.
Figure 1.4. Intracellular concentrations of *A. baumannii* 17978 DNA damage-inducible proteins increase upon UV irradiation. There is 40-fold more RecA protein at 160 J m$^{-2}$ compared to untreated, 2.5-fold more UvrA, and 3-fold more DinB, while RpoB remains constant. *A. baumannii* cultures were grown to exponential phase as indicated in Materials and Methods section and irradiated with increasing amounts of UV (J m$^{-2}$). Equal amounts of whole cell lysates per treatment were probed with polyclonal anti-RecA, polyclonal anti-UvrA, polyclonal anti-DinB, and monoclonal anti-RpoB (refer to Materials and Methods). Antibodies used were raised against the *E.coli* proteins. A comparative experiment using the isogenic *recA::Km* strain could not be performed due to its extreme sensitivity to UV irradiation.
Figure 1.5. **Mutation frequency is elevated upon treatment with DNA damaging agents or upon desiccation in a recA-dependent manner.** (A) *A. baumannii* 17978 strain has higher frequency of rifampicin mutants upon both UV- and MMS-treatment compared to untreated cultures. There is no significant increase in induced mutation frequency for isogenic recA::Km. The isogenic dinB::Km shows a modest, but significant, decrease (3.5-fold) in spontaneous mutants compared to parental, but has the same frequency of induced rifampicin mutants as parental upon both treatments. There is also no significant difference between recA::Km and dinB::Km untreated spontaneous mutation frequency. Error bars represent the standard error of the mean for at least 3 independently tested cultures and statistical significance was determined using a student t-test. A statistically significant increase in mutation frequency between treated and untreated cultures (P≤0.02) is marked by *. (B) *A. baumannii* 17978 has a dramatically increased frequency of rifampicin mutants after desiccation only in a recA+ background. The *A. baumannii* 17978 recA::Km strain shows no difference in pre-desiccation to post-desiccation rifampicin mutants (P=0.2). *A. baumannii* ATCC 19606, a strain containing fewer isogenic umuD and umuC genes than the *A. baumannii* 17978 strain, has increased desiccation-induced RifR frequency, but fewer RifR mutants than the *A. baumannii* 17978 recA+ strain. A statistically
significant increase in mutation frequency between post-desiccation and pre-desiccation cultures (P<0.01) is marked by *. *A. baumannii* 17978 and 19606 cells were desiccated for 24 hours resulting in 3-5 fold killing compared to non-desiccated cells. Cells were then rehydrated in LB medium, outgrown and deposited on plates with rifampicin (100 µg mL⁻¹). The *recA::Km* strain, treated for 6 hours, was killed 15-fold compared to non-desiccated cells. *A. baumannii* 17978 *recA⁺* cultures at 15-fold killing show no difference in mutation frequency compared to the cultures that resulted in 3-5 fold killing (not shown). Error bars represent the standard error of the mean for at least 5 independently tested cultures. Statistical significance was determined using a student t-test.

**Figure 1.6.** *A. baumannii* DinB shares sequence similarity to *E. coli* DinB. An alignment of *E. coli* DinB and a DinB consensus sequence from 21 strains of *A. baumannii*. Known *E. coli* catalytic residues D8, F12, F13, Y79, and D103 are highlighted in boxes. Bar graph represents conservation with full bars as 100%. Dashes in overall consensus sequence represent ambiguity. Alignment was generated using the CLC Main Workbench (CLC Bio).
**Figure 1.7. Predicted UmuC proteins from *A. baumannii* 17978 are similar to *E. coli***

**UmuC.** Full alignment of 17978 UmuC sequences with *E. coli* UmuC. Conserved catalytic residues are highlighted in boxes. Bar graph represents conservation with full bars as 100%.

Dashes in overall consensus sequence represent ambiguity. E values are all less than or equal to 7×10⁻²². Alignment was generated using the CLC Main Workbench (CLC Bio).
Figure 1.8. Plasmid-borne *A. baumannii* *dinB* complements certain phenotypes of *dinB*-deficient *E. coli*. (A) Wild-type P90C *ΔdinB* cells bearing *A. baumannii* 17978 *dinB* on a plasmid (pAb-*dinB*<sup>lac</sup>) are rescued as well as those with *E. coli* *dinB* (pEc-*dinB*) upon nitrofurazone (NFZ) and 4-nitroquinolone-1-oxide (4-NQO) treatment. There is no rescue of *ΔdinB* strains upon methyl methanesulfonate (MMS) treatment. Ab-*dinB* expression is driven by the *lac* promoter and Ec-*dinB* expression is driven by its native promoter. Percent survival was determined by calculating the fraction of colony forming units (CFUs) that grew on LB medium supplemented with NFZ (7.5 µM), 4-NQO (8 µM), or MMS (7.5 mM) per total number of
untreated CFUs. (B) Similar results are found using MG1655 ΔalkA tag dinB, an E. coli strain deficient in base-excision repair, using pAb-dinB\textsuperscript{native} (expression driven by the E. coli dinB native promoter) and pAb-dinB\textsuperscript{lac} (not shown). In addition to MMS, there is no rescue of strains upon ethyl methanesulfonate (EMS) treatment. P90C ΔdinB cells were not sensitive to EMS. Percent survival was calculated as described in (A) using NFZ (5 \(\mu\)M), 4-NQO (6 \(\mu\)M), MMS (0.08 mM), or EMS (3.4 mM). Error bars represent the standard deviation of the mean from 3 independent experiments for both graphs.

![Graph showing mutation frequencies](image)

**Figure 1.9. There is no effect of Ab-dinB on the frequency of MMS-induced rifampicin mutants.** E. coli MG1655 ΔalkA tag dinB mutS cultures bearing Ab-dinB on a plasmid have a similar mutation frequency to those carrying the empty vector after treatment with MMS. Those carrying Ec-dinB on a plasmid have reduced mutation frequency upon treatment, indicating proficient and accurate bypass of alkylation lesions. Deletion of umuDC has no effect on mutation frequencies. The frequency of mutation was calculated by counting the total number of colonies that grew on LB supplemented with and without rifampicin (100 \(\mu\)g mL\(^{-1}\)) after 2 hours of treatment with 0.3 mM MMS. Error bars represent the standard error of the mean from 5 independent cultures.
### Table 1.1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’ to 3’)</th>
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<tr>
<td><em>umuDC</em>(0636-0637)-F</td>
<td>GGCTGAAATCCAGATTAC</td>
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<tr>
<td><em>umuDC</em>(0636-0637)-R</td>
<td>CATTGCCATCATCGAGG</td>
</tr>
<tr>
<td><em>umuDC</em>(1173-1174)-F</td>
<td>CGTTATGTGATGAACAATG</td>
</tr>
<tr>
<td><em>umuDC</em>(1173-1174)-R</td>
<td>GTCAATGGCTTTAAGCAG</td>
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<tr>
<td><em>umuD</em>(1389)-F</td>
<td>GTGAATGGAGGCGATATGCCAAAG</td>
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<tr>
<td><em>umuD</em>(1389)-R</td>
<td>GCAATTTTCGACTCGTTGAC</td>
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<tr>
<td><em>umuC</em>(2008)-F</td>
<td>CGTTATGTTGATGAACAATG</td>
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<tr>
<td><em>umuC</em>(2008)-R</td>
<td>GTCAATGGCTTTAAGCAG</td>
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<tr>
<td><em>umuC</em>(2015)-F</td>
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<td><em>umuC</em>(2015)-R</td>
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<td>16S-518R</td>
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<td>kan-R</td>
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Table 1.2. Comparison of number of putative TLS DNA polymerase genes from select isolates of *A. baumannii*.

<table>
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<tr>
<th><em>A. baumannii</em> Strain</th>
<th>GenBank accession number</th>
<th>Number of putative genes</th>
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<td></td>
<td></td>
<td>umuC</td>
</tr>
<tr>
<td>ATCC 17978</td>
<td>CP000521</td>
<td>4</td>
</tr>
<tr>
<td>TCDC-AB0715</td>
<td>CP002522</td>
<td>3</td>
</tr>
<tr>
<td>AB059</td>
<td>ADHB00000000</td>
<td>3</td>
</tr>
<tr>
<td>ATCC 19606</td>
<td>ACQB00000000</td>
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</tr>
<tr>
<td>AB0057</td>
<td>CP001182</td>
<td>2</td>
</tr>
<tr>
<td>AB058</td>
<td>ADHA00000000</td>
<td>2</td>
</tr>
<tr>
<td>ABNIH3</td>
<td>AFTB00000000</td>
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<tr>
<td>ACICU</td>
<td>CP000863</td>
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</tr>
<tr>
<td>AYE</td>
<td>CU459141</td>
<td>1</td>
</tr>
<tr>
<td>MDR-ZJ06</td>
<td>CP001937</td>
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</table>
### Table 1.3. Mutation signatures of desiccation-induced *A. baumannii* 17978 Rif<sup>R</sup> mutants

<table>
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<tr>
<th>Mutation type</th>
<th>rpoB nucleotide change</th>
<th>RpoB amino acid substitution</th>
<th>Mutation frequency (%)&lt;sup&gt;a&lt;/sup&gt; (N=42)</th>
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<tr>
<td><strong>Transversion</strong></td>
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<tr>
<td>1564 CAG→AAG</td>
<td>522 Gln→Lys&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
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<tr>
<td>1565 CAG→CTG</td>
<td>522 Gln→Leu&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
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<tr>
<td>1573 GAC→TAC</td>
<td>525 Asp→Tyr&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>1574 GAC→GTC</td>
<td>525 Asp→Val&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>535 His→Asp&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>1604 CAT→CTT</td>
<td>535 His→Leu&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12</td>
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<tr>
<td>1619 TCT→TAT</td>
<td>540 Ser→Tyr&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>1741 ATC→TTG</td>
<td>581 Ile→Phe&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td><strong>Transition</strong></td>
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<td>1565 CAG→CGG</td>
<td>522 Gln→Arg&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td>1603 CAT→TAT</td>
<td>535 His→Tyr&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1619 TCT→TTT</td>
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<td>566 Arg→Cys&lt;sup&gt;a,c&lt;/sup&gt;</td>
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<sup>a</sup> Indicates novel *Acinetobacter* substitution

<sup>b</sup> Indicates mutation was also found in an *A. baumannii* 17978 dinB::Km strain

<sup>c</sup> Indicates mutation was unique to *A. baumannii* 17978 dinB::Km strain
CHAPTER 2

Functional analysis of multiple, putative *Acinetobacter baumannii* 17978 DNA polymerase V gene products in *Escherichia coli*
**ABSTRACT**

*Acinetobacter baumannii* is a clinically important and dangerous opportunistic pathogen. It quickly gains antibiotic resistances through horizontal gene transfer and a DNA damage response that we have recently discovered. Many *A. baumannii* strains have acquired multiple *umuD* and *umuC* orthologues, which encode putative subunits of DNA Polymerase V (Pol V). We have previously shown that these gene products are induced upon DNA damage in *A. baumannii* 17978, and we hypothesize that multiple DNA Pol Vs are likely to contribute to DNA damage-induced mutagenesis and thus enhance *A. baumannii*’s mutational capacity. To test this, we cloned the *umuD* and *umuC* genes of *A. baumannii* 17978 into *E. coli* to measure in vivo translesion synthesis (TLS) activity using established DNA damage assays. We find that these genes are expressed in *E. coli*, but ΔumuDC, ΔumuD, or *umuC*-deficient *E. coli* strains are not complemented by expression of the *A. baumannii* genes. Therefore, they are still sensitive to UV-irradiation and incapable of UV-induced mutagenesis. Our results indicate that *A. baumannii umuD* and *umuC* gene products are not active in *E. coli*. It is likely that an *A. baumannii* component required for DNA Pol V-mediated TLS is missing in *E. coli*, the implications of which are discussed.
INTRODUCTION

*Acinetobacter baumannii* is a nosocomial pathogen that has become a serious health threat worldwide (41). Notably, its resistance to desiccation and disinfection allows it to live for long periods of time in intensive care units, where it infects immunocompromised patients (40-42). Strains of *A. baumannii* are inherently resistant to many classes of antibiotics (40-42) and some resistant to every antibiotic available have emerged (58). It appears that *A. baumannii* readily incorporates foreign DNA through horizontal gene transfer processes (59), which permit the acquisition of new antibiotic resistances. Although bacteria, such as *Escherichia coli*, possess DNA damage response systems known to increase mutagenesis and therefore the evolution of antibiotic resistances (1, 15, 36), it was unknown whether *A. baumannii* did the same. In our previous work (Chapter 1; (111)) we uncovered a regulated system in *A. baumannii* capable of inducing mutagenesis upon DNA damage and thus increases the evolution of antibiotic resistance.

We found that in *A. baumannii*, DNA-damage induces classic DNA repair and error-prone DNA polymerase genes, such as those encoding Y-family DNA Pol IV and Pol V (Chapter 1, Figs. 1.3 & 1.4). RecA, the main activator of the SOS response in *E. coli*, mediates this response and is required for increased frequency of antibiotic resistant mutants after desiccation, UV-irradiation, or alkylation damage (Chapter 1, Fig. 1.5). These results are consistent with Aranda et al., who used DNA microarrays to show a RecA-dependent increase in DNA damage response genes following mitomycin-C treatment (112).

Intriguingly, *A. baumannii* strains have acquired multiple *umuD* and *umuC* orthologues (Chapter 1, Table 1.1), encoding putative subunits of DNA Pol V. In particular, *A. baumannii* strain ATCC 17978 has acquired two *umuDC* operons, two unlinked *umuCs*, and one unlinked
*umuD* (Chapter 1, Fig. 1.1). These genes may have been acquired through horizontal gene transfer (76). One of the *umuDC* operons (1174-1173) encodes a DNA Pol V with high sequence similarity to Pol V$_{R391}$, encoded by *rumA'B* from the conjugative IncI transposon R391 (113). This Y-family DNA polymerase is capable of generating even higher mutation rates than *E. coli* DNA Pol V (113). Indeed, we found that desiccation-induced rifampicin resistant mutants contained base-pair substitutions that match the signature of *E. coli* DNA Pol V and Pol V$_{R391}$ (Chapter 1, Table 1.3; (111, 113)). However, while we and others have shown that *A. baumannii* 17978 upregulates these multiple *umuD* and *umuC* orthologues upon DNA damage (111, 112), it is still unclear whether or not they are all active in this strain. *E. coli* contains one single *umuDC* operon, which is highly regulated at the levels of both transcription and post-translation (5).

DNA Pol V (UmuD’$_2$C) complex requires UmuC, the catalytic DNA polymerase subunit, a homodimer of the amino terminally cleaved form of UmuD, UmuD’$_2$, and RecA* for activity (114). It bypasses UV photoproducts, abasic lesions, and guanine oxidation products through a process termed translesion-synthesis (TLS) (115, 116). It also participates in TLS past cytotoxic lesions produced by alkylating agents such as methyl methanesulfonate (87). Because DNA Pol V is very error-prone, it is the major cause of UV- and SOS-induced mutagenesis in *E. coli* (1, 9).

Our evidence suggests that the *A. baumannii* 17978 DNA Pol Vs are involved in DNA-damage induced mutagenesis (Chapter 1, Fig. 1.5, Table 1.3). The acquisition of multiple genes encoding DNA Pol V orthologues could potentiate an increase in *A. baumannii*’s ability to gain antibiotic resistance. In this work, we examined the TLS activities of *A. baumannii* 17978 *umuD* and *umuC* gene products in *E. coli* to gain a better understanding of TLS-mediated mutagenesis in *A. baumannii*. 

52
MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study are listed in Table 2.1. *Acinetobacter baumannii* ATCC 17978 was purchased from The American Type Culture Collection (ATCC). *E. coli* strains were routinely grown at 37°C in Luria Broth (LB) or on LB agar. For plasmid maintenance, 200 µg mL⁻¹ of ampicillin (Ap; Sigma), 30 µg mL⁻¹ of kanamycin (Km; Sigma), or 12 µg mL⁻¹ tetracycline (Tet; Sigma) were used. When required, 100 µg mL⁻¹ of rifampicin (Rif; Calbiochem) was added to LB agar.

Construction of plasmids. *E. coli umuDC* and *A. baumannii* 17978 *umuDC* genes were cloned in the low copy-number vector, pWSK29, all under the native *E. coli umuDC* promoter. First, *pumuDC1* (Table 2.1) was constructed by using the In-fusion (Clontech) kit and by following the manufacturer’s protocol. Briefly, *E. coli umuDC* and its native promoter region were amplified by PCR from strain P90C (Table 2.1) using fusionDC-F and fusionDC-R oligonucleotides (Table 2.2). *pumuDC1* was then used as the template for the construction of *pEc-umuDC* (Table 2.1), which contains a NheI restriction site inserted precisely between the promoter region and the start codon of *umuD*. To insert the NheI restriction site, the GeneTailor (Life Technologies) site-directed mutagenesis protocol was followed using *umuCNheIF* and *umuCNheIR* (Table 2.2) oligonucleotides. Next, *A. baumannii* 17978 (Table 2.1) *umuDC* genes were amplified using oligonucleotides containing NheI and SacI restriction sites (Table 2.2). Gene loci amplified from *A. baumannii* 17978 are as follows: *umuD(A1S_0636)umuC(A1S_0637), umuD(A1S_1174)umuC(A1S_1173), umuC(A1S_2008),* and *umuC(A1S_2015).* *A. baumannii umuDC* genes were cloned into the NheI and SacI sites of *pEc-umuDC,* effectively creating isogenic plasmids under the Ec-*umuDC* promoter (Table 2.1). All constructs were confirmed by sequencing (Tufts University Core Facility).
pNLAC1-Ec-umuD and pNLAC-Ab-umuD(1389) plasmids (Table 2.1) were constructed by cloning the respective amplification products from P90C or *A. baumannii* 17978 into the PstI and PvuI sites of pNLAC1 (Table 2.1; generous gift from Tom Russo, University at Buffalo). These sites are within the *bla* gene (encodes β-lactamase), putting the *umuD* genes under the control of the *bla* promoter and disrupting β-lactamase production. Oligonucleotides sets used for the PCR amplifications were *umuD*PvuI-F and *umuD*PstI-R and 1389PvuI-F and 1389PstI-R (Table 2.2). pWSK129-Ec-umuD and pWSK129-Ab-umuD(1389) (Table 2.1) were constructed by cloning the respective amplification products (as above) into the SacI and ApaI sites of pWSK129 (Table 2.1). Control of these genes is under the lac promoter. Oligonucleotide sets used for the PCR reactions were *umuD*SacI-F and *umuD*ApaI-R and 1389SacI-F and 1389ApaI-R (Table 2.2). Constructs were confirmed by sequencing (Tufts University Core Facility).

**UV-irradiation and induced-mutagenesis.** For UV-survival, saturated *E. coli* P90C cultures (~10⁹ cells) were serially diluted in SMO (100 mM NaCl, 20mM Tris-HCl pH 7.5) and 10 µL spots were deposited on LB-Ap plates. Plates were individually irradiated in the dark under a UV germicidal lamp with increasing amounts of UV-exposure (J m⁻²). Survival was calculated by dividing the number of colony forming units (CFUs) that grew on each UV-treated plate by the number of CFUs grown on LB-Ap alone after 20-24 hours.

For UV-induced mutagenesis, *E. coli* P90C cultures were started from ≤100 cells to minimize the probability of preexisting mutants in the starting inoculum. Saturated cultures were spun down, resuspended in equal volume of SMO, and 2 mL samples were spread evenly in a petri dish. Samples were irradiated in the dark under a UV germicidal lamp with 55 J m⁻² and immediately transferred to LB-Ap medium at a 1:10 dilution following irradiation. Upon saturation, appropriate cell dilutions were deposited on LB-Ap with and without rifampicin to
assess, respectively, the number of rifampicin resistant mutants (Rif$^R$) and total number of CFUs. Colonies were counted after 24 hours of incubation. Mutation frequency was calculated by dividing the number of Rif$^R$ mutants by the total number of CFUs.

**MMS-induced mutagenesis.** *E. coli* MG1655 (Table 2.1) cultures were started from $\leq$100 cells, grown to saturation, then diluted 1:100 and grown for 4 hours. Cultures were then treated with 0.3 mM methyl methanesulfonate (MMS; Sigma) for 1 hour. Cells were washed with SMO, serially diluted, and deposited on LB-Ap plates with and without rifampicin. After 24 hours of incubation, mutation frequency was determined as described above.

**Semi-quantitative RT-PCR.** Cultures were treated as described in the UV-induced mutagenesis section (see above) with three exceptions. First, to ensure cells were in exponential phase growth, saturated cultures were diluted 1:1000 and grown for 2.5 hours, then diluted 1:50 and grown for 2 hours 3 consecutive times. Second, after UV-irradiation, samples were incubated for an additional 1 hour at 37º C prior to RNA extraction to allow for gene expression. Third, untreated cultures were not used. The procedure was carried out as described in Chapter 1 Materials and Methods (111). *E. coli rpoB* RNA expression was used as the control and was equal in each sample. Gene specific RT-PCR oligonucleotides are listed in Table 2.2.
RESULTS

*A. baumannii umuDC* and *umuC* genes are expressed upon UV-irradiation from their respective plasmid constructs in *E. coli*. To test the activity of *A. baumannii* 17978 *umuDC* (Ab-*umuDC*) gene products in *E. coli*, we constructed isogenic low copy-number plasmids containing each *umuDC* operon or unlinked *umuC* from the 17978 strain (See Materials and Methods). *E. coli umuDC* (Ec-*umuDC*), Ab-*umuDC*s and Ab-*umuC*s were cloned into pWSK29 (Table 2.1) under the control of the SOS-inducible Ec-*umuDC* native promoter (Table 2.1). We choose not to use the Ab-*umuDC* or Ab-*umuC* promoters because it is unknown whether these are functional in *E. coli*. Use of the Ec-*umuDC* promoter ensures relatively equal expression and induction by the SOS response (1).

Figure 2.1 shows mRNA expression levels of each *umuDC* construct (pWSK29; grey bars) as determined by semi-quantitative RT-PCR after ∆*umuDC* cells were treated with UV-light, a strong inducer of the SOS response (95). Ec-*umuDC*, Ab-*umuDC*s, and Ab-*umuC*s are expressed at similar levels (within an approximately 10-fold range (Fig. 2.1; grey bars)).

Ec-*umuD* and the unlinked *umuD*(1389) from *A. baumannii* were also cloned into a higher copy-number plasmid, pNLAC1 (Table 2.1), which should result in constitutive expression from the *bla* promoter. We find that Ec-*umuD* and Ab-*umuD*(1389) are equally expressed in *E. coli* ∆*umuDC* (Fig. 2.1; white bars) upon UV-irradiation. Relative expression levels of these two genes are on par with the *umuDC*s and *umuC*s expressed from the Ec-*umuDC* promoter in pWSK29 (Fig. 2.1; grey bars). These data suggested to us that we would be able to assay *A. baumannii umuDC* gene products for activity in *E. coli*.

*A. baumannii umuDC* gene products do not rescue *E. coli* ∆*umuDC* from UV-sensitivity. We first wanted to test *A. baumannii umuDC* gene products for their ability to form
active DNA Pol V in vivo and thus bypass UV-induced lesions. This is one of the main functions of *E. coli* DNA Pol V and a conserved feature across domains (5, 21). *E. coli ΔumuDC* cells carrying the pWSK29-*umuDCs* were exposed to increasing amounts of UV-light. As expected, Ec-*umuDC* rescues cells from UV-sensitivity (Fig. 2.2A; compare pEc-*umuDC* to pWSK29). However, cells carrying the plasmids with *A. baumannii umuDC*(0636-0637), *umuDC*(1174-1173), *umuC*(2008), or *umuC*(2015) did not have improved survival over the vector alone (Fig. 2.2A). We did not expect the Ab-*umuC* gene products to rescue *E. coli ΔumuDC*, because formation of a typical DNA Pol V complex would not happen in the absence of Ab-*umuD* or Ec-*umuD*.

To determine whether the *A. baumannii umuC* gene products are able to form an active DNA Pol V hybrid in *E. coli*, we provided them with Ec-*umuD*+. We hypothesized that the presence of native Ec-*umuD*+ would facilitate DNA Pol V formation if the Ab-UmuCs are similar in structure and function to Ec-UmuC. This strategy eliminates the variable of both Ab-UmuD and Ab-UmuC needing to be active in *E. coli*, since it is possible that these particular Ab-UmuDs might not be cleaved to Ab-UmuD’ in *E. coli*. Moreover, the dependence of RecA* might be particular for the *A. baumannii* gene products (9). We used the strain, *umuC122::Cm* (Table 2.1), which is *umuD*+ and phenotypically ΔumuC to test our hypothesis. We find that the Ab-*umuCs* and Ab-*umuDCs* do not rescue cells from UV-sensitivity (Fig. 2.2B), in stark contrast to Ec-*umuDC* (Fig. 2.2B). The results thus far suggest that *A. baumannii* UmuC are either unable to form active DNA Pol V complex in *E. coli* or are unable to perform TLS past a UV-induced DNA lesion.

**A. baumannii umuD and umuC gene products do not confer UV-induced mutagenesis in E. coli.** Although cells expressing the *A. baumannii umuDC* gene products were
sensitive to UV-irradiation, survival and mutagenesis are not always linked (93). Therefore, we
next tested UV-induced mutagenesis. *E. coli* Δ*umuDC* strains carrying the pWSK29 *umuDC*
plasmids were provided a second plasmid harboring either Ec-*umuD*, Ab-*umuD*(1389), or
nothing (pNLAC1; Table 2.1). In this way, the *A. baumannii* *umuDC* gene products could be
tested for their ability to promote UV-mutagenesis in the presence and absence of either *umuD*
gene product. *A. baumannii* *umuD*(1389) was provided because we hypothesized that this
unlinked *umuD*, which is conserved in *Acinetobacter* spp. (91), may act as a universal *umuD* to
all the *umuCs* in *A. baumannii* 17978.

In the absence of either *umuD* plasmid (Fig. 2.3; white bars), we find that expression of
neither the Ab-*umuDC* operons nor the unlinked Ab-*umuCs* conferred UV-induced mutagenesis.
The rifampicin resistance (Rif<sup>R</sup>) mutation frequencies for strains carrying these plasmids are
equal to those carrying the empty vector (Fig. 2.3; white bars). However, Ec-*umuDC* confers a
dramatic increase in Rif<sup>R</sup> mutation frequencies (~100-fold) as would be expected for wild-type
*E. coli* cells (1, 5, 71).

The addition of the pNLAC1 plasmid bearing Ec-*umuD*, although properly expressed
(Fig. 2.1), does not change any of the *E. coli* Δ*umuDC* Rif<sup>R</sup> mutation frequencies (Fig. 2.3; grey
bars). Moreover, there are no significant changes in mutation frequencies with the addition of
Ab-*umuD*(1389) (Fig. 2.3; black bars), which we have also shown to be expressed (Fig. 2.1).
This result prompted us to assess whether the *A. baumannii* *umuD* gene products alone can
confer UV-induced mutagenesis through formation of a hybrid DNA Pol V complex in vivo with
Ec-UmuC.

We used an *E. coli* Δ*umuD* (*umuC<sup>+</sup>*; Table 2.1) strain to test the activity of the *A.
buannii* *umuD*(1174), *umuD*(0636), and *umuD*(1389) gene products. We first measured UV-
induced Rif\textsuperscript{R} mutation frequencies using strains carrying the Ab-\textit{umuDC} operons. The presence of \textit{umuC} on the plasmids seemed to have no ill effects, because we find a significant increase in Rif\textsuperscript{R} mutation frequency (>100-fold) when cells are provided with Ec-\textit{umuDC} (Fig. 2.4A). In contrast, cells carrying Ab-\textit{umuDC}(1174-1173) or Ab-\textit{umuDC}(0636-0637) operons have Rif\textsuperscript{R} mutation frequencies equal to those with the empty vector (Fig. 2.4A). Furthermore, similar results were found when we tested Ab-\textit{umuD}(1389) in the \textit{E. coli} Δ\textit{umuD} strain using two different plasmid constructs (pNLAC1 and pWSK129; Table 2.1). We find that with either plasmid, \textit{umuD}(1389) does not confer UV-induced mutagenesis (Fig. 2.4B), in contrast to the significant increase in mutation frequency obtained using Ec-\textit{umuD} (Fig. 2.4B). This result suggests that Ab-\textit{umuD}(1389) is unable to form active DNA Pol V with Ec-UmuC, even though it has been shown to be cleaved to a UmuD’-like form in \textit{E. coli} (91). Taken together, these results provide further evidence for the lack of activity of \textit{A. baumannii umuD} and \textit{umuC} gene products in \textit{E. coli}, at least upon conditions of UV-damage.

**MMS-induced mutation frequencies are unaffected by the expression of \textit{A. baumannii umuDC}s.** Lastly, we wanted to see if we could detect TLS activity using a different DNA-damaging agent; we chose the alkylating agent methyl methanesulfonate (MMS). Using the \textit{E. coli} Δ\textit{umuDC} strain, it was difficult to detect significant differences in MMS-induced mutagenesis between those carrying Ec-\textit{umuDC} and the empty vector control (data not shown). We therefore used the Δ\textit{alkA tag mutS umuDC dinB} background (Table 2.1) that we (Chapter 1, Fig. 1.9) and others (87) have successfully used to measure differences in MMS-induced mutagenesis. Here, the presence of \textit{dinB} dramatically reduces the frequency of Rif\textsuperscript{R} mutants because DinB accurately bypasses DNA alkylation lesions produced by MMS (87). Therefore, the accuracy of TLS past alkylation lesions can be measured in vivo. Interestingly, we find a
modest decrease in mutation frequency (thus detectable TLS activity) in cells with Ec-umuDC compared to those with the vector alone (Fig. 2.5), and as expected, a large decrease in mutation frequency in cells with Ec-dinB (Fig. 2.5). We find that mutation frequencies for cells with A. baumannii umuDCs or umuC s remain equal to those with the vector alone (Fig. 2.5). We would expect to observe Rif\(^R\) frequencies greater than those with the Ec-umuDC strain if the A. baumannii gene products were actively performing highly mutagenic TLS past alkylation lesions. While this could be the case here, the results are more likely indicating a lack of DNA polymerase activity.
DISCUSSION

*Acinetobacter baumannii* integrates foreign DNA from its environment and other bacteria through horizontal gene transfer processes such as plasmid acquisition, transposons, and homologous recombination (40, 42, 59). We have previously shown that *Acinetobacter baumannii* isolates have acquired varying numbers of *umuD* and *umuC* genes, which encode putative subunits of DNA Pol V (Chapter 1, Figs. 1.1 & 1.7, Table 1.2). It is believed that these genes were acquired through horizontal gene transfer (76), but the question of whether or not they are active still remains. We set out to answer this question by assaying these genes for classic signs of TLS activity in *E. coli*. *A. baumannii* 17978 contains two *umuDC* operons, two unlinked *umuCs*, and one unlinked *umuD* (Chapter 1, Fig. 1.1), bringing the total number of putative DNA Pol V component genes to seven; five more than *E. coli*. The potential for DNA damage-induced mutagenesis is thus greater than *E. coli*’s, if the systems of regulation are identical. However, we know that the mechanisms regulating the DNA-damage response in *A. baumannii* 17978 are different than *E. coli*’s (Chapter 1 & (112)).

Multiple lines of evidence suggest that the *A. baumannii* 17978 *umuD* and *umuC* genes are inactive in *E. coli*. We tested different combinations of *A. baumannii* (Ab-) and *E. coli* (Ec-) *umuDs* and *umuCs* to see if active DNA Pol V complex could be formed in vivo. Ab-*umuDCs* alone or combined with Ec-*umuD* failed to rescue cells from UV-irradiation (Fig. 2.2) or confer UV induced-mutagenesis (Fig. 2.3). This suggests that Ab-*umuC*’s are unable to form active DNA Pol V with either Ab-*umuD* or Ec-*umuD*. We also tested the opposite combination, Ab-*umuD*’s with Ec-*umuC*, and failed again to observe UV-induced mutagenesis (Fig. 2.4A). These results imply that some factor may be missing for these enzymes to be active in *E. coli*; i.e. a native *A. baumannii* protein.
We hypothesized that Ab-umuD(1389), whose gene product has been shown to be cleaved to a UmuD’-like protein in *E. coli* (91), was the missing component necessary for activity. This Ab-UmuD(1389) has characteristics of both UmuD and LexA, and has recently been shown to repress error-prone DNA polymerase genes in *A. baumannii* (112). While this could be the main function of Ab-UmuD(1389), the evidence for DNA damage-inducible cleavage (91) led us to believe it could still be functioning like Ec-UmuD’ in the DNA Pol V complex. Our results suggest that Ab-UmuD(1389), unlike Ec-UmuD, cannot complement ΔumuD *E. coli* cells (Fig. 2.4B). Furthermore, the addition Ab-UmuD(1389) does not change the mutation frequencies of *E. coli* ΔumuDC cells carrying any of the Ab-umuDC operons or Ab-umuCs (Fig. 2.3). Therefore, even though Ab-umuD(1389) is cleaved to a UmuD’ form, our evidence suggests that it does not form DNA Pol V in *E. coli* with either Ab-UmuCs or Ec-UmuC. The data point to a larger issue of whether the Ab-UmuDs or Ab-UmuC proteins have even the potential to be active in *E. coli*, independent of their ability to form DNA Pol V molecules.

Ec-UmuC is quickly degraded by the Lon protease, and the umuD gene products by Lon and ClpXP (117), a level of post-translational regulation that ensures minimal time for potential mutagenesis. Although we have shown that the *A. baumannii* genes are transcribed in *E. coli* nearly as well as native genes (Fig. 2.1), it is unknown whether they are being translated efficiently or how quickly their gene products are being degraded. Since they are foreign genes in *E. coli*, it is entirely possible that they are translated at lower levels (rare codons) and/or are degraded faster than native Ec-umuDC gene products. We would need to have specific antibodies made against each of these *A. baumannii* gene products in order to know the levels of protein in the cell at multiple time points. Remarkably, we have previously shown that Ab-DinB
is active in *E. coli*. Cells bearing plasmid-borne Ab-*dinB* are rescued upon treatment with either nitrofurazone or 4-nitroquinolone-1-oxide (Chapter 1, Fig. 1.8). While the DinB family of enzymes are more evolutionarily conserved than the UmuC family (21), these results demonstrate that some *A. baumannii* enzymes do have the potential to be active in *E. coli*.

One factor that could be missing from our transplanted *A. baumannii* DNA Pol V TLS system in *E. coli* is the *A. baumannii* β-binding clamp of the DNA Pol III holoenzyme (encoded by *dnaN*). This essential replication factor is important for recruitment and management of DNA polymerases at the replication fork (118). In order to access the replication fork and perform TLS, *E. coli* TLS DNA polymerases, as well as UmuD and UmuD’, require interaction with the β-clamp through conserved motifs (119-123). The UmuC canonical β-binding motif is required for UV-induced mutagenesis (124), and mutating the β-binding motif of DinB (DNA Pol IV) abolishes its activity in vivo (93). The system of governing access to the replication fork is delicate, and even a single residue change in the β-binding motif can dramatically alter the activity of Ec-UmuC (124). We therefore hypothesize that even if Ab-*umuDC* gene products are successfully produced in *E. coli*, then they may be inactive because of their inability to bind to the native *E. coli* β-binding clamp.

The Ab-DinB β-binding motif (QLSLW_) is similar to the QLVLGL motif found in Ec-DinB (Chapter 1, Fig. 1.6; (125)). One residue is missing in the *A. baumannii* motif and only two residues differ between the two. However, an alignment of all the *A. baumannii* 17978 *umuC* gene products shows that the *E. coli* β-binding motif is not as conserved as DinB’s (Chapter 1, Fig. 1.7). Residues 357-361 (QLNLF) at the C-terminal end of Ec-UmuC (118, 123) do not align with a similar motif in the Ab-UmuC sequences. A consensus sequence of TYDLL at this location (Chapter 1, Fig. 1.7) could very well be an *A. baumannii* alternative β-binding motif.
Therefore, if Ab-UmuCs are translated and stable in *E. coli*, then a proper β-clamp interaction could be a limiting factor in our assays. We plan on providing an *E. coli* dnaN temperature sensitive mutant with Ab-dnaN to further test the TLS activity of the Ab-umuDC gene products. Preliminary evidence suggests that Ab-dnaN is able to complement growth proficiency in this mutant *E. coli* strain at high temperatures (data not shown).

This work offers an interesting perspective on how we think of bacterial foreign gene acquisition. It is easy to presume that bacteria, such as pathogens, uptake foreign DNA through horizontal gene transfer processes and immediately use it to their advantage. It is alarmingly common for pathogens like *A. baumannii* to acquire resistance islands, transposons, and plasmids containing antibiotic resistance genes or virulence factors (41, 58, 126, 127). However, our results suggest that successful utilization of a new gene, such as a mutagenic DNA polymerase, may require modification of that gene or other components of the TLS system to make it work – an evolutionary process that could take many generations. It remains unclear at this point whether the multiple *A. baumannii* 17978 DNA Pol Vs are truly active polymerases. Nevertheless, independent of whether or not Ab-DnaN or some other component will confer activity in *E. coli*, the complexity of the situation lends support to this perspective.

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Figure 2.1. All plasmid-borne *A. baumannii* genes are expressed upon UV-irradiation in *E. coli* Δ*umuDC*. Relative expression level in arbitrary units is shown for each pWSK29 (gray) and pNLAC1 (white) construct. pWSK29 genes are all expressed from the native promoter of the *E. coli* *umuDC* operon. pNLAC1 genes are expressed from the *bla* (encoding β-lactamase) promoter. Semi-quantitative RT-PCR was performed on total RNA purified from 55 J/m² UV-irradiated cultures. See Materials and Methods section for details. Gene specific RT-PCR primers were used to amplify approximately 300bp regions of each *umuD*, *umuC*, or *umuDC* operon. *E. coli* genes are denoted by “Ec-”. *A. baumannii* 17978 gene names include genomic locus tags (“A1S_” not included before number) in parenthesis. Data from a representative experiment is shown.
Figure 2.2. *A. baumannii* 17978 *umuDCs* do not rescue *E. coli* *ΔumuDC* from UV-sensitivity. (A) *E. coli* *ΔumuDC* cells carrying pWSK29 with *A. baumannii* 17978 *umuDCs* are sensitive to increasing amounts of UV-light. In contrast, native Ec-*umuDC* confers survival. (B) *umuC122::Cm* cells are *umuD*⁺ but have a C-terminal UmuC truncation, rendering them sensitive to UV (see pWSK29). Cells with native Ec-*umuDC* survive much better than those with *A. baumannii* 17978 *umuDCs*, even though the cells are *umuD*⁺. Error bars represent the standard deviation of the mean from at least 3 independent experiments.
Figure 2.3. *A. baumannii* 17978 *umuDC*s do not confer UV-induced mutagenesis in *E. coli* Δ*umuDC*. All strains carry both pWSK29 constructs (X-axis) and pNLAC1 *umuD* derivatives. Strains with plasmid-borne *A. baumannii* 17978 *umuDC*s and Ec-*umuDC* were assayed for UV-induced mutagenesis in the absence (white bars) and presence of Ec-*umuD* (grey bars) or Ab-*umuD*(1389) (black bars). Ec-*umuDC* confers an increase in rifampicin resistance (Rif\(^R\)) mutation frequency regardless of the presence of Ec-*umuD* or Ab-*umuD*(1389). *A. baumannii* *umuDC*s do not confer any increase in Rif\(^R\) mutation frequency compared to the vector. The addition of either Ec-*umuD* or Ab-*umuD*(1389) has no significant effect on mutation frequency. Error bars represent the standard deviation of the mean from at least 3 independent experiments.
Figure 2.4. *A. baumannii* 17978 *umuDs* do not complement *E. coli ΔumuD* for UV-induced mutagenesis. (A) *A. baumannii* 17978 *umuDC*(1174-1173) and *umuDC*(0636-0637) operons were used to assess whether pWSK29 plasmid-borne *umuD*(1174) or *umuD*(0636) would complement *E. coli ΔumuD*. Cells expressing Ab-*umuDCs* show no increase in UV-induced mutagenesis compared to those with the vector. Ec-*umuDC* confers a significant increase in mutation frequency. (B) Ab-*umuD*(1389) expressed in *E. coli ΔumuD* cells from either the *lac* promoter in pWSK129 (white bars) or the *bla* promoter in pNLAC1 (grey bars) does not confer a significant increase in Rif\(^R\) mutation frequency compared to the control (Vector). In contrast, Ec-*umuD* expressed from either plasmid significantly increases the mutation frequency. * denotes statistical significance of P < 0.01 as determined by T-test. Error bars represent the standard deviation of the mean from at least 3 independent experiments.
Figure 2.5. *A. baumannii* 17978 *umuDC* s do not affect MMS-induced mutation frequencies in an alkylation damage-sensitive strain of *E. coli*. MG1655 Δ*alkA tag mutS umuDC dinB* cells bearing *A. baumannii* 17978 *umuDC*s have Rif<sup>R</sup> mutation frequencies no different than the vector (pWSK29). In this background, Rif<sup>R</sup> mutation frequencies are lower in the presence of *dinB* because DinB accurately bypasses DNA alkylation lesions (compare pEc-<i>dinB</i> to pWSK29). The Rif<sup>R</sup> mutation frequencies of cells with Ec-*umuDC* are modestly lower than those with the vector alone. Error bars represent the standard deviation of the mean from at 6 independent experiments.
## TABLES

### Table 2.1. Strains and plasmids

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CHAPTER 3

Examining the role of the carboxy-terminal domain of *Escherichia coli* DNA polymerase V subunit, UmuC
ABSTRACT

Cells have a variety of mechanisms to cope with DNA replication fork stalling. One of these is translesion DNA synthesis (TLS), in which cells alleviate fork stalling by way of specialized, low-fidelity DNA polymerases. *Escherichia coli* TLS DNA polymerase, DNA Pol V, is composed of the accessory subunit, UmuD’, and the catalytic subunit, UmuC. DNA Pol V is responsible for the majority of UV- and SOS- induced mutagenesis and is therefore regulated at both the level of transcription and post-translation. The carboxy-terminal domain of UmuC may mediate such protein-protein interactions, but this is poorly understood. To gain insights into the role of this domain, we constructed an IPTG inducible plasmid that expresses a C-terminal fragment of UmuC lacking its catalytic domain. We hypothesized that this domain may be involved in UmuC regulatory interactions, and expression of this fragment would reveal these interactions through sequestration or modulation of certain factors. We find that expression of the C-terminus lowers the frequency of induced mutagenesis upon hydroxyurea treatment in a mutagenic strain that lacks the C-terminus of UmuC. We also find that expression of this C-terminal fragment in an UmuC+ background has diverse effects on cell viability and is only detectable upon SOS-inducing conditions. We hypothesized that bacterial Hsp90 (HtpG), an enigmatic heat shock protein, may have a role in this phenomenon since it is known to directly interact with and regulate eukaryotic TLS DNA polymerases. Results suggest that HtpG does not interact with UmuC at the C-terminus; therefore, the role of this heat shock protein in *E. coli* TLS regulation remains elusive. Together these data support the notion that the C-terminus of *E. coli* UmuC is likely involved in DNA Pol V regulatory protein-protein interactions under conditions of stress.
INTRODUCTION

Replicative DNA polymerases copy templates proficiently and with high fidelity, but they cannot incorporate nucleotides opposite of lesions on the template DNA. Thus, DNA damage can cause replication fork stalling and therefore genomic instability and cell death by hindering the progress of replicative DNA polymerases. In a process termed translesion synthesis (TLS), specialized DNA polymerases can take over replication at stalled forks and continue synthesis past DNA lesions (1). These DNA polymerases, in particular those of the Y-family, have a comparatively open active site, which allows for accommodation of damaged bases (30). Because of this feature, Y-family DNA polymerases have poor processivity and are, in general, error prone on undamaged DNA compared to replicative, high-fidelity DNA polymerases (1). This low fidelity, potentially mutagenic incorporation of nucleotides may be helpful for bacteria to evolve and survive conditions of stress (15), but it also makes it necessary for these DNA polymerases to be extensively regulated (1). Y-family DNA polymerases are highly conserved throughout all domains of life (1, 21, 129, 130). They have been implicated in human cancers (131, 132) and in the growing problem of bacterial antibiotic resistance (15, 133). Therefore, studying the function and regulation of these DNA polymerases in the model system of *Escherichia coli* is translatable and significant.

*E. coli* has two Y-family DNA polymerases, DNA Pol IV (DinB) and DNA Pol V (UmuD’2C), encoded by the *dinB* and *umuDC* genes, respectively. They are both transcriptionally regulated by the LexA repressor as part of the SOS gene network and induced upon DNA damage or replication stress (1). UmuC is the catalytic subunit of DNA Pol V, and it has the ability to insert nucleotides on templates with abasic sites and UV-photoproducts such as thymine-thymine dimers and thymine-thymine cyclobutane pyrimidine dimers (115, 116). It is
responsible for the majority of UV- and SOS-induced mutagenesis in E. coli, thus it is highly regulated at the levels of transcription and post-translation (1). While the number of DinB molecules in the cell increases from approximately 25 to 2,500 on SOS induction, the number of DNA Pol V molecules increases from nearly zero to approximately 15 (19). Indeed, the umuDC promoter has the highest affinity for the LexA repressor (18), meaning it is tightly repressed and one of the last SOS genes to be turned on. The umuC gene is translated less efficiently from the umuDC operon than umuD, because its start codon is out of the umuD reading frame in the messenger RNA by one nucleotide (1). Active UmuC requires both the active form of UmuD, UmuD’, and RecA* for efficient translesion synthesis (114). Chaperone proteins such as GroEL (Hsp60), DnaK (Hsp70) and DnaJ have also been shown to be required for DNA Pol V activity (134-136); furthermore, both UmuC and UmuD are rapidly degraded in the cell by proteases (117). Full-length UmuD is translated from the umuDC operon first and undergoes cleavage of the first 24 amino acids of the amino terminus to become UmuD’. Cleavage of UmuD to UmuD’ requires RecA bound to single stranded DNA, which builds up when the replicative DNA polymerase stalls. RecA::ssDNA nucleoprotein filament (RecA*) then stimulates the autocleavage of UmuD to UmuD’ (1). Strikingly, though these requirements are clearly understood, there is still nothing known about the regions on UmuC that interact with UmuD’ or with RecA*, assuming the latter is via direct interaction with UmuC (137).

The β-sliding clamp is an essential replication factor important for recruitment and management of DNA polymerases at the replication fork (118). Replicative DNA polymerases as well as TLS polymerases benefit from interactions with the β-clamp by increased polymerase processivity (119, 122). Four of E. coli’s DNA polymerases as well as UmuD and UmuD’ are able to bind to the β-clamp through protein-protein interactions at their β-binding motifs (119-
The UmuC canonical β-binding motif, residues 357-361 (QLNLF) at the C-terminal end (118, 123), has been shown to be necessary for UV-induced mutagenesis (124).

Chaperone proteins, also known as heat shock proteins, mitigate the detrimental effects of heat and other stresses by maintaining protein homeostasis. They facilitate folding of newly synthesized proteins to allow for active conformations, assemble and disassemble protein complexes, and help the refolding of misfolded proteins (138). Hsp90, known as HtpG in bacteria, is a highly conserved chaperone protein (139) that is well understood and essential in eukaryotes (140). However, its function in bacteria is obscure (141) and only recently has it begun to be understood. It is known that HtpG stabilizes phycobilisome protein in cyanobacterium (142). In E. coli it cooperates with DnaK in client protein remodeling (143) and is essential for the activity of the CRISPR/Cas system (144). Intriguingly, Hsp90 has been found to chaperone folding of two eukaryotic orthologues of UmuC, DNA polymerase eta and REV1, into their active forms through direct interactions, thus mediating mutagenic TLS (145, 146). These findings led us to hypothesize that HtpG may also do the same for UmuC in bacteria.

DNA replication fork progress can be stalled in vivo in a damage-independent manner with hydroxyurea (147). Hydroxyurea causes a depletion in the pool of deoxyribonucleotide triphosphates (dNTPs) by inhibiting class I ribonucleotide reductases in E. coli (148). This leads to cell death by apparently increasing intracellular hydroxyl radical formation (149). DNA Pols IV and V are involved in the response to DNA damage-independent replication fork stalling (61). A unique allele of UmuC, UmuC122, which lacks 102 residues of the carboxy-terminus (150), confers to cells a remarkable resistance to hydroxyurea (61). This may mean that when the C-terminus is intact, interactions occur in a pathway that leads to cell death during hydroxyurea induced replication fork stalling. The catalytic activity of both UmuC122 and DinB are required
for the resistance phenotype, suggesting that DNA Pols IV and V are responsible for most of the DNA replication that occurs during low dNTP conditions (61); a phenomenon distinct from TLS. Mutagenesis is also elevated upon hydroxyurea treatment, suggesting that Pols IV and V may generate mutations to permit cell survival during this stressful, low dNTP condition (28, 61).

Important questions arise from the UmuC122 data. What does the C-terminus of UmuC normally interact with and what is its function in vivo? Environmental bacterial samples have revealed the existence of natural C-terminal truncations of UmuC homologues, suggesting an evolutionary benefit to losing this domain in some bacteria (Godoy, V.G., unpublished data). We hypothesize that if the C-terminus of UmuC is involved in the regulation of DNA Pol V activity, then expressing high intracellular concentrations of a UmuC C-terminal fragment in E. coli cells will reveal inherent UmuC interactions and possibly functions of the C-terminal domain. In vivo excess of the C-terminal fragment of a TLS polymerase already has precedence as a molecular tool. D’Souza et al. demonstrated that the C-terminus of yeast polymerase Rev1 is involved in protein-protein interactions and is required for S. cerevisiae Rev1-dependent survival and mutagenesis (151).

Our hypothesis that the C-terminus may be involved in the regulation of DNA Pol V activity is outlined in Figure 3.1. Unknown factors (X, Y, Z; Fig. 3.1A) may interact with UmuC at its C-terminus, albeit transiently. With excess concentrations of a C-terminal fragment in the cell, these transient interactions may be magnified and possibly result in sequestration or modulation of these factors (Fig. 3.1B). An exaggeration of transient or regulatory interactions should result in an observable phenotype, indicating the involvement of the C-terminus of UmuC.
Due to their conserved function, studying TLS DNA polymerases in *E. coli*, in particular the error-prone UmuC, will enhance our understanding of other prokaryotic and eukaryotic TLS polymerases and their roles in mutagenesis. In this study, we looked for phenotypes generated by the expression of a C-terminal domain fragment of *E. coli* UmuC to gain a better understanding of its role. We also analyzed the solubility of the C-terminal fragment to determine factors that may help stabilize UmuC, such as HtpG, and/or to determine C-terminal binding partners. We ultimately provide evidence that the C-terminus of UmuC interacts with SOS-induced factors.
MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* strains used in this study are listed in Table 3.1. Bacterial cultures were routinely grown at 37° C in Luria Broth (LB) or on LB agar and supplemented with 20 µg mL⁻¹ of chloramphenicol (Sigma; Cm) for plasmid maintenance unless otherwise specified.

P90C ΔumuDC and ΔhtpG::kan were made using a standard P1 bacteriophage transduction method (152). The Cm⁻ cassette was deleted from P90C ΔumuDC::cat by introducing pCP20 (expressing the flippase recombinase) into the cells by transformation, plating on LB agar containing ampicillin (Ap), and incubating at 30 °C. Colonies obtained were streaked on LB agar, grown overnight at 42 °C, and then streaked on LB agar containing Ap or Cm to test for sensitivity.

P90C ΔhtpG(D80N) was constructed using the SOE-LRed method (153). Briefly, *htpG* and its native promoter were amplified from P90C using *htpG*-fwd-PstI and *htpG*-rev-SacII oligonucleotides (Table 3.2), GoTaq Green 2X Master Mix (Promega), and the manufacturer’s protocol. The approximately 2 Kb PCR product was cloned into the PstI and SacII sites of pWSK29 (Ap⁺; Table 3.1). GeneTailor™ Site Directed Mutagenesis System (Life Technologies) was performed on *phtpG* (Table 3.1) using the oligonucleotides *htpGD80N*-fwd and *htpGD80N*-rev (Table 3.2) to introduce the mutation (base pair 347 G to A). The procedure was performed following the manufacturer’s procedure. *phtpGD80N* (Table 3.1) was then used to carry out the SOE-LRed procedure to move the *htpG*(D80N) mutation onto the chromosome of P90C. The mutant was confirmed by sequencing using oligonucleotides: *htpG*-F-flank, *htpG*-R-flank, *htpG*-mid-F, and *htpG*-mid-R (Table 3.2).
Construction of pVector, pC-terminus, and pC-terminus(β). JW1173 was a gift from Dr. Kim Lewis and is originally from the ASKA collection (154). This plasmid (pCA24N; Table 3.1) contains a N-terminal 6xHis-tagged *umuC* with a C-terminal green fluorescence protein (GFP) fusion. Located between the His-tag and GFP fusion are two SfiI restriction sites for removal of *umuC*. Using SfiI (New England Biolabs), pCA24N was digested and the vector was re-ligated to itself to create the pVector control plasmid (containing 6xHis-tagged GFP; Table 3.1). The pC-terminus plasmid (Table 3.1) was created using N175SfiI-F and pCA24N-SfiI-R (Table 3.2) to amplify approximately half of the *umuC* gene, resulting in 175 codons deleted from the N-terminus. PCR was performed using GoTaq Green 2X Master Mix (Promega) following the manufacturer’s protocol. PCR fragments were cleaned using QIA Quick PCR Purification Kit (Qiagen) and cut with SfiI restriction enzyme. Digested products were ligated into SfiI digested pCA24N vector using T4 DNA ligase (Promega). The *umuC* β-binding motif variant (pC-terminus(β); Table 3.1) was constructed using the GeneTailor™ Site Directed Mutagenesis System (Life Technologies) and by following the manufacturer’s procedure. Oligonucleotides used to mutate the *umuC* gene product’s residues 357-361 (QLNLF) to alanines (AAAAA) were *umuC* beta5xA-F and *umuC* beta5xA-R (Table 3.2). All constructs were confirmed by sequencing using pCA24N-Fwd and pCA24N-Rev (Tufts Core Facility; Table 3.2). Sequences were analyzed using CLC Main Workbench (CLC Bio).

Survival Assays. Three independent cultures were grown to saturation then serially diluted in SMO. 10 µL spots were deposited on LB-Cm agar containing 0.1mM IPTG and hydroxyurea (HU; Calbiochem), methyl methanesulfonate (MMS; Acros Organics), or ciprofloxacin (Cip; Sigma) at concentrations specified in the figure legends. For UV treatment, samples were spot plated as described on LB-Cm plates and irradiated in the dark under a UV
germicidal lamp. Percent survival was determined by calculating the fraction of colony forming units (CFUs) grown with the DNA-damaging agent (or after UV irradiation) per total number of CFUs grown on untreated LB-Cm. HU, Cip, and UV-treated plates were incubated in the dark for 20 hours and MMS plates were incubated in the dark for 40 hours. The *umuC122::Tn5* strain cultures were grown to saturation, diluted 1:1000 in 10 mL LB-Cm, then either left untreated or treated with 100 mM HU for 24 hours. At time points specified in the figure legends, samples were taken, diluted in SMO and deposited on LB-Cm plates. CFUs were determined as described.

**Mutagenesis Assays.** For MMS and HU-induced mutagenesis, independent cultures were grown to saturation then sub-cultured 1:1000 in 125 mL baffled flasks containing 10 mL of LB-Cm, 0.1mM IPTG and either 7.5 mM MMS or 75 mM HU. Cultures were incubated for 24 hours with shaking then serially diluted in SMO and plated at the appropriate dilutions on tetrazolium galactose agar (TG). TG contains Cm, 1% galactose (Sigma), and 75 μM 2,3,5-Triphenyltetrazolium chloride (tetrazolium indicator dye). For UV mutagenesis, appropriate dilutions of saturated cultures were deposited on TG plates and irradiated with 46 J m⁻² under a UV germicidal lamp. After 18-20 hours of incubation, Gal⁺ colonies appear white in color, and *gal* mutants appear dark pink or red. Only those colonies that were indistinguishably dark pink/red in color were counted. Mutation frequency was determined by dividing the total number of red CFUs by the total number of both white and red CFUs. Between 1,000 and 20,000 colonies were screened for each culture.

**Immunoblotting.** Cells were spun down and lysed with Bugbuster (Novagen) after MMS treatment (as described for MMS-induced mutagenesis). Total protein concentration was measured for each sample using a spectrophotometer nanodrop at A₂₈₀ (Nanodrop 2000, Thermo
Scientific). Equal amounts of total protein per sample, mixed 1:1 with Laemmli Sample Buffer (2x; Sigma), were separated by SDS-PAGE on a 4-12% Bis-Tris gel (Life Technologies) with either 1x MOPS buffer (Life Technologies) or 1x MES buffer (Life technologies). After electrophoresis, proteins were transferred to a PVDF membrane (Immobilon-P; Millipore). Incubation with primary or secondary antibodies was carried out according to published procedures (83) and the QIAexpress Detection and Assay Handbook (Qiagen).

Polyclonal mouse anti-penta-His antibody (Qiagen) was used at a 1:2000 dilution. Polyclonal rabbit anti-*E. coli* HtpG was the generous gift of Dr. Costa Georgopoulos (University of Utah) and was used at a 1:1000 dilution. Horse anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technologies) was used at a 1:3000 dilution. HRP conjugated goat anti-rabbit secondary antibody (Pierce) was used at a 1:50,000 dilution.
RESULTS

A plasmid encoding the C-terminus of UmuC was constructed to investigate the role of this functionally unknown domain. The amino terminus of UmuC contains the palm and finger domains (Fig. 3.2A), which includes conserved catalytic residues such as D6, Y11 and D101 (90, 155). The middle region of UmuC contains the thumb and some of the little finger domains, while the carboxy terminus is composed of the rest of little finger domain and an unknown region (Fig. 3.2A; (90)). To try and gain insights into the enigmatic role of this domain, we constructed a plasmid that encodes only the C-terminus of UmuC. As diagramed in Figure 3.2B, the pC-terminus encodes a hexahistidine tagged C-terminal derivative of UmuC fused to green fluorescent protein (GFP). The first 738 basepairs of umuC were removed by cloning only the last 528 basepairs of the gene into the ASKA plasmid pCA24N (154). The gene product is catalytically inactive because it is missing the N-terminal catalytic residues (90). The GFP fusion was originally included to facilitate visual confirmation of gene expression. pCA24N is a high copy number plasmid with an IPTG inducible promoter and it contains lacI^q for strict repression of expression (154). The control plasmid, pVector, encodes hexahistidine tagged GFP (Table 3.1; Fig. 3.2C).

Complementation of a mutator strain, umuC122::Tn5, with pC-terminus affects both mutagenesis and survival. The umuC122::Tn5 (umuC122) strain has a unique allele of umuC whose gene product lacks 102 residues of the C-terminus (61), mainly the unknown domain diagrammed in Figure 3.2. When cells are treated with hydroxyurea (HU), umuC122 confers rescue of cell viability compared to the wild type but also leads to a significant increase in mutagenesis (i.e. 100x; (61)). When the C-terminus is intact, interactions occur in a pathway that lead to cell death during HU-induced replication fork stalling. UmuC122 may not be
subjected to these normal regulatory protein-protein interactions mediated by the missing C-terminal domain (61). We hypothesized that the C-terminal truncation protein that we constructed would complement this UmuC allele because it contains the missing domain. Because this is a non-catalytic fragment, the roles we are looking for are structural and likely involved in protein-protein interactions. Thus, one of the first questions was whether there would be any effect on mutagenesis. We find that for the umuC122 strain bearing pC-terminus, HU-induced galactose utilization deficient (gal) mutants are undetectable (Fig. 3.3A), which is in stark contrast to the high frequency of gal mutants observed for the strain with the vector only (Fig. 3.3A). To our surprise, we also find that the strain with pC-terminus survives approximately 15-fold and 300-fold better than the control after 12 and 24 hours, respectively (Fig. 3.3B). These results suggest that the C-terminus is likely sequestering or modulating factors involved in both the UmuC122 mutagenesis pathway and a general HU-induced lethality pathway. It has been previously shown that dinB is required for UmuC122 mediated survival (61), and here we show that the increase in survival gained by pC-terminus is also dependent on dinB (Fig. 3.3B). This result implies that DinB is also involved in the C-terminus survival pathway, at least in the umuC122 background.

**UmuC C-terminus fragment causes diverse changes in cell viability in an umuDC⁺ background.** We tested the survival of wild-type (umuDC⁺) cells bearing pC-terminus and pVector using a variety of different DNA-damaging treatments. Similar to the results found in the umuC122 background, we find that umuDC⁺ cells with pC-terminus are dramatically rescued from HU-induced cell death (Fig. 3.4A). Deletion of umuDC (Fig. 3.4A) or dinB (data not shown) has no effect on this trend, suggesting survival in an umuDC⁺ background involves a different pathway compared to umuC122. In contrast, we find that cell viability of the strain
carrying pC-terminus is decreased when cells are treated with an SOS-inducing DNA alkylating agent (MMS; Fig. 3.4B; (93)) or ciprofloxacin (Fig. 3.4C), an antibiotic that potentiates double strand breaks in E. coli (94) and is a strong inducer of the SOS response (11, 93). These trends are independent of the presence of umuDC (Figs. 3.4B & C; ΔumuDC). Lastly, cells carrying pC-terminus display a modest sensitivity to UV-irradiation in the umuDC\textsuperscript{+} background (Fig. 3.4D), but no effect on viability is seen in a ΔumuDC background (Fig. 3.4D).

We next tested the hypothesis that the observed phenotypes may be due to C-terminus-DNA Pol III β-clamp subunit interactions. DNA polymerases, including both UmuC and DinB, interact with the β-clamp subunit of the holoenzyme, giving them access to the replication fork (118, 121, 122). In fact, this interaction is necessary for in vivo activity of these translesion DNA polymerases (118, 124, 156) and is mediated by a universal β-binding motif, which for E. coli UmuC is residues \textsuperscript{357}QLNFL\textsuperscript{361} (123). We constructed a β-binding motif variant of C-terminus::GFP (pC-terminus(β)) using site directed mutagenesis and by changing the basepairs encoding QLNLF of the C-terminus to encode alanines (AAAAA). The umuDC\textsuperscript{+} strains carrying pC-terminus(β) are only partially rescued upon hydroxyurea treatment compared to those carrying pC-terminus (Fig. 3.4A) and they are also completely viable upon MMS treatment (Fig. 3.4B). In addition, upon UV-treatment the strains carrying pC-terminus(β) survive the same as those with pC-terminus in both backgrounds (Fig. 3.4D; umuDC\textsuperscript{+} and ΔumuDC). These results suggest that the survival phenotypes may depend on different degrees of C-terminus-β-clamp interactions occurring at the replication fork. The C-terminal fragment dependent increase in cell viability upon HU treatment may be partially dependent on this interaction; MMS sensitivity may be completely dependent on it; and UV sensitivity may be independent of this interaction.
Interestingly, these data may reveal different degrees of replication fork access for UmuC, depending on the type of replication fork stalling or DNA damage.

**The UmuC C-terminus fragment alters SOS-induced mutagenesis in umuDC cells.** We next measured the mutation frequencies of strains bearing pC-terminus upon treatment with various DNA damaging agents. We find that umuDC cells carrying pC-terminus have a significantly increased frequency of MMS-induced gal mutants compared to those carrying the empty vector (Fig. 3.5A). Conversely, spontaneous gal mutation frequency is not significantly different between strains carrying each plasmid (Fig. 3.5D). Furthermore, induction with IPTG is required for this phenotype (Fig. 3.5A), strengthening the case that expression of C-terminus protein is the cause of this mutator phenotype. This mutator effect is not observed in either a ΔumuDC or ΔdinB background (Fig. 3.5B), indicating that both DNA Pol V (or UmuD alone) and DinB are required, along with C-terminus protein, in this mutagenic pathway.

To test our hypothesis that the bacterial Hsp90 homologue, HtpG, may mediate TLS and mutagenesis in *E. coli*, we introduced a deletion of htpG into the umuDC strain and measured gal mutation frequency. We still find an increase in gal mutants for the ΔhtpG strain carrying pC-terminus compared to the vector alone (Fig. 3.5C), indicating that the pathway remains intact without a requirement for HtpG. However, we do find that the use of geldanamycin, which specifically inhibits Hsp90/HtpG by inhibiting ATP binding (157), modestly reduces mutagenesis in umuDC cells bearing pC-terminus (Fig. 3.5C). These data suggest that inactivation of HtpG may affect this mutagenesis pathway more than deletion of HtpG.

Upon ciprofloxacin treatment, there is a dramatic increase in mutation frequency when comparing umuDC strains bearing pC-terminus versus pVector (Fig. 3.6A). Indeed, we were unable to detect ciprofloxacin-induced gal mutants in cells carrying the vector (Fig. 3.6A). In
contrast, we find a modest but significant decrease in UV-induced mutagenesis in \textit{umuDC}^+ strains with pC-terminus compared to the vector (4-fold; Fig. 3.6B). In the \textit{ΔhtpG} background, the trend is similar (11-fold; Fig. 3.6B), suggesting no involvement of HtpG in this UV-mutagenesis pathway. These data demonstrate that expression of the C-terminus of UmuC has a diverse array of cellular effects in vivo. Depending on the type of DNA damage, the C-terminus may sequester different factors or disrupt normal stress response interactions, resulting in changes in both survival and induced-mutagenesis.

\textbf{The UmuC C-terminus protein is only detected upon SOS-inducing conditions.} In order to confirm that the phenotypes we had thus far observed were due to C-terminus protein expression, we performed a series of immunoblots to detect protein levels. \textit{umuDC}^+ Cells were treated as they were for MMS-induced mutagenesis (see Materials and Methods section) and the soluble fraction of the whole cell lysates were probed with anti pentahistidine antibodies. As shown in Figure 3.7A, the hexahistidine (his)-tagged C-terminus::GFP fusion protein is detected only in the lane with both MMS treatment and IPTG induction (indicated with arrow). As expected, equal amounts of his-tagged GFP are detected in both lanes (Fig. 3.7A; pVector), indicating GFP production is independent of MMS treatment and our in vivo findings are independent of GFP. The results strongly infer that C-terminus stability and solubility are dependent on SOS factors, thus the in vivo phenotypes are observed only upon SOS inducing conditions.

We next wanted to test if inhibition of HtpG would have an effect on in vivo solubility by repeating the experiment with geldanamycin added to the cultures. Since an increase in MMS-induced mutagenesis appeared to be mitigated by the addition of geldanamycin (Fig. 3.5C), we were not surprised to observe undetectable levels of C-terminus protein here as well (Fig. 3.7B).
Furthermore, by using a HtpG specific antibody, we find equal amounts of soluble HtpG in all cultures (Fig. 3.7B), indicating that geldanamycin does not affect the concentration of HtpG protein. There is, however, a significant decrease in soluble his-GFP suggesting perhaps the addition of geldanamycin has non-specific effects on GFP or all cellular protein concentrations. This makes sense, since HtpG is a chaperone protein that works in concert with other chaperones to fold and stabilize client proteins (142, 143). GFP is a non-native protein in *E. coli* and likely requires the concerted effort of multiple chaperone proteins for folding and stability (158, 159).

**Expression of an inactive mutant of HtpG does not have an effect on UmuC C-terminus solubility.** Based on the geldanamycin results, we hypothesized that active HtpG could be required to help stabilize and/or fold the UmuC C-terminus during SOS-inducing conditions, resulting in detectable levels of soluble protein. We repeated the experiments using MMS (as in Fig. 3.7) to induce the SOS response and looked for soluble C-terminus in a ΔhtpG background. We also constructed a chromosomal point mutant of *htpG* that would render HtpG inactive, the idea being it would mimic the effect of geldanamycin. An aspartic acid to asparagine mutation (D79N) in the yeast Hsp90 orthologue, Hsp82, has been shown to render the chaperone catalytically inactive by inhibiting binding of ATP (157). Using a multi-sequence alignment of yeast Hsp82 and HtpG (data not shown), we determined that this aspartic acid residue at position 79 was conserved at position 80 in HtpG, thus we constructed *htpG*(D80N). Again we find that soluble C-terminus protein is detectable only with MMS treatment in the *umuDC*⁺ *htpG*⁺ (wild-type) background (WT; Fig. 3.8A). Intriguingly, the results are similar in both the ΔhtpG and *htpG*(D80N) backgrounds. Nearly equal amounts of HtpG protein were found in both the wild-type and *htpG*(D80N) strains, while no HtpG is detected in the ΔhtpG (Fig. 3.8A). MMS treatment has no effect on the concentration of HtpG (Fig. 3.8A), which strengthens the notion
that C-terminus solubility is dependent on MMS treatment (i.e. SOS induction). Although the amount of C-terminus protein appears to be less in the \textit{htpG}(D80N) strain (Fig. 3.8A), similar immunoblots using these strains show no differences in detectable protein (data not shown).

Lastly, a similar experiment (as Figs. 3.7 & 3.8A) was done to determine if UmuD was a required factor for C-terminus stability/solubility. Here we find no change in detectable C-terminus protein concentration between the \textit{ΔumuDC} and \textit{umuDC}+ cultures (Fig. 3.8B). Taken together, these data suggest that neither UmuD nor HtpG are required to stabilize the C-terminus protein, at least upon MMS treatment. However, these data do not rule out the possibility that the C-terminus could sequester these factors in vivo, resulting in the phenotypes we have observed.
DISCUSSION

DNA Pol V is a highly regulated, evolutionarily conserved DNA polymerase (1, 129, 130). The N-terminal catalytic domain is most conserved (90) while the poorly understood C-terminal domain has an unknown, but likely important function (160). Our analysis of *E. coli* cells expressing a C-terminal fragment of UmuC provides evidence that this domain is of functional importance. We hypothesized that excess C-terminal fragment would reveal structural interactions normally made by this domain of UmuC and lead us to uncover the function of this domain.

It is likely that the UmuC122 protein is deregulated in some way, which in turn leads to an increase in mutagenesis in conditions of nucleotide starvation (i.e. hydroxyurea treatment; (61)). We determined that the C-terminus complements mutagenesis in the *umuC122* background (Fig. 3.3A). Because UmuC122 is missing its C-terminal domain, reduction of mutagenesis in this strain by replacement of the missing component makes sense. Complementation with the C-terminus brings back interactions in some pathway leading to a decrease in mutagenesis. The effect on survival in the *umuC122* background is unexpected (Fig. 3.3B), but suggests that the C-terminus of UmuC is involved in various pathways that affect cell survival. Additionally, we show that DinB is part of this pathway and is necessary for survival in the *umuC122* background with or without the C-terminus. It is possible that the C-terminus either allows DinB greater access to the replication fork, or blocks other factors from preventing DinB from gaining access. Increased DinB replication fork access during conditions of HU-induced nucleotide starvation may cause sustained cell viability. Another possibility is that the C-terminus prevents prolonged fork access by UmuC122, which would decrease mutagenesis (Fig. 3.3A) and increase viability over the longer time frame (Fig. 3.3B).
The diverse effects of high intracellular C-terminus expression are especially evident in the umuDC\(^+\) background. Here, we can assume that DNA Pol V is normally produced upon SOS-inducing conditions. Interestingly, deletion of umuDC does not have an effect on the C-terminus-dependent phenotypes. For instance, cells expressing C-terminus survive equally in both umuDC\(^+\) and \(\Delta{umu}DC\) backgrounds when treated with hydroxyurea (HU), MMS, or ciprofloxacin (Fig. 3.4). The different effects on cell viability between the treatments may be due to different forms of replication stress. HU causes replication fork stalling in the absence of DNA damage (61), MMS and UV-light produce lesions on the DNA, and ciprofloxacin causes DNA double strand breaks. Only upon DNA damaging conditions does expression of the C-terminus result in a decrease in survival (Fig. 3.4B, C, D). The C-terminus may be modulating or activating factors involved in cell death under these conditions; but upon HU-induced replication fork stalling, the C-terminus interacts with or signals factors in a pathway that promotes cell survival. Therefore, there may be more than one UmuC pathway that has become apparent using the C-terminal fragment.

We had theorized that expression of the C-terminus fragment might cause changes in the cell by sequestering factors such as UmuD. N-terminal UmuC derivatives missing residues of the C-terminus are unable to interact with UmuD/UmuD’ (160-162), suggesting that UmuD/UmuD’ interacts with UmuC via the C-terminal domain (160-162). In our studies, UmuD or UmuD’ may be sequestered by the C-terminus under certain conditions. We find a modest decrease in viability when cells expressing C-terminus are UV-irradiated, with survival approaching \(\Delta{umu}DC\) levels (Fig. 3.4D). The fact that no change in survival is seen in \(\Delta{umu}DC\) cells expressing C-terminus compared to the vector suggests that the effect depends on umuDC. Decreased survival in this case may be due to the excess concentrations of C-terminus titrating...
out UmuD or UmuD’, limiting the available pool for DNA Pol V formation, and decreasing TLS past UV-induced lesions.

Another factor that appears to be involved in this complex story is the β-clamp of the DNA Pol III holoenzyme. It is known that the TLS DNA polymerases of *E. coli* require the β-clamp for access to the replication fork, and in turn, TLS activity (118, 121). DinB variants containing mutations in the β-binding motif have been shown to lack activity in vivo (93). Likewise, direct interactions with the β-clamp are critical for UmuC TLS participation (124, 163), and mutations in the canonical β-binding motif affect the ability of UmuC to interact with the β-clamp (124, 155, 163). Using this knowledge, we constructed the β-clamp mutant, C-terminus(β), to inhibit binding of the fragment to the β-clamp.

We found that the β-binding motif of the C-terminus is partially required for HU survival (Fig. 3.4A) and completely required for MMS sensitivity (Fig. 3.4B). In contrast, the β-binding motif mutation had no effect on UV-treated cells (Fig. 3.4D). Assuming that the C-terminus(β) mutant is expressed as well as the wild-type C-terminus, we can conclude two things. Either the mutant is more unstable than the wild-type fragment and therefore has less of an effect in vivo, or C-terminus fragment produces some of its effects due to β-clamp binding. During conditions of nucleotide starvation (Fig. 3.4A), DNA Pol V and DinB are recruited to the replication fork because of their increased K_m for dNTPs (61). In these conditions, the C-terminus fragment may help recruit one or both of these TLS polymerases to the replication fork through an unknown mechanism that partially involves the β-clamp. When cells are treated with MMS, binding of the β-clamp may inhibit DNA polymerases such as DinB from accessing the replication fork, thus causing sensitivity to alkylation lesions (87, 93). Upon UV damage, *umuDC* cells expressing C-terminus(β) are just as sensitive as those expressing C-terminus, indicating the effect is β-clamp
independent and mainly due to something else (i.e. UmuD sequestration).

It is intriguing that we found increased MMS-induced mutagenesis in strains expressing the C-terminus fragment (Fig. 3.5). We also demonstrate that MMS treatment itself is required for the detection of soluble C-terminus (Figs. 3.7 & 3.8) in comparison to untreated cells. Since UmuC is quickly degraded in vivo and the Lon-protease signal may be located on the C-terminus (117), the C-terminal fragment may also be quickly degraded. Taken together, we conclude that an SOS-induced factor is required to stabilize intracellular C-terminus protein levels, possibly by prevention of degradation. In this model (Fig. 3.9), cells are flooded with excess C-terminal fragment by IPTG induction. Upon treatment with an alkylating agent, SOS protein concentrations increase and there is a need for UmuC (DNA Pol V) in the cell. Participation in the SOS-induced mutagenesis pathway is the most likely role of UmuC in this scenario (1). Without interacting SOS factors, the C-terminus is likely degraded and undetectable by immunoblot (Fig. 3.9). Indeed, it is thought that the C-terminus of UmuC contains a Lon-protease signal, which targets UmuC for degradation (117). These SOS-induced protein factors may not only bind to the C-terminus and structurally stabilize it, but they might also cover the signal, preventing Lon-targeted degradation and leaving UmuC more vulnerable to Lon.

We have show that both umuDC and dinB are required for the MMS-induced increase in mutagenesis (Fig. 3.5B). To explain this mechanism there are a few interesting scenarios: (i) sequestering of UmuD/UmuD’ by C-terminus modulates DinB-dependent mutagenesis (63), (ii) C-terminus-β-clamp interactions alter the TLS DNA pols access hierarchy to the replication fork (164), (iii) C-terminus sequesters or modifies an unknown factor that alters the TLS pols activity or access to the fork. Furthermore, upon UV-irradiation, where mutagenesis is completely dependent on DNA Pol V (1), we find that mutagenesis is significantly decreased (Fig. 3.6B).
This lends more weight to the idea that UmuD’ is likely a factor sequestered by the C-terminus fragment. But to make matters complicated, *umuD* is not required for C-terminus solubility (Fig. 3.8B). This may be explained by solubility/stability being dependent on factors other than UmuD such as chaperones or RecA. A C-terminus-RecA interaction could account for some of the observed in vivo effects. This interaction could not only change basic RecA functions such as homologous recombination (62), but could alter the modulation of DinB (63) and DNA Pol V activity (114, 165), thus changing SOS-induced mutagenesis and survival.

Throughout our studies we envisioned a scenario where the bacterial Hsp90 homologue, HtpG, would be one such factor involved in UmuC stability and mutagenesis. Since Hsp90 has been shown to regulate UmuC homologue-mediated TLS in eukaryotes (145, 146), the perfect scenario would be filling the evolutionary gap with similar evidence in prokaryotes. However, our data did not support this idea. Notably, there were no significant changes in MMS- or UV-induced mutagenesis in the *htpG* deletion strain (Figs. 3.5C & 3.6B). If HtpG was required for DNA Pol V-induced mutagenesis, we would have at least seen a difference between the *htpG*+ and Δ*htpG* strains carrying the vector upon UV-irradiation (Fig. 3.6B).

Interestingly, when geldanamycin was added to cultures along with MMS (Fig. 3.5C), mutation frequency in the *umuDC*+ *htpG*+ strain expressing C-terminus was modestly lowered. Additionally, C-terminus protein is not detected in soluble cell lysate from these geldanamycin cultures (Fig. 3.7B). This led us to think that there may be differences between a complete absence of HtpG, and a present but inactive HtpG. We hypothesized that the *htpG*(D80N) mutant would mimic the results of geldanamycin treatment, since both should inhibit HtpG’s ability to bind ATP (143, 157). We found that soluble C-terminus protein levels were relatively unchanged in the D80N background (Fig. 3.8A), leading us to conclude that HtpG is probably not involved
in C-terminus solubility/stability. Indeed, we also saw a reduction in soluble GFP protein (Fig. 3.7B), suggesting that the effect of geldanamycin may not be as HtpG specific as it is thought to be (157). It would be interesting to see the effects of geldanamycin and the D80N background on native UmuC protein levels, however native levels of UmuC are notoriously difficult to detect. There is also the possibility that the D80N mutation does not render bacterial HtpG inactive even though the amino acid is conserved. Testing this theory would require arduous in vitro ATP-binding studies and/or an in vivo phenotype for HtpG that has yet to be discovered in *E. coli*.

To summarize, the results of this work demonstrate that the C-terminal domain of UmuC is important to the overall structure and function of UmuC. Protein-protein interactions during SOS- or other stress response-induction are likely responsible for maintaining stability, allowing UmuC to form DNA Pol V complex with UmuD’, access the replication fork, and induce mutations. The regulation of DNA Pol V’s error-prone activity is of the utmost importance to the cell. Learning all we can about the complexities of UmuC regulation enhances our understanding of how mutagenesis as a whole is regulated. Since UmuC related proteins are found in all domains of life, the knowledge gained studying *E. coli* UmuC can be beneficial to the study of mutagenesis in bacterial pathogens, and even to the study of human cancer.

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**Figure 3.1. Model of hypothesis and method.** If the C-terminus of UmuC is involved in the regulation of DNA Pol V activity, then studying the effects of excess concentration of a C-terminal fragment will reveal the existence of inherent C-terminal interactions. (A) The C-terminus of UmuC may interact with and possibly be regulated by factors X, Y, and Z normally in vivo. UmuD’ interacts with UmuC to form DNA Pol V but is not specifically represented in this diagram. (B) Excess C-terminal fragment may result in sequestration or activation of factor(s) X, Y, or Z. The result may be an altered effect on a UmuC pathway and an observable downstream phenotype. Figures are not drawn to scale and are only representative of hypothetical structures.
Figure 3.2. Schematic of UmuC carboxy terminus construct. (A) Diagram of full length UmuC and its five structural domains. Proposed domains are adapted from Boudsocq et al. (2002) and are approximations. (B) pC-terminus is a high copy number plasmid (pCA24N) that expresses His$_6$-C-terminus::GFP fusion protein from an IPTG inducible promoter. The C-terminus::GFP fusion protein contains approximately half the residues of full length UmuC; all N-terminal catalytic residues are deleted. (C) The control, pVector, expresses His$_6$-GFP protein from the same IPTG inducible promoter.
Figure 3.3. Complementation of the mutator strain, *umuC122::Tn5*, with UmuC carboxy terminus results in decreased mutagenesis and increased hydroxyurea resistance. (A) Frequency of galactose deficient mutants is decreased for the *umuC122::Tn5* strain bearing pC-terminus compared to the control (pVector). Undetectable frequency of mutation is represented by * symbol. (B) Survival over time is increased in the *umuC122::Tn5* strain bearing pC-terminus compared to the control (pVector). Deletion of *dinB* results in decreased survival for both strains. Liquid cultures were treated for 24 hours with 75 mM (A; mutagenesis) or 100mM (B; survival) hydroxyurea. Averages of at least 3 independent cultures are shown with error bars representing the standard deviation.
Figure 3.4. Cell viability of $umuDC^+$ strains bearing pC-terminus varies depending on the treatment. (A) Both wild-type (WT; $umuDC^+$) and $\Delta umuDC$ cells bearing pC-terminus (pC-term) are dramatically rescued upon treatment with 7.5mM hydroxyurea compared to the control (pVector). Cells bearing the β-binding motif variant of pC-terminus (pC-term(β)) are only partially rescued. (B) Decreased cell viability is observed in both wild-type and $\Delta umuDC$ backgrounds bearing pC-terminus compared to the vector upon treatment with DNA alkylating agent MMS (7.5mM). Sensitivity is not observed in cells bearing the β-binding motif variant. (C) WT and $\Delta umuDC$ cells bearing pC-terminus are also sensitive to treatment with ciprofloxacin (0.016 µg/ml), a strong inducer of the SOS response. (D) A modest decrease in cell viability is observed in wt cells bearing pC-terminus compared to the vector upon increasing doses UV light irradiation. $\Delta umuDC$ ($\Delta DC$) cells are more sensitive to UV light irradiation than WT cells, as
expected, however there is no significant difference between cells bearing pC-terminus compared to pVector in this background. No differences in survival in either background are seen when comparing pC-terminus to pC-terminus(β). The average of 3 replicates is shown with error bars representing the standard deviation of the mean for all experiments.

Figure 3.5. The C-terminus construct increases the frequency of mutagenesis upon treatment with MMS in a manner requiring dinB and umuDC but independent of htpG. (A) The frequency of galactose deficient (gal) mutants for the umuDC⁺ strain bearing pC-terminus increases compared to the umuDC⁺ strain carrying the control plasmid upon treatment with MMS (9mM). The uninduced strain carrying pC-terminus (IPTG⁻) has a mutation frequency comparable to the control. (B) Deletion of htpG has no effect on the C-terminus mutator phenotype. The umuDC⁺ strain carrying pC-terminus but supplemented with 30µM Geldanamycin (Gm) has decreased mutation frequency; geldanamycin inhibits the activity of HtpG. (C) Deletion of either umuDC or dinB mitigates the mutator effect of C-terminal fragment. (D) The frequency of gal mutants is the same between IPTG induced wild-type strains.
carrying pVector and pC-terminus. The difference seen is not significant (P>0.05). For all experiments shown, the average of at least 3 independent cultures is shown with error bars representing the standard error of the mean.

Figure 3.6. UmuC C-terminus increases mutagenesis upon treatment with ciprofloxacin and decreases mutagenesis upon UV irradiation. (A) *umuDC* strain carrying pC-terminus has an increased frequency of *gal* mutants compared to the control upon treatment with 0.016 µg/ml ciprofloxacin. Undetectable frequency of mutation is represented by *** symbol. (B) In both wild-type (*umuDC* ∆htpG) and ∆htpG backgrounds, the strains carrying pC-terminus have decreased mutation frequency compared to the strains carrying pVector upon UV treatment (46 J/m²). The average of at least 3 independent experiments is shown. Error bars represent the standard error of the mean. P<0.01 = *, P≤0.01 = **.
Figure 3.7. **SOS induction is required to detect soluble UmuC C-terminus protein.** (A) Soluble C-terminus protein expressed from pC-terminus is only detectable upon MMS treatment and IPTG induction in *umuDC*+ cells. Soluble his-tagged GFP is detectable at the expected KDa size. 6xHis protein ladder (Qiagen) is shown on the left side of the immunoblot. (B) Separate experiment performed as in (A) but with geldanamycin (Gm) added to the cultures where indicated. Soluble C-terminus protein is no longer observed in *umuDC*+ cells treated with MMS and Gm; Gm inhibits HtpG activity but does not affect the amount of HtpG protein. A significant decrease in soluble GFP is also observable. Equal amounts of soluble protein were separated by SDS-PAGE and probed with α-HtpG and α-pentaHis antibody.
Figure 3.8. Solubility of UmuC C-terminus protein is not dependent on *htpG*, active HtpG, or *umuDC*. (A) Soluble C-terminus protein expressed from pC-terminus is detected upon MMS treatment in the wild-type (WT; *umuDC*), ΔhtpG, and *htpG*(D80N) strains. The *htpG*(D80N) contains a chromosomal mutation of *htpG* and produces inactive HtpG. Soluble HtpG protein is seen in the WT and *htpG*(D80N) strains, but not the ΔhtpG. (B) Soluble C-terminus protein is detectable in both *umuDC* (WT) and Δ*umuDC* strains. Equal amounts of soluble protein were separated by SDS-PAGE and probed with α-HtpG or α-pentaHis antibody.
Table 3.1. Strains and plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype/Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P90C</td>
<td>$\Delta(lac-pro)_{\text{XIII}}$ thi ara</td>
<td>(86)</td>
</tr>
<tr>
<td>$umuC122::\text{Tn5}$</td>
<td>As P90C, but with Tn5 insertion in the $umuC$ gene</td>
<td>(61)</td>
</tr>
<tr>
<td>$umuC122::\text{Tn5}$</td>
<td>As $umuC122::\text{Tn5}$ with deletion of $\text{dinB}$</td>
<td>(61)</td>
</tr>
<tr>
<td>$\Delta\text{dinB}::\text{kan}$</td>
<td>As P90C, but with precise deletion of $\text{dinB}$ and replacement by Kan$^R$ marker</td>
<td>Lab stock</td>
</tr>
<tr>
<td>$\DeltaumuDC$</td>
<td>As P90C, but with precise deletion of the $umuDC$ allele; transduction from MG1655 $\DeltaumuDC::\text{Cm}$; Cm$^R$ marker has been removed</td>
<td>This work</td>
</tr>
<tr>
<td>$\DeltahtpG::\text{kan}$</td>
<td>As P90C, but with precise deletion of $htpG$ and replacement by Kan$^R$ marker; transduction from JW0462 $\DeltahtpG::\text{kan}$</td>
<td>This work; (166)</td>
</tr>
<tr>
<td>$htpG(D80N)::\text{kan}$</td>
<td>As P90C, but with $htpG(D80N)$ mutation</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JW1173</td>
<td>pCA24N; pBR322 origin, Cm$^R$; $lacI_q$; bearing N-terminal His$_{6\times}$-tagged $umuC$ with C-terminal GFP fusion</td>
<td>(154)</td>
</tr>
<tr>
<td>pCA24N</td>
<td>pVector; as JW1173 but with $umuC$ excised from SfiI sites; encodes His$_{6\times}$-tagged GFP</td>
<td>This work</td>
</tr>
<tr>
<td>pC-terminus</td>
<td>As JW1173 but with 525 bp deletion of $umuC$ N-terminus</td>
<td>This work</td>
</tr>
<tr>
<td>pC-terminus($\beta$)</td>
<td>As pC-terminus; with $umuC$ beta binding motif mutation</td>
<td>This work</td>
</tr>
<tr>
<td>pWSK29</td>
<td>pSC101 replicon with pBluescript II SK$^+$ multiple cloning site, Ap$^R$</td>
<td>(88)</td>
</tr>
<tr>
<td>phtpG</td>
<td>As pWSK29; $htpG$ with native promoter inserted into PstI and SacII sites</td>
<td>This work</td>
</tr>
<tr>
<td>phtpGD80N</td>
<td>As phtpG; bearing $htpG(D80N)$</td>
<td>This work</td>
</tr>
<tr>
<td>pCP20</td>
<td>Ap$^R$; temperature-sensitive replication; encodes FLP recombinase</td>
<td>(167)</td>
</tr>
<tr>
<td>pKD46</td>
<td>Ap$^R$; $\lambda$ Red recombinase expression</td>
<td>(167)</td>
</tr>
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</table>
Table 3.2. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
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</thead>
<tbody>
<tr>
<td>N175SfiI-F</td>
<td>GAAAAAGGCCCTGAGGCCCATGTCTGCTTCCCC</td>
</tr>
<tr>
<td>pCA24N-SfiI-R</td>
<td>CTTTTGGCGCAGCATAGGCCTTTGAAGCAGCTCAGAAATCAC</td>
</tr>
<tr>
<td>pCA24N-Fwd</td>
<td>CGAGGCCCTTTGCATTCCACC</td>
</tr>
<tr>
<td>pCA24N-Rev</td>
<td>CAGGTGCACCCCTTAGGCG</td>
</tr>
<tr>
<td>umuCbeta5xA-F</td>
<td>TCTTCACTCAAGGGAGTGGAGCGCAGCAGGCTGCGAGATGACAACG</td>
</tr>
<tr>
<td>umuCbeta5xA-R</td>
<td>CGCGACTCCCTGACCTGAAAGAAATCCCGAGCATCAGCC</td>
</tr>
<tr>
<td>htpG-fwd-PstI</td>
<td>ATATCTGCAGCTTGCAGGGGCATCAGTATGGGATCAGGTCGAGGCGAGG</td>
</tr>
<tr>
<td>htpG-rev-SacII</td>
<td>ATATCCGCCGCGCTCAAGAAAGGACCTGAGG</td>
</tr>
<tr>
<td>htpGD80N-fwd</td>
<td>AGCGTACGCTGACCATCTCCAATAACGCGGTAGGGG</td>
</tr>
<tr>
<td>htpGD80N-rev</td>
<td>GGAGATGCGGTAGCTACGGTACTGGTCTTATCGAAAGAG</td>
</tr>
<tr>
<td>htpG-F-flank</td>
<td>GCAACGAGACAGCAGGATCACC</td>
</tr>
<tr>
<td>htpG-R-flank</td>
<td>GGCTGAAACGGGTATGGCAG</td>
</tr>
<tr>
<td>htpG-mid-F</td>
<td>GTACATCCGCCATCCAGGCTC</td>
</tr>
<tr>
<td>htpG-mid-R</td>
<td>GGAGTCAATCAGAGCCAGC</td>
</tr>
</tbody>
</table>
CONCLUDING REMARKS

Over the past two decades, great strides have been made in *Escherichia coli* DNA damage and Y-family DNA polymerase research (70). It has become apparent that these DNA polymerases are involved in not only error-prone translesion DNA synthesis (TLS), but also a multitude of activities and diverse roles (9, 28). Their precise regulation is critical to cell survival and maintenance of genomic integrity. Because DNA damage and environmental stress is ever present for bacteria, they have evolved sophisticated and interconnected coping mechanisms (2, 3). The remarkable process of evolution gave rise to stress-responses and repair systems that can actually allow bacteria to further increase genetic diversity and fitness during these stressful times (2, 8, 29, 46). This balancing act of maintaining genomic integrity and increasing genetic diversity has been fine tuned over the course of billions of years (168). Likewise, bacteria have also had hundreds of thousands of years to either become experts at infecting the human species (169), skillfully evading our immune response, or to survive in a human host when given the opportunity (170). Moreover, over time they have also evolved resistance mechanisms (8) to combat antibiotics first produced by their fellow bacterial neighbors (171), and now produced by us (39).

In this work we have uncovered a DNA damage response in the opportunistic pathogen, *Acinetobacter baumannii*. This emerging pathogen is a worldwide source of nosocomial infections and has become resistant to almost every antibiotic available to clinicians (40-42, 44). We have shed light on the mechanisms of its regulation, including the involvement of the highly conserved bacterial protein, RecA (1). We demonstrated that in the absence of *recA*, *A. baumannii* does not induce putatively mutagenic Y-family DNA polymerases nor does it mutate upon DNA damaging conditions such as desiccation. Others have shown that the *recA* mutant is
sensitive to a variety of DNA damaging agents in addition to decreased pathogenicity (66). The implications of these findings are clinically important because *A. baumannii* survives desiccation in the hospital setting; enduring on equipment and surfaces and getting transmitted between staff and patients (40, 41, 43). RecA thus becomes an attractive target for specific treatment and control of *A. baumannii* outbreaks. A stable chemical inhibitor that could be universally used as part of the disinfection process would be an ideal scenario. Similarly, a stable drug that could be taken along with antibiotics to increase the potency (110) and prevent further mutation is also ideal. The current development of these inhibitors will hopefully yield efficacious candidates, as the need for new antimicrobials has never been greater (39).

The Y-family DNA polymerases are also interesting target candidates for a compound that could prevent bacterial evolution (15, 36). Although they are dispensable in bacteria under ideal laboratory conditions (9, 70), in long-term stationary phase competition experiments, bacteria that lack translesion DNA polymerases lose (in both survival and evolutionary fitness) to those that have them (172). Therefore, in theory, it seems as though inhibiting them could prevent growth and/or mutagenesis under natural conditions, though bacteria are still left with other ways to increase genetic diversity such as spontaneous mutations (1), horizontal gene transfer mechanisms (59), and copious other mechanisms of mutagenesis/evolvability (173-179). Thus, the challenge of preventing bacteria from evolving in the hospital or in the host is an uphill battle. A highly conserved target such as RecA, which modulates the activity of these DNA polymerases and activates the entire DNA damage response, is clearly one of the more useful directions to take (15, 36).

The insights we have gained in studying the Y-family DNA polymerases and DNA damage response of both *A. baumannii* and *E. coli* have nevertheless been worthwhile. Our work
in *A. baumannii* has shed new light on establishing DNA damage response mechanisms different than the *E. coli* paradigm. Although *E. coli* works well as a model system and we should continue our efforts, we must not be constrained by this one species, or what we can accomplish and learn will be severely impedes. Finally, there is still much to be learned about how mutagenic DNA polymerases are regulated and what their functional roles are. It is not only about finding a drug or inhibitor to combat pathogenic bacteria. Every scientific contribution made to the greater pool of knowledge leads us closer to understanding mutagenesis, and hence molecular evolution, in both bacteria and humans. Additionally, the more that is learned in bacteria the more we are able to translate those findings to the diagnosis (131, 132) and some day prevention of cancer.
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