Evaluation of Potential Inhibitors of *Escherichia coli* RecA to Attenuate the Rate of Antibiotic Resistance Development and to Sensitize *Escherichia coli* to Current Antibiotics

by

KERI A. FLANAGAN

A thesis submitted to the faculty of the University of North Carolina at Chapel Hill
in partial fulfillment of the requirements for the degree of
Master of Science in the UNC Eshelman School of Pharmacy

CHAPEL HILL
2009

APPROVED BY:

Scott F. Singleton, Ph.D.

Jian Liu, Ph.D.

Qisheng Zhang, Ph.D.

Alexander Tropsha, Ph.D.

ABSTRACT

Keri A. Flanagan

Evaluation of Potential Inhibitors of *Escherichia coli* RecA to Attenuate the Rate of Antibiotic Resistance Development and to Sensitize *Escherichia coli* to Current Antibiotics

Under the direction of Scott F. Singleton, Ph.D.

Antibacterials alike have proven to be an invaluable breakthrough in the treatment of infectious diseases. Upon their introduction, countless lives were saved. However, bacteria have a profound ability to alter their susceptibility to antibiotics, rendering themselves resistant to one or more of the current antibiotics available[4,7,26,27]. As in the beginning of the twentieth century, thousands of people are again dying from infectious diseases that were once kept at bay due to the rise in antibiotic resistant bacterial strains. Bacteria become resistant to newly introduced antibiotics very quickly[5]. Therefore, a new approach to treating bacterial infections is needed and may be accomplished by attenuating bacterial resistance mechanisms and sensitizing them to current antibiotics. RecA, a recombinase enzyme that is involved in various aspects of DNA repair, horizontal gene transfer and the induction of SOS mutagenesis[19-25], seems to be a promising target whose inhibition would preclude the RecA-dependent activities used by bacteria to reduce their susceptibility to antibiotics. Previous work completed by the Singleton research group has identified various classes of small molecules, peptides, inorganic cations and nucleotide analogs have been able to diminish the SOS response [50-52,55, 71-78]. Furthermore, unpublished results from our laboratory have identified cell-permeable small molecules that are bona fide inhibitors of

purified *E. coli* RecA and demonstrated that such inhibitors can attenuate SOS in live *E. coli* and potentiate E. coli killing by bactericidal agents. In order to enhance the data validating RecA as a pharmaceutical target, it was of interest to assess whether such RecA inhibitors could also serve to block its function in horizontal gene transfer[27,27,37]. In this study, we evaluated whether cell-permeable RecA inhibitors could prevent the transfer of genetic material from heat-killed antibiotic-resistant *E. coli* to live, susceptible *E. coli*. It was demonstrated that one inhibitor identified from a previous screen (A1) attenuated the rate at which *E. coli* developed resistance to chloramphenicol in both the presence and absence of heat-killed chloramphenicol-resistant cells. However, the results also suggest that *E. coli* may have a RecA-indepedent pathway for developing resistance. Regardless of this possibility, the hit compound may reveal yet another unique means of impeding the spread of antibiotic resistance genes that could be unraveled in future studies, but does not discredit RecA from being a novel and promising target for battling antibiotic resistance. Although the latter results may obfuscate the critical path for developing RecA inhibitors as pharmaceutical agents, it should be emphasized that the results also demonstrate the power of biologically active small molecules to help elucidate complex biological processes. To initiate the process of discovering next-generation RecA inhibitors suited to the task of teasing apart contributions of RecA to antibiotic resistance, collaborative studies were undertaken to identify prospective inhibitors among virtual compound libraries.

To my parents and sister, Joseph, Crystal and Susan Flanagan, who have always loved and supported me in every decision I have made. Whatever I have ever wanted in life, they have done everything in their power to help me make it happen. I love them dearly and thank them for making my life wonderful. Also, to Mert Sedef, my rock to cling to when waters were rough, I am so thankful to have you in my life. Lastly, despite the fact that they cannot read this, to my cats, Tyler and Orion, have always loved me unconditionally and comforted me in times of need. Their companionship has been and is invaluable to me.

ACKNOWLEDGMENTS

Despite the fact that I have chosen to leave this amazing and opportunistic program and school, I am grateful for having the opportunity to study at the University of North Carolina under the direction of many talented and respectable faculty members who have shared their abundance of knowledge and experience with me. They have always been supportive and wish to see all of their students succeed. I have a strong foundation to enter the work force and am confident they have provided the necessary tools for me to thrive in life, no matter what endeavors upon which I embark.

Firstly, I would like to thank Dr. Scott Singleton. He has always been extremely kind to me and concerned about my wellbeing. I will never forget when I was about to come to North Carolina all the way from New Jersey how he personally stopped by an apartment complex I was considering to move to just to ensure its safety and integrity. He accepted me as a summer rotation student so I could even start the program early. When I needed extra help in my coursework, his door was always open. For him, there were never too many questions to ask. Lastly, he always trusted and believed in my abilities, even when I did not. I cannot express how much this meant to me.

I need to extend a special thanks to Dr. Tim Wigle, who was my mentor when I first arrived in the Singleton laboratory. Most of what I learned in my experiences in lab was from Tim. I always appreciated his brotherly concern for me and his need to challenge me to figure out the answers on my own. Despite our minor confrontations, I am thankful to have learned under such a talented and aspiring scientist.

To the rest of the Singleton lab members, Dr. Anna Gromova, Dr. Daniel Cline, Dr. Malinath Hadamani and Demet Guntas, their support has been invaluable to me. I am glad to have worked with Anna to learn some chemical synthesis. I am sure I will use it in my future career endeavors at some point. Dan was always there to patiently answer any questions I had, no matter how rudimentary they may have seemed to him. He is the most patient person I have ever met. Mali was the first person to whom I have ever taught lab techniques, so I was glad to be able to share my knowledge with him. Lastly, Demet was always my pool of emotional support inside and outside of lab. Not only did she teach me many techniques in the lab, she was always there to give me encouragement and a hug when I was feeling discouraged. She was more than a co-worker, she was a wonderful friend and I will miss her dearly when I leave. I will miss them all when I leave.

My dissertation committee, Dr. Jian Liu, Dr. Qisheng Zhang, and Dr. Alexander Tropsha, have all been kind and generous in their advice and desire to aid me in my studies. All I wish is that I utilized their knowledge base more because I know they would have many more insights into my research to share if I had just asked. Also, Dr. M. Karthikeyan, whom I collaborated with in the Dr. Tropsha laboratory, has been instrumental in furthering the research I wished to accomplish. He ran the molecular simulations and explained all of their complexities to me. I greatly valued and appreciated his efforts.

Briefly, to Renita Patel and Heidi Clarke, a pharmacy student and undergraduate student, whom I have had the pleasure to mentor, I thank them for giving me the opportunity to do something I love: teach. I thoroughly enjoyed sharing my knowledge with them and hopefully I passed along some skills and wisdom they will be able to use.

To my friends: I have always valued their love, support, advice and companionship. As my family always has (and I thank them for the same reasons), they have always been there for me and wanted the best for me. They have pushed me through some rough spots and I cannot thank them enough. To Tom Riley especially, who is one of my few friends in the same field, I am glad to have his knowledge just a phone call away.

And, to everyone whom with I practice kung fu, whether in Hungary or the United States, I thank them for teaching me discipline, self respect, ultimate and divine love, beauty, patience, strength and for opening my eyes to see what I truly want from life. They have supported and positively changed me in more ways than they will ever know. Hinár Polczer: he has been more than an instructor, but a most wonderful friend. Encouraging me to go to kung fu camp was the best act he could have ever done for me. It was truly the most wonderful experience of my life and I thank him for that opportunity. And to my master, Marin Vélin, who is the most spiritual and kind-hearted man I have ever met. I am greatly obliged to be his student and to have had the pleasure of meeting him.

To Mert Sedef, my significant other, who has been an endless supply of emotional support and love, I thank him for being there for me to give me a hug when I needed it, to grocery shop when I lacked the time, and for being my cheerleader on the sideline always rooting for me.

Last, but not least, to my family: I know deep down they are disappointed that I was not the first in the family to get a PhD. But I also know they are proud of me nonetheless and will always be proud. They have always wanted me to be happy, no matter how that happiness manifests itself. Thank you for always loving me and caring for me throughout all of the years of my life.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Amp	Ampicillin
ATPγS	Adenosine 5′-[γ-thio]triphosphate
Cam	Chloramphenicol
Cam^r	Chloramphencial resistance/Chloramphenicol resistant cell supernatant
CFU	Colony-forming units
Cipro	Ciprofloxacin
cssDNA	circular single-stranded DNA
DHF	dihydrofolate
DSB	Double-strand break
dsDNA	double-stranded DNA
DTT	Dithiothreitol
Em	Emission
Ex	Excitation
HGT	Horizontal Gene Transfer
HTS	High-throughput screening
IRA	Inhibitor of RecA
Kan	Kanamycin
LB	Luria Bertani
MMC	Mitomycin C
MOE	Molecular Operating Environment
MRSA	Methicillin-resistant *Staphylococcus aureus*

NAD^+	β-Nicotinamide adenine dinucleotide, oxidized
NADH	β-Nicotinamide adenine dinucleotide, reduced
NCI	National Cancer Insititute
NER	Nucleotide excision repair
NMP	Nucleotide monophosphate
NDP	Nucleotide diphosphate
NTP	Nucleotide triphosphate
NXP	Nucleotide mono-, di- or triphosphate
NPF	Nucleoprotein filament
nts	nucleotides (in reference to DNA oligomer concentration)
OD	Optical density
ONPG	2-Nitrophenyl-β-D-galactopyranoside
P_i	Inorganic phosphate
P-loop	Phosphate binding loop
PMB	Phosphomolybdate blue
PNP	Purine nucleoside phosphorylase
PolIV	DNA polymerase IV
PolV	DNA polymerase V
QSAR	Quantitative structure-activity relationship
RDR	Recombinational DNA repair
SMILES	Simplified Molecular Input Line Entry Specification
ssDNA	Single-stranded DNA
SSB	Single-stranded DNA binding protein

TCA cycle	Tricarboxylic acid cycle
THF	Tetrahydrofolate
tsDNA	Triple-stranded DNA
UV	Ultraviolet
VRE	Vancomycin-resistant *enterococci*
wt	wild-type

CHAPTER I

INTRODUCTION

Implications of Bacterial Drug Resistance

Limited organisms in existence have the ability to thrive in every imaginable habit this earth has to offer. Bacteria, being unicellular microorganisms, are able to survive in water, soil, radioactive waste, acidic hot springs, even in the very living bodies of plants and animals. Millions of bacteria are present on the skin and within the human body at any given time. Bacteria are perceived as disease-causing, undesirable living organisms, despite the fact that their existence is vital to the cycles of the earth due to their capability to recycle nutrients. They are even beneficial to humans in that they aid in the health of the digestive tract by performing fermentation of unused energy substrates, controlling the growth of harmful bacterial species and producing valuable vitamins for their hosts. Most bacteria living in the human body are regulated by the immune system, but pathogenic strains do arise and cause infections diseases, thus bestowing bacteria with their ominous reputation.

However, before the introduction of antimicrobials, communicable infectious diseases, including bacterial infections, claimed the lives of thousands of people every year. At the start of the twentieth century, diseases caused by bacteria ranked at the top among the top ten causes of death in the United States,

which included pneumonia (viral and bacterial), tuberculosis and diarrhea. Maintaining personal hygiene, reliance on the immune system and the use of crude plant extracts were not enough to contend with the microorganisms that caused these diseases. Attempts to discover agents that could have antibacterial effects were unsuccessfully made in the early 1900's. Sulfa drugs were introduced in the early 1930's as the first antimicrobial drugs, but due to adverse effects and toxicity issues, other antimicrobial agents were desired. Sulfa drugs paved the way for revolutionary agents like penicillin. Also in the 1930's, Sir Alexander Fleming accidentally stumbled upon a petri dish growing *Staphyloccocus* contaminated with mold[1]. However, this plate showed lack of growth of the bacteria near the mold, thus indicating that it released a natural product that was lethal to the bacteria. Upon analysis of the mold, it was determined to be *Penicillium notatum*, and thus, the natural product being released from the mold was coined penicillin. Through collaborative efforts with Howard Florey and Ernst Chain, penicillin was shown to have bactericidal effects in vivo and proved effective in treating bacterial infections in mice[2]. In the 1940's, penicillin was finally able to be mass-produced, which enabled it to be extensively used to treat countless soldiers in World War II, allowing them to treat otherwise fatal and devastating wounds. The Nobel Prize was awarded to Fleming, Florey and Chain in 1945 in recognition of their discoveries. They paved the road for the introduction of other antibiotics which would lead to a great improvement of the quality of life by combating the infectious diseases that claimed the lives of so many in the beginning of the century.

The introduction of antibiotics, which were viewed as “miracle drugs”, was considered to be one of the most imperative medical advancements of the twentieth century, giving a false sense of hope that humans had triumphed in the war against infectious diseases. Fleming himself foresaw and warned against the dangers of resistance, stating that underdosage of an antibiotic easily caused microbes to develop resistance in the laboratory and in humans by not exposing them to a lethal dosage of the drug, thus allowing microbes to “educate” themselves on how to resist antibiotics[3]. Many battles had been victorious with the introduction of new and more effective agents, thus keeping bacterial resistance from becoming a major medical problem for decades, but resistance to antibacterials escalates today at an alarming rate.

According to the Center for Disease Control and Prevention, two million people acquire bacterial infections in hospitals each year[4]. Ninety thousand of those people die from these infections due to approximately seventy percent of those infections being caused by a strain of bacteria that are resistant to one or more of the one-hundred or so available antibiotics[4]. These statistics account only for hospitalized patients. Millions more acquire infections outside of the hospitals. Because of the prevalence of resistant strains and the rapid onset of resistance to newly introduced antibiotics (Figure 1.1[5]), many doctors find difficulties and limitations in treating patients.

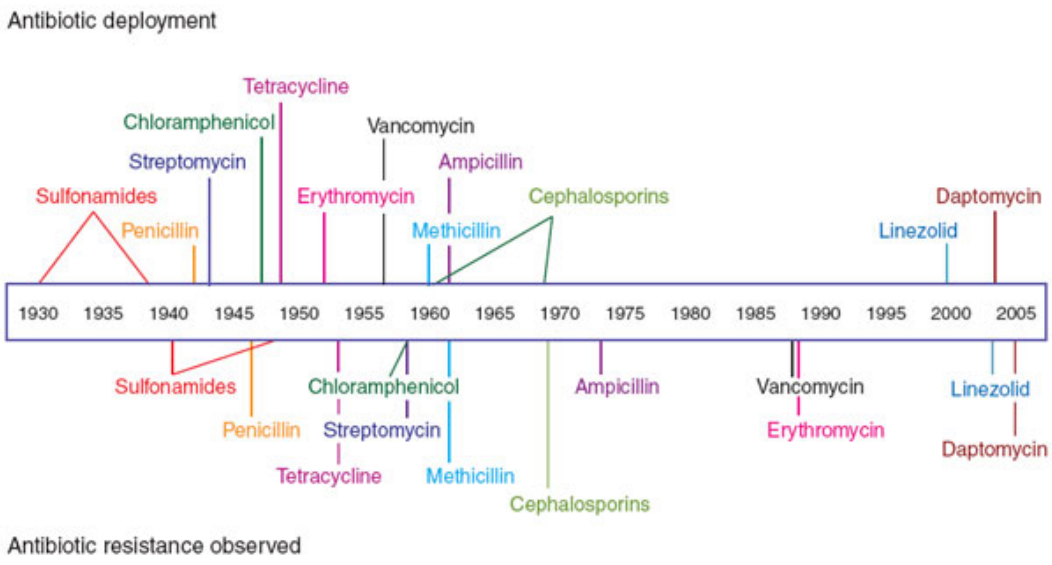

Figure 1.1. Timeline of the introduction of new antibiotics vs. the reported resistance to the antibiotics. Many antibiotics introduced to the market often acquire resistance quickly after their introduction. The figure was reproduced from Clatworthy, et al. (2007) *Nat. Chem. Biol. 3*, 541-548.[5]

A positive trend has been observed when reporting the amount of resistant strains infecting people in the United States. Starting from about 1980, the percent incidence of resistant strains was very low, less than 5% (Figure 1.2, left panel). However, over the course of twenty years, the percent incidence of resistant strains has drastically increased to about 60% incidence for MRSA and 25-30% incidence for VRE and FQRP. The need for novel antibacterials is quite apparent, however, an inverse correlation is seen in the number of antibacterials approved in the United States over the same time span. A total of three new antibiotics were approved for administration between 2003-2007 (Figure 1.2, right panel). How can this be when the need for such treatments is so great? Many large pharmaceutical companies, including Glaxo Smith-Kline, Wyeth, Aventis and

Bristol-Myers Squib, have eliminated or downsized their antibacterial research[6]. This is due to the increased cost of drug discovery research, short-comings of target based drug discovery to unravel novel antibacterials, a shift of interest from short-term acute diseases to long-term chronic diseases and because the rapid onset of resistance to antibacterial agents gives them a short –lived market time frame. The cost to develop and market the drugs is far greater than the revenue they generate[6].

The moral implications of this biological predicament outweigh the monetary demand. Therefore, the goal of this thesis was to further understand the implications of the RecA protein in bacterial resistance development and utilize it as a potential novel target in battling resistance onset and therefore battling bacterial infections diseases that are claiming the lives of so many.

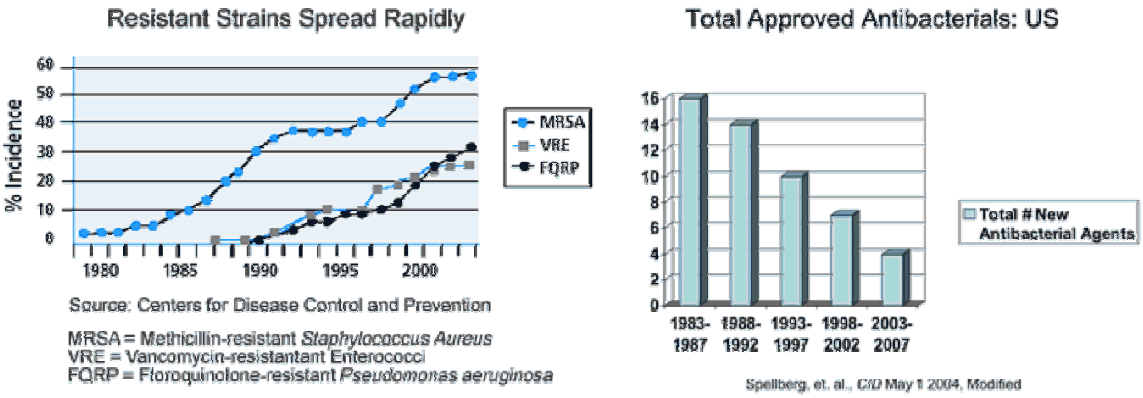

Figure 1.2 Trends in the spread of bacterial resistant strains and approved antibiotics in the United States. Left panel: Displays the increase of percent incidence of MRSA, VRE and FQRP vs. time in years. Right panel: Displays the declining number of approved antibiotics vs time in years. Figures were reproduced from the Infections Disease Society of America, *Bad Bugs, No Drugs* website: http://www.idsociety.org/badbugsnodrugs.html

Targets and Mechanisms of Antibiotic Resistance

Antibiotics are able to function and kill bacteria by inhibiting enzymes and proteins that are necessary for their survival. Ideal antibiotics have low affinity for host enzymes and proteins and have low toxicity levels within hosts. Four major categories of targets into which most antibiotics can be classified are inhibitors of cell wall synthesis, nucleic acid synthesis, protein synthesis and agents that impair the cell membrane[7]. Specifically for protein synthesis, antibiotics can bind to the 50S and 30S subunits of ribosomes preventing the translation of RNA to protein. Nucleic acid synthesis can be blocked three ways: antibacterials can inhibit RNA polymerase, which is needed to initiate transcription; antibacterial agents can act by inhibiting folic acid biosynthesis, which stops the formation of folate that is required to synthesize DNA bases; and lastly can prevent DNA topoisomerases (including DNA gyrases) from functioning, which renders the enzyme incapable of catalyzing the supercoiling of DNA necessary for metabolic processes[7]. Figure 1.3 illustrates the possible targets of antibiotics, as well as lists common antibiotics used for each target.

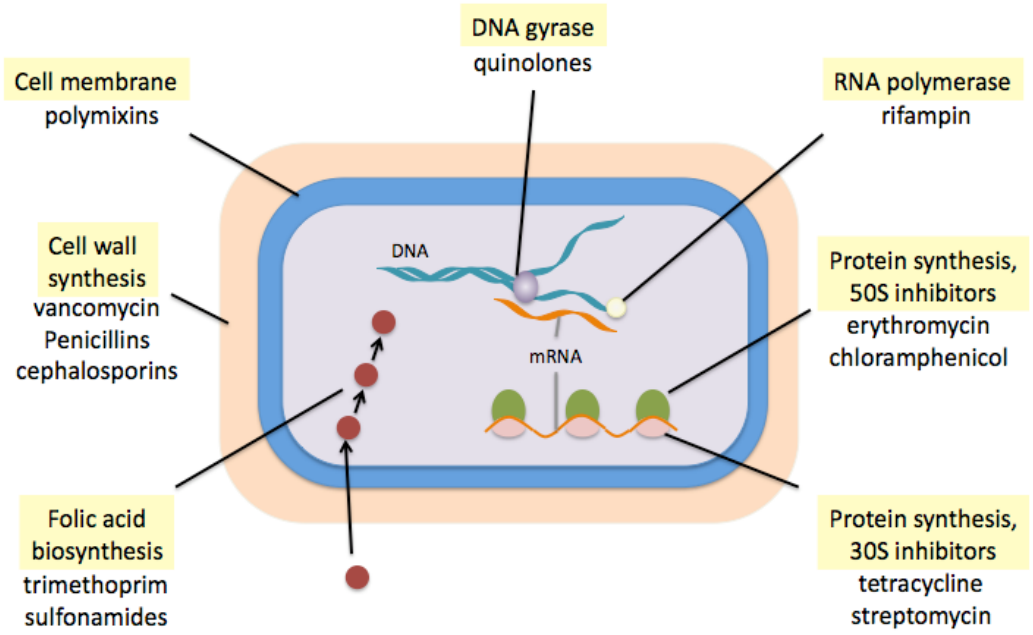

Figure 1.3 Targets of antibiotics with representative agents. The various targets of antibiotics are highlighted in yellow. A few representative antibiotics are listed for each target. This figure was reproduced from Molecular Biology of the Cell, 4th ed., Ch. 25, fig. 8 . [8]

Since antibiotics function by inhibiting necessary processes within bacteria, in order to survive, bacteria must find means of bypassing the inhibition capabilities of antibiotics. Therefore, bacteria exhibit three phenotypes in relation to antibiotics: susceptibility, intrinsic resistance and acquired resistance. Any bacteria species that is susceptible to an antibiotic may develop or acquire resistance to that antibiotic, either through *de novo* mutations or the inheritance of DNA encoding resistance either from transformation or mobile genetic elements (including plasmids and transposons)[4]. Intrinsic resistance refers to a bacteria's natural ability to be resistant to an antibiotic due to the make up of the organism, meaning the hydrophobic lipopolysaccharide outer membrane of gram-negative bacteria repel hydrophobic antibiotics and large compounds cannot transverse the

size-excluding porins in the outer membrane[9]. Basically, no genetic manipulations are needed for intrinsic resistance. On the other hand, acquired resistance, which is present in only select isolates of certain species, exhibit resistance to antibiotics through four mechanisms, which are alteration of the antibiotic through enzymatic degradation or modification, acquisition of a drug-insensitive enzyme that functions in place of the drug-sensitive enzyme thus altering the metabolic pathway, decreased accumulation of the antibiotic through reduced membrane permeability and/or increased efflux and mutation of the target site rendering the antibiotic unable to bind its target[7] (Figure 1.4).

Due to the fact that acquired resistance requires resistant genes from exogenous DNA, either from transformation or mobile genetic elements, or de novo mutations, a mechanism for incorporating these genes or inducing mutations is necessary. RecA, a recombinase protein, has been implicated in the acquisition and incorporation of bacterial resistant genes[19, 23-27] and through the induction of mutations leading to resistance[21,22, 28-36].

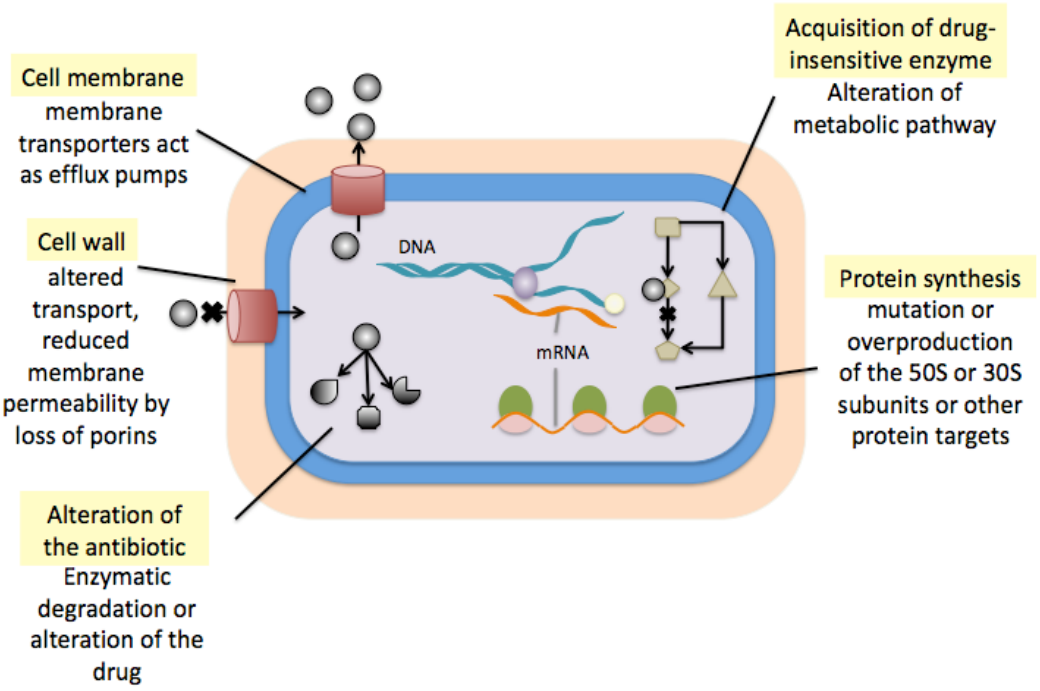

Figure 1.4 Antibiotic resistance mechanisms. The four major mechanisms or resistance are outlined: mutation or over production of the target, altered metabolic pathway, degradation or alteration of the drug or decreased drug accumulation due to reduced membrane permeability and/or active efflux pumps.

Mechanisms of Chloramphenicol Resistance

Chloramphenicol was first announced in 1948 by Ehrlich and coworkers[10]. What made this antibiotic so unique is it was the first natural product to contain a nitro group and to be a derivative of dichloroacetic acid[11]. It is mainly a broad-spectrum bacteriostatic agent with the ability to inhibit all bacteria tested and organisms that are closely related to bacteria[11]. Chloramphenicol inhibits protein synthesis in concentrations of 10 μM and above, but does not inhibit the activity of proteins[11]. During protein synthesis, aminoacyl tRNA molecules are synthesized in the cytoplasm and are transported

to the ribosome where they bind to associated mRNA at the P site[7]. Another tRNA then binds to the A site that corresponds to the next codon in the mRNA sequence and a transpeptidation reaction utilizing peptidyl transferase allows for the linkage of the growing peptide chain to the next amino acid to be added to the chain in the A site[7]. Lastly, the growing chain translocates to the P site, which frees the A site for the next tRNA and this will continue until a stop codon is reached[7] (Figure 1.5). Specifically, chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal subunit of the 70S ribosome and impedes the peptidyl transferase reaction, thus stopping peptide chain formation[7].

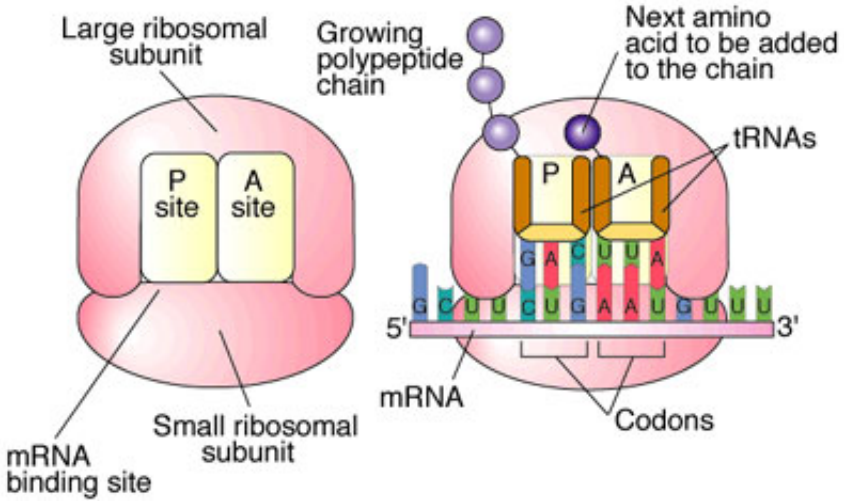

Figure 1.5 Protein synthesis. Image was reproduced from the Biology Active Learner, 6th ed. website: http://www.dwm.ks.edu.tw/bio/activelearner/index.html

Three of the common mechanisms of antibiotic resistance have been implicated in the development of chloramphenicol resistance: 1) reduced accumulation of chloramphenicol in the cell through reduced membrane permeability and active efflux pumps[9,12-14] 2) altered target[15] and 3) enzymatic degradation of chloramphenicol[7,17]. Chloramphenicol, being a small and

hydrophilic antibiotic, transverses the outer membrane through porin channels,[9] which span the outer membrane and are water-filled open channels that allow the passive diffusion of hydrophilic molecules[12]. The three major porins of *E. coli* are the OmpF, OmpC and PhoE porins and *E. coli* exhibits modifications of its porins to become resistant to antibiotics by reducing the amount of OmpF and OmpC porins or mutating OmpC porins[12]. This kind of mutation involves the alteration of the internal loop 3, which in return alters the ability of antibiotics to transverse the porin channels[12]. Chloramphenicol is believed to pass through the OmpF channel due to its larger size and because of its expression being regulated by marA, which is de-repressed in response to chemical and antibiotic stress and through downstream signaling, porin synthesis is downregulated while efflux pumps are overexpressed[9,12-13]. Figure 1.6[13] outlines this cascading event. The AcrAB-TolC is a common efflux pump used to export commonly used antibiotics, chloramphenicol included[13,14,15]. The *acrA* gene encodes a membrane fusion protein, *acrB* encodes a cytoplasmic membrane efflux pump and *tolC* encodes for an outer membrane channel[13,14]. MdfA (also known as CmlA and Cmr) is another multi-drug pump that is chromosomally encoded and it provides resistance to chloramphenicol[13,15].

Also reported, a mutation of guanine to adenine at position 2057 of the 23S RNA gene in the rRNA operon confers resistance to chloramphenicol[16]. This mutation causes an alteration in the sequence in a region of the 23S secondary structure, which is a part of the peptidyl transferase region, and therefore hinders chloramphenicol to bind the 50S subunit due to the peptidyl

transferase active site being located near the peptidyl transferase region containing the 23S RNA region[16]. Thus, alterations of the target site of chloramphenicol results in resistance development to chloramphenicol.

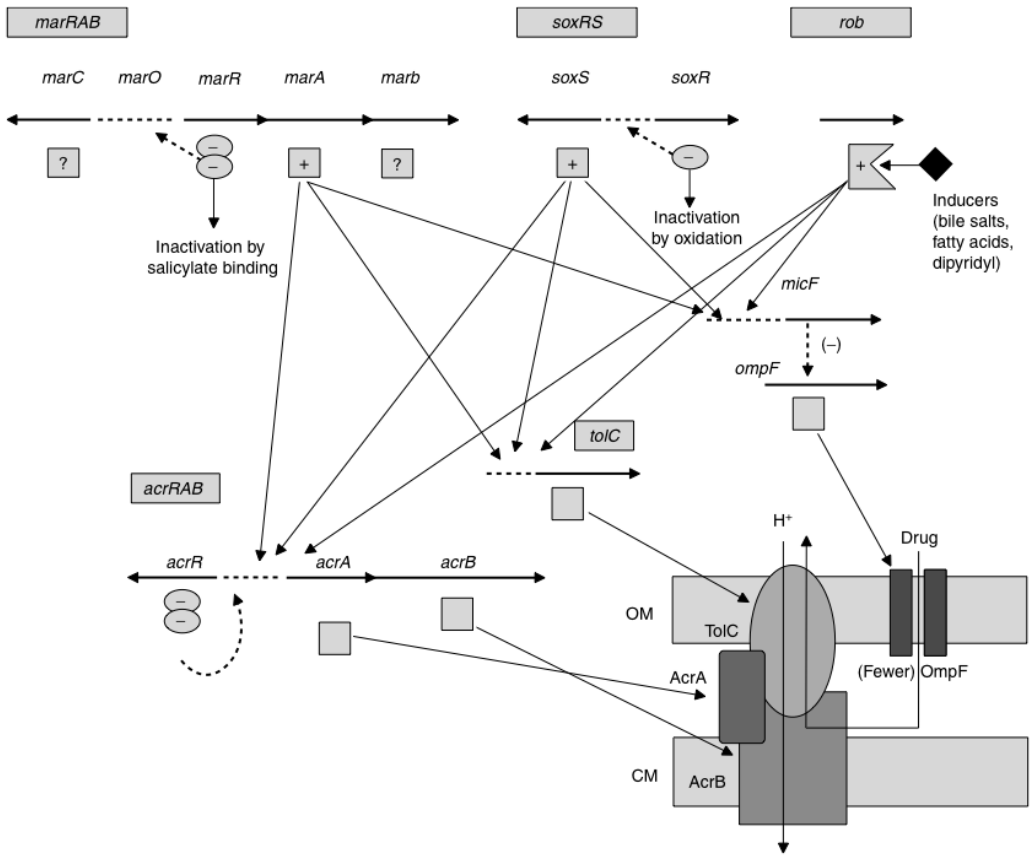

Figure 1.6 Signaling cascades responsible for loss of porins and increased drug efflux. Activation of *marA* through de-repression of *marR* due to antibiotic induced stress activates *micF*, which inhibits *ompF* expression, and activates *acrA*, *acrB* and *tolC*, genes that express proteins necessary for the assembly a common efflux pump in *E. coli*. This figure was reproduced from[13].

Lastly, genes that are responsible for enzymatic degradation of chloramphenicol are *cat* genes, which encode for chloramphenicol acetyltransferase[7,18]. This enzyme functions by acetylating the hydroxyl group on

position three on the carbon side chain, utilizing an acetyl group from acetyl-coenzyme A, giving rise to the 3-acetyl derivative; the acetyl group can replace the hydroxyl group on the carbon on position one on the carbon side chain, thus allowing for a second acetylating reaction to the hydroxyl group on the third carbon on the side chain[7,17,18]. Some of the *cat* genes found in *E. coli* include *catI*, *catB2*, *catB3*[18], which encode for different types of CATs (types A and B, type A being native to the organism, type B being xenobiotic).

The Functions of the RecA Protein

Genetic preservation and variation are two necessary processes all living organisms must utilize and balance in order to survive. The genome must be preserved in order for an organism to function properly while it also must be varied in order for organisms to adapt to changing environments. RecA is a key player in maintaining the balance in bacteria: all bacteria contain RecA and its function is implicated in processes that lead to either genomic preservation or variation[19, 20]. Metabolic and physiological stresses caused by antibiotic treatment, heat shock, starvation, exposure to UV or harmful chemicals and pressure changes either directly or indirectly lead to DNA damage, which effectively activates RecA[19,21].

Within the cell, RecA exists as a protein monomer and is biologically inactive; however, in the presence of ssDNA and ATP, RecA monomers polymerize to coat the ssDNA to form an activated RecA nucleoprotein filament (NFP) and begin ATP-hydrolysis driven DNA repair processes[19,23], which

included homologous recombination and replication for restart[23], as well as signaling the initiation of the SOS response[22]. RecA-mediated repair processes, whether they initiate preservation or variation of the genome, aid in the survival of bacteria by providing means of maintaining genetic integrity as well as adapting to environmental changes. Figure 1.7 summarizes the activities of RecA involved in preservation and adaptation.

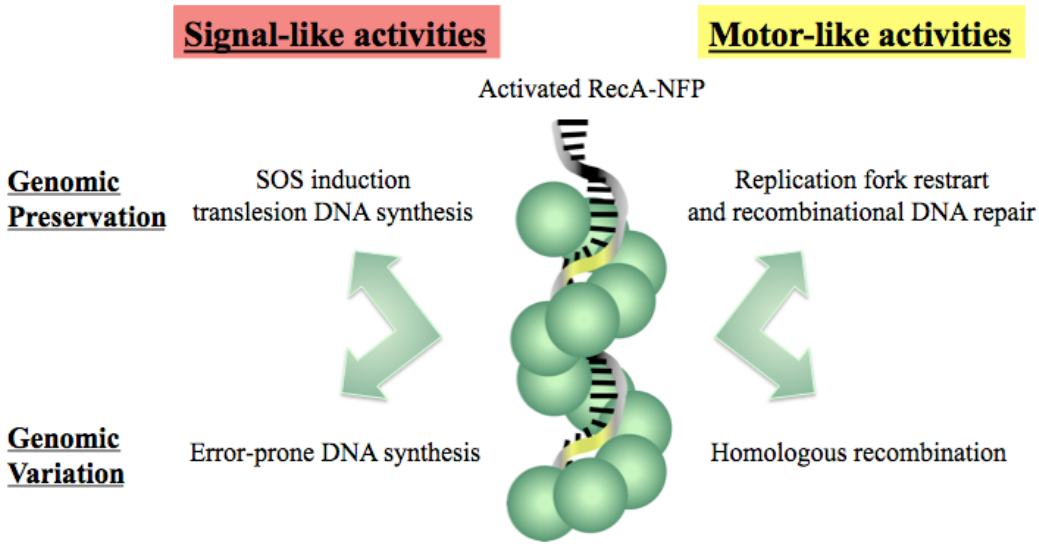

Figure 1.7 Signal-like and motor-like activities of RecA. Upon activation of the RecA-NPF, RecA-mediated activities that lead to genomic preservation are SOS induction translesion synthesis, replication for restart and recombination DNA repair, while activities that lead to genomic variation are error-prone DNA synthesis and homologous recombination.

The Involvement of RecA in Recombination

Recombination in its simplest meaning is the breaking and rejoining of genetic materials, most often DNA. It is implicated in the genetic diversification of offspring during sexual unions and horizontal gene transfer between microorganisms. Homologous recombination, or genetic exchange between similar or identical strands of DNA, is needed for recombinational repair in bacteria. However, recombinational repair is only implicated in certain types of DNA damage due to DNA being double stranded; for example, lesions occurring on one strand can easily be excised and the other strand can be used as a template to fill in the gap, which does not require recombination[19]. Cross-links, double-strand breaks and lesions in ssDNA would be cases of DNA damage that would require recombination to obtain reliable sequence information from a homologous strand and RecA is at the heart of recombinational activity[19].

During each of the three repair mechanisms mentioned, ssDNA is either exposed (stalled replication forks caused by lesions) or generated by the actions of other accessory proteins (UvrA,B, and C and nucleases in cross-link repair and RecBCD and nucleases in double-strand break repair)[19]. Once ssDNA is present, RecA polymerizes on the ssDNA to form the NPF[19,23], conducts a homology search by recruiting a linear dsDNA molecule, then aligns the ssDNA with a homologous sequence of dsDNA[23] and proceeding to facilitate strand exchange[24]. Combined with RuvA,B and C, branch migration occurs, which eventually leads to resolved, repaired dsDNA[25]. Figure 1.8 summarizes the role of RecA in homologous recombinational repair.

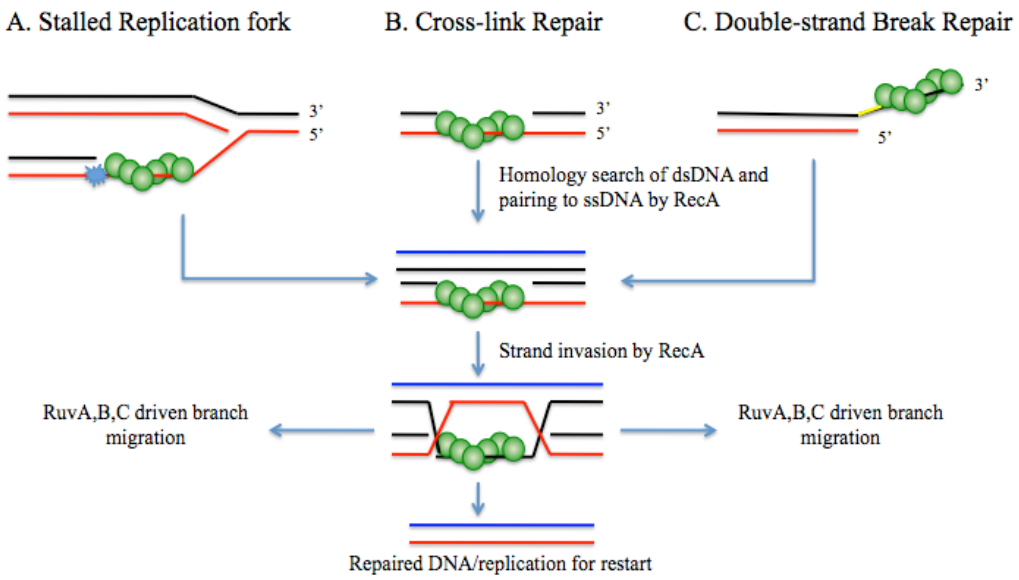

Figure 1.8 Role of RecA in homologous recombination. RecA polymerizes onto ssDNA formed during stalled replication forks, cross-link repair and double-strand break repair. RecA then performs a homology search of dsDNA and pairs the ssDNA with homologous sequences. RecA then performs strand invasion and RuvA,B and C drive branch migration, which leads to repaired dsDNA and the restart of replication forks. Figure was adapted from Roca and Cox, *Prog Nucleic Acid Res Mol Biol 56*, 129-223[19]

Not only is this process useful for DNA repair, bacteria can utilize RecA-mediated homologous recombination to incorporate genes into their genome that they acquired through horizontal gene transfer[26]. Horizontal gene transfer can occur in three ways: transformation, transduction and conjugation[27] (Figure 1.9[27]). Transformation is the process in which bacteria uptake exogenous DNA from their environment, usually excreted by a donor bacteria during lysis (usually upon death of the bacteria). Transduction occurs through a bacteria phage, which carries the genes from the phage-infected donor cell to the recipient cell and injects the DNA into the recipient cell. Conjugation is the process by which donor bacteria assemble a sex pilus (F factor) and inject DNA into the recipient cell. Once bacteria cells have taken up the exogenous DNA, whether from the same or

different species, RecA can pair this DNA, which is heteroduplex DNA, with homologous ssDNA from the host cell (Figure 1.10). Through strand exchange during a tsDNA intermediate, genes from the donor bacteria can be incorporated into the genome of the host cell. This process has had tremendous effects on the evolution of bacteria, especially in regards to the spread of antibiotic resistant genes.

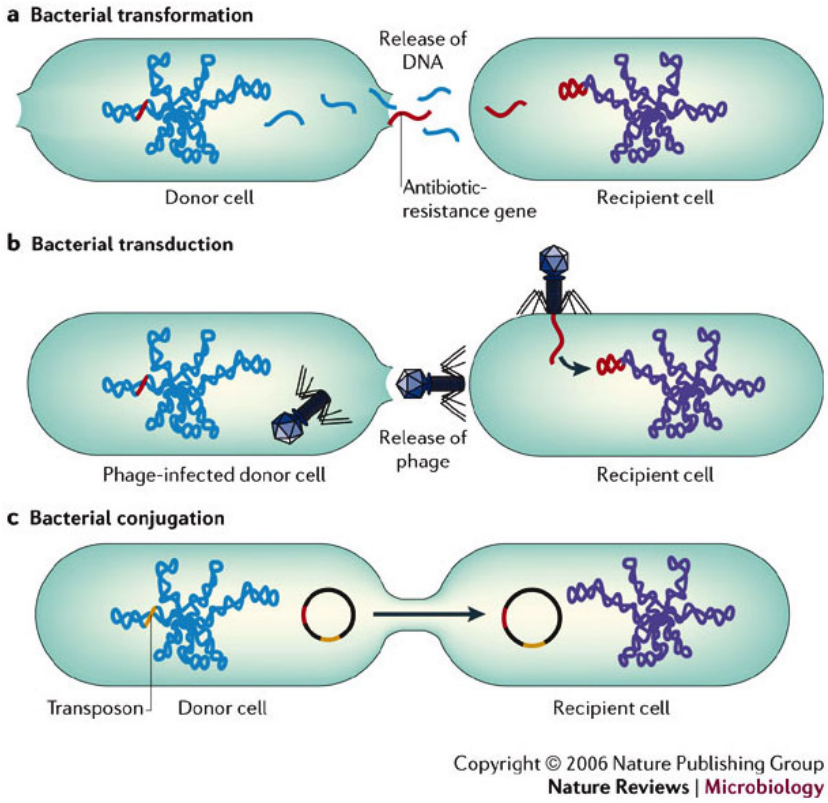

Figure 1.9 Horizontal gene transfer in bacteria. This figure displays the three mechanisms for HGT: transformation, transduction and conjugation and was reproduced from Furuya et al., *Nature Reviews Microbiology 4*, 36-45.[27]

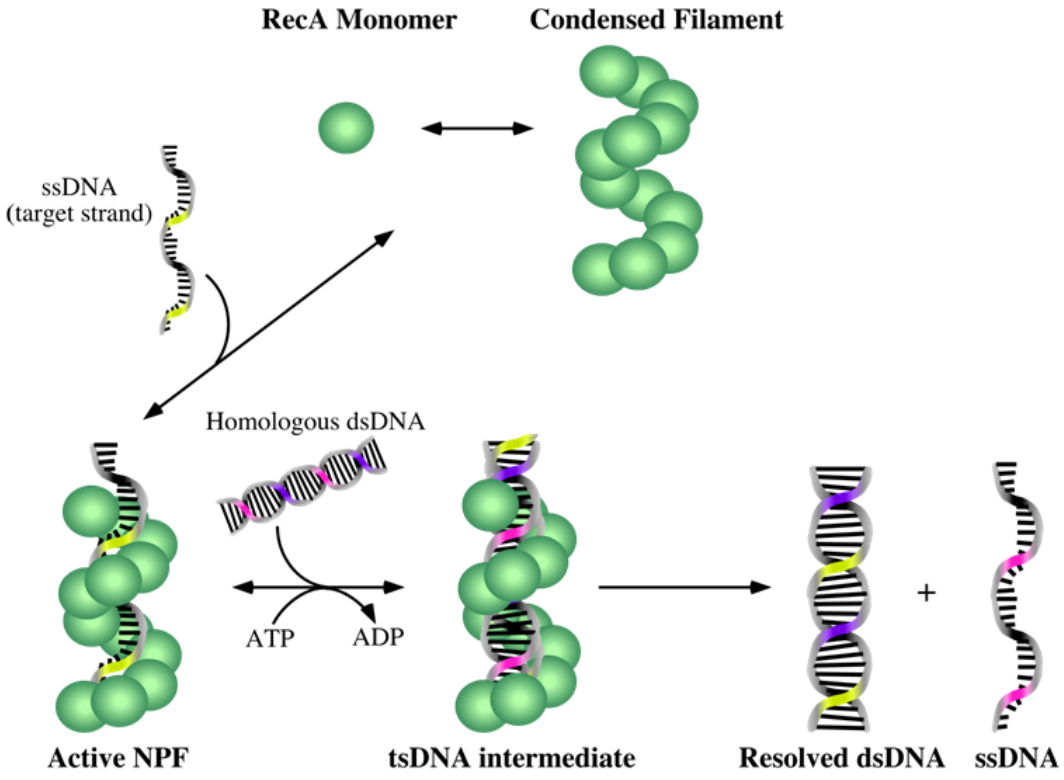

Figure 1.10 Homologous recombination during horizontal gene transfer. In this case, the homologous dsDNA was donated from a donor cell. The host cell acquires that DNA and can use it to pair its own ssDNA to homologous sequences in the acquired donor DNA. When this happens, a tsDNA intermediate is formed, during which RecA can perform strand exchange, which eventually leads to resolved dsDNA incorporating genes from the donor.

The Role of RecA in the SOS Response to DNA Damage

RecA has proven to be a vital participant in genomic preservation in bacteria by playing a key role in homologous recombination to repair DNA[19,23] that has experienced a stalled replication fork, double-strand break or cross-linkage, as well as in the diversification of the bacterial genome through a partial "mutagenic" role utilizing homologous recombination to incorporate genes acquired during horizontal gene transfer[26,27]. Yet another vital role of RecA in

genomic preservation and variation is through induction of the SOS response[21,22].

The expression of SOS genes are regulated by LexA, a repressor protein dimer, which binds to the SOS box of the promoter region of the SOS genes, thus preventing their expression by preventing RNA polymerase from accessing the promoter[22]. When cells are experiencing normal physiological conditions, the SOS genes are repressed by LexA. However, when DNA damage occurs and ssDNA accumulates, RecA polymerizes on the ssDNA and is activated, which in return interacts with the LexA repressor protein[28,29,30]. This interaction with LexA facilitates the autoproteolysis of LexA, thus allowing the downstream DNA repair proteins to be turned on[28,29]. It is important to note that the induction of the SOS to genomic damage is a graded response in which different mechanisms of repair are sequentially activated, starting with excision repair and recombination and eventually leading to mutagenesis if the damage persists (Figure 1.11). Sequential activation of approximately forty DNA repair proteins depends on the location of the genes to their specific SOS box and the binding affinity of LexA to that SOS box[22]. Generally, proteins can be classified into three main groups: early, middle and late genes.

Early genes consist of *uvrA, B, C* and *D,* which encode for endonucleases that participate in nucleotide excision repair[31]. Small amounts of DNA damage can be repaired this way by simply excising a small length of ssDNA containing the damage and this process allows the cell to attempt to repair the damage without fully committing to full-fledged SOS response.

However, if the damage is not repaired after a short amount of time, the amount of de-repressed LexA increases, thus allowing for the expression of the middle SOS genes, which include *recA* and *recBCD*[32,33], the genes required for the expression of recombinase proteins used in double-strand break and cross-linkage repair. Much larger amounts of DNA can be repaired through recombination as compared to nucleotide excision repair. Because RecA is included in the group of middle SOS genes, the SOS response can be propagated at a very accelerated rate due to the influx of RecA expression.

DNA damage that still persists even after NER and recombinational repair attempts will eventually lead to the activation of the late SOS genes, which encode for Polymerase IV, UmuC and D, as well as SulA[22,35]. SulA is responsible for binding FtsZ, an essential protein that initiates cell division[34]. By binding FtsZ, cell division is arrested, thus diverting all of the cell's efforts to repairing the damage. This includes the atuoproteolysis of UmuD whose byproducts can bind to UmuC, therefore forming Polymerase V[22]. Both translesion Polymerases IV and V lack the ability to proof read DNA and therefore cannot detect lesions within the DNA, thus allowing the insertion of any base into gaps across from the site of the lesion[22, 35]. And thus SOS mutagenesis is induced, which allows for the introduction of spontaneous mutations that can produce new resistant genes or strengthen already existing resistant genes.

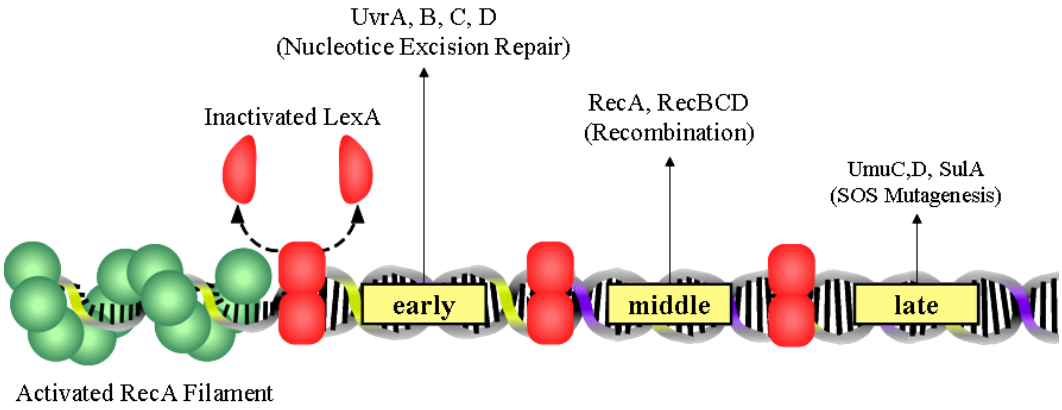

Figure 1.11 Bacterial SOS response. The induction of the SOS to genomic damage is a graded response in which different mechanisms of repair are sequentially activated after the de-repression of the LexA repressor protein, starting with excision repair (UvrA, B, C and D) and recombination (RecA and RecBCD) and eventually leading to mutagenesis if the damage persists (SulA repression of FtsZ, UmuC and D for translesion synthesis).

Responsibility of RecA in the Development of Resistance to Antibiotic Treatment

RecA has profound roles in the repair of DNA through homologous recombination and also in inducing the SOS response in bacteria by signaling for the expression of DNA repair proteins, which both processes are imperative in maintaining bacterial genomic integrity. RecA-mediated homologous recombination is also used by bacteria to incorporate genes acquired through horizontal gene transfer by pairing the heteroduplex DNA from the donor cell with homologous ssDNA from the host cell, which leads to integration of the donated DNA into its own genome. SOS induction by RecA can also lead to SOS mutagenesis as a last resort when nucleotide excision repair and recombination

fail to repair DNA damage. SOS mutagenesis and homologous recombination of horizontally acquired genes are therefore essential processes for genomic variation, which provide mechanisms for bacteria to survive in changing environments.

Antibiotics severely alter the environment of bacteria, thus imposing great stress on the bacteria to survive. Antibiotic induced stress has been shown to induce the SOS response through RecA activation[4,26]. As shown in Figure 1.3, there are six major types of antibiotics categorized by their targets: protein synthesis inhibitors, RNA polymerase inhibitors, inhibitors of DNA synthesis and function, inhibitors of cell wall synthesis, agents that disrupt cell membrane integrity and folic acid biosynthesis inhibitors. Besides DNA damaging agents, other antibiotic classes that have been shown to activate the SOS response are folic acid biosynthesis inhibitors (since folate is needed for the synthesis of nucleic acids) and inhibitors of cell wall synthesis. It is also pertinent to classify antibiotics according to their mechanism of action: either bacteriostatic (inhibiting cell growth) or bactericidal (killing > 99.9% of bacteria). Bacteriostatic agents mostly consist of protein synthesis inhibitors.

Recently, it has been demonstrated that all classes examined of bactericidal agents including quinolines, β-lactams and aminoglycosides, produce hydroxyl radicals in *E. coli* through the reduction of hydrogen peroxide by ferrous iron, which contribute to the killing action of these antibiotics, thus indicating that there is a common mechanism that induces cellular death[36]. However, bacteriostatic agents do not produce these radicals. Specifically, this process was

proposed to occur by bactericidal agents stimulating the depletion of NADH by hyperactivation of the electron transport chain, which in return stimulates formation of superoxide that damages iron-sulfur clusters. Iron released from these clusters is oxidated, giving hydroxyl radicals as by products that damage DNA, lipids and proteins, ultimately leading to cell death[36]. As expected, the SOS response was also stimulated via RecA, as demonstrated by a fluorescence assay measure LexA-driven GFP expression and a cellular assay monitoring cell death in wild type and *ΔrecA* cells. In the fluorescence assay, quinolines and β-lactams showed a significant increase in the GFP reporter, thus indicating the activation of RecA and the SOS response[36]. Kanomycin, the aminoglycoside tested, did not show an increase in the GFP reporter, but this is expected since kanomycin blocks translation of proteins and therefore the translation of GFP expression. By disabling the SOS response, the killing effect of bactericidals would be expected to increase. This was demonstrated with all three agents in *ΔrecA* cells, which stresses the importance of the induction of the SOS response to bypass the killing effects of hydroxyl radicals[36].

The results from the Collins group also suggest that bacteriostatic agents, since they do not produce hydroxyl radicals, may not induce the SOS response. Experiments to prove this were not done in this study. However, as demonstrated by our laboratory (unpublished data), studies done with chloramphenicol (a bacteriostatic agent) show that at sub-lethal dosages of chloramphenicol, wild type *E. coli* cells develop resistance to chloramphenicol at a slower rate then *E. coli* cells with RecA constitutively turned on in cultures (Figure 1.12, top panel)

and relativly at the same rate during passaging (Figure 1.12, bottom panel). *E. coli* cells with RecA knocked out are not able to develop resistance in culture to chloramphenicol at sub-lethal dosages (Figure 1.12, top panel) and developed resistance much more slowly during passaging (Figure 1.12, bottom panel). These results imply that the SOS response is needed to confer chloramphenicol resistance.

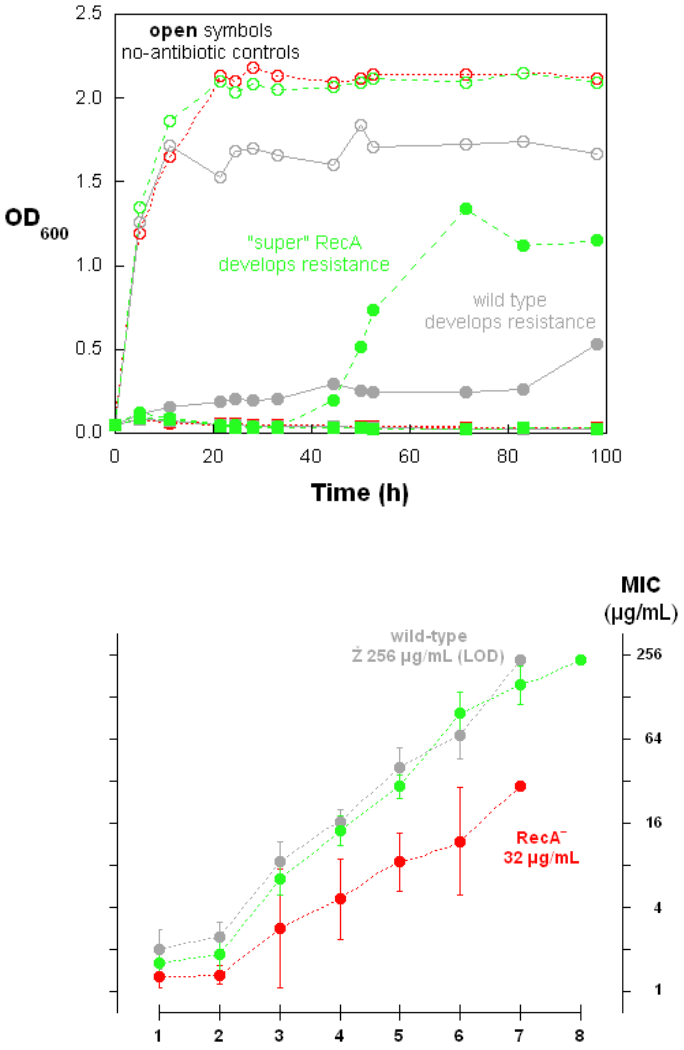

Figure 1.12 Development of chloramphenicol resistance in wild type, super RecA and *ΔrecA-ΔlexA E. coli* cells. The top panel shows resistance development to chloramphenicol at 5 µg/mL in super RecA and wild type cells in the one-flask resistance assay. The bottom panel shows the results of a passaging assay: after 8 passages lasting 24 hrs. each, wild type and super RecA cells develop resistance at relatively the same rate while there is a lag in resistance development in ΔrecA cells.

The Griffith Experiment:
Potential Role of RecA in Incorporation of Exogenous DNA

In the 1920's, pneumonia was a prevalent cause of death, which is an infectious disease affecting the lungs and is caused by a bacterial species *Streptococcus pneumoniae*, or pneumococci. Dr. Frederick Griffith, while studying the distribution of different pneumococcal types obtained from people infected with pneumonia, noticed four distinct types: Types I, II and III and Group IV. Upon compilation of the data of two two year periods and one three year period, he discerned a decrease in the number of Types I and II pneumococci infections and a significant increase in the incidents of Group IV infections[37]. However, it was also noted that Group IV pneumococci was always found in conjunction with another type and was not shown to cause the disease on its own. Therefore, Griffith devised a series of experiments involving the injection of these strains into mice in various combinations of the types in heat-killed virulent, or smooth (S), versions of the strains with living avirulent, or rough (R), versions of the strains to see if reversion of the R strain to S form could occur. It is also important to note, what defined the S strain from the R strain was a mucous film around the S strain, which was a polysaccharide capsule, referred to as S antigen by Griffith[37]. The polysaccharide capsule protects the pneumococci from attack by the immune system of its host. What he observed was mice injected with an S strain alone died of pneumonia while mice injected with a R strain alone lived and did not produce R or S cultures when obtained from blood of the mice[37]. Also dually noted, when mice were injected with only heat-killed S strains, the mice

also lived and did not produce either S or R cultures when obtained from blood of the mice.

But perhaps the most significant observations of this study when mice were injected with both R and heat-killed S strains were five fold: 1) the mice died of pneumonia 2) cultures recovered from the blood of the mice were S strains 3) inoculation of the mice of the R strain of on type of pneumococci and the heat-killed S strain of a different type of pneumococci displayed the type of the heat-killed culture 4) the most successful reversions of the R strain to the S strain were the inoculation with same type of pneumococci (for example, Type II R and heat-killed Type II S) and 5) reversion is not observed when incubated in vitro, meaning passage through mice is necessary for the reversion[37]. From these results, it can be concluded that the R strain is able to make use of the remnants of the dead S culture for the synthesis of S antigen. It can also be hypothesized that since this only occurred in mice and not in vitro that the pressure of the immune system is needed to drive the R strain to survive and seeks the information on how to make S antigen from the killed S cells.

Precisely how transformation occurred from the R strain to the S form was elucidated by Avery, MacLeod, and McCarty. In their attempts, they used the R36A strain, which was an R Type II pneumococci strain, which was derived from an S Type II strain that had its capsule removed[38]. Crude extracts were obtained from the S strain (A66, Type III pneumococci) by washing with saline after they were killed with heat and was purified by appropriate methods[38]. These extracts were subjected to treatment with trypsin, chymotrypsin and

ribonuclease and when these extracts were used to transform the R strain, transformation to the S form was observed[38]. Therefore, it can be concluded that proteins and ribonucleic acid, normally broken down by these substances, are not responsible for transformation. But when the extracts were subjected to treatment with deoxyribonucleases from various sources, the transformation activity of the R strain to the S form was obliterated[38]. Finally, it was demonstrated that DNA was responsible for the transformation principle.

Since DNA is responsible for transformation, the cell must have a method of uptake and incorporation of virulent genes in the case of pneumococci studies by Griffith and Avery et al. In the case of this particular study, a method for the incorporation of resistant genes is necessary. Exogenous DNA is taken up by the cell and once ssDNA is present, RecA is activated and forms the nucleoprotein filament with the ssDNA[19]. From here, RecA will facilitate recombinational repair and integration of the exogenous DNA into the host genome. This process was necessary for the R strain of the pneomococci to incorporate the DNA encoding for the polysaccharide capsule from the heat-killed S strain, thus making it virulent. This principle can be applied to cultures of *E. coli* inoculated with heat-killed antibiotic resistant cells. Theoretically, if *E. coli* can demonstrate natural competence as *S. pneumoniae* does (either by a similar or unique pathway), then *E. coli* cells should be able to uptake the exogenous DNA encoding antibiotic resistance genes and incorporate it into its own genome through the use of RecA mediated recombination.

Observation of Natural Competence in *Escherichia coli*

Competence, the ability of a cell to take up exogenous DNA from its environment, can be classified as artificial or natural. Artificial induction of competence is a standard laboratory technique in recombinant DNA technology and is used to incorporate desired genes into host cells[39]. It involves the use of ice-cold $CaCl_2$ or other divalent cations, which is necessary to fluidize the membrane (cold temperature) and aid DNA adsorption and binding to the cell surface by forming coordination complexes with DNA and the LPS (Ca^{2+}); a brief heat shock that causes the membrane to become rigid, allows for the release of lipids, which possibly forms pores for DNA to enter and mediates the depolarization of the membrane, therefore reducing its negative charge inside the cell allowing DNA to pass into the cell; and incubation on ice that allows the pores to close and trap the DNA[39].

Natural competence, on the other hand, is a cell's natural ability to take up exogenous DNA from the surrounding medium. Genetic transformation through the utilization of natural competence involves four steps: (1) development of competence through a stimulus; (2) binding of DNA to the cell surface; (3) processing and uptake of the DNA and (4) integration of the DNA into the chromosome by recombination and expression[40]. Problems that arise from the translocation of DNA are hydrophobic bacterial cytoplasmic membranes act as barriers for DNA, the outer membrane of gram-negative bacteria (like *E. coli*) is negatively charged due to the LPS content and hinders negative molecules like DNA from crossing the outer membrane and nucleases in the periplasmic space

may attack DNA during transfer. However, certain strains of gram-positive and gram-negative bacteria have means of uptaking DNA and protecting it while it transverses. For example, *Bacillus subtilis* forms a pilin complex with ComGC proteins (competence proteins), allowing the DNA-binding protein complex ComEA to bind DNA, which delivers the DNA to a nuclease (unidentified) for degradation of one strand of the DNA and the compliment strand is driven into the cytosol by a DNA translocase complex, ComFA, through a channel in the cytoplasmic membrane constructed from ComEC proteins[41]. *S. pneumoniae* has a very similar mechanism. An example of a gram-negative bacteria natural competence pathway is N. gonorrhoeae, which consists of a pilin complex of PilQ proteins that bind DNA and allow it to cross the cell surface[41]. Another pilin complex consisting of PilE allows the DNA to cross the periplasm with the aid of ComE, a DNA-binding protein, and a nuclease at the cytoplasmic membrane degrades one strand while allowing the other strand to enter the cell through a channel consisting of ComA proteins[41]. Figure 1.8 demonstrates this[41]. Claverys and Martin also demonstrated that there are some homologous proteins in *E. coli* to DNA uptake machinery proteins and pore assembly proteins found in other species[41], which suggests that *E. coli* may just be naturally competent, contrary to popular believe.

Other authors have demonstrated convincing evidence that *E. coli* is naturally competent. Maeda et al. showed that when colonial SURE *E. coli* cells (kan^r and tet^r) grown on various solid media (including LB, water, and $CaCl_2$ agar, as well as food stuffs) harboring kanomycin and tetracycline were introduced to a

pBluescript KS^- plasmid (amp^r) were re-grown on solid media harboring all three antibiotics (kan, tet and amp), the cells were able to grow[42]. These results indicated that *E. coli* colonies on various solid medias can develop moderate competence, as well as that this process was either Ca^{2+} independent or trace amounts of Ca^{2+} from the agar or dead cells could act as competence inducing factors. Maeda and collaborators also further demonstrated this possibility by utilizing conjugative deficient strains of *E. coli*, CAG18439 harboring tet^r and DH5a harboring the pHSG299 plasmid with kan^r, to co-culture them on LB, water or $CaCl_2$-agar lacking antibiotics then transferring colonies to LB, water or $CaCl_2$-agar containing both antibiotics[43]. Since colonies grew, it can be concluded that nonconjugative, nonviral horizontal gene transfer is possible in *E. coli*, which would indicate the need for natural competence.

Baur et al. also have contributed some convincing evidence that *E. coli* is able to develop natural competence. JM109 cells were incubated in various natural waters in which the Ca^{2+} concentrations were known and pUC18 plasmid DNA was added to the culture, which encoded for amp^r[44]. After sufficient incubation, samples were plated on LB-amp-agar plates and the transformation frequencies were determined[44]. The results showed a positive correlation between the calcium concentrations of the waters and the transformation frequency (meaning as the calcium concentration increased, so did the transformation frequency)[44]. What these results demonstrated was calcium concentrations exceeding 1 mM, concentrations associated with natural waters,

are sufficient to induce competence in *E. coli* without the addition of any other competence-inducing factor.

Lastly, Sun et al. observed natural transformation in *E. coli* without the aid of cations and temperature shifts[45]. In shaking cultures of HB101 *E. coli* cells, cultures were incubated for 12 hours and statically cultured for up to 12 hours. A plasmid harboring amp^r (pDsRED) was added and mixed into the cultures at various incubation times, which were then plated on LB-amp-agar and the transformation efficiency was determined[45]. The transformation efficiency was shown to be dependent on time in static culture, not on viability, cations or temperature shifts.

Much more research has been conducted in this area, but significant works were summarized above. This work is important because it demonstrates the possibility that Griffith's transformation principle can be applied to cultures of *E. coli*. The work presented here demonstrates that antibiotic resistant genes can be horizontally transferred between *E. coli* cells and they are capable of taking up exogenous DNA from their environments (plasmids, for example), which implies that DNA released from dead cells, especially heat-killed cells, can be transferred to *E. coli* cells in culture.

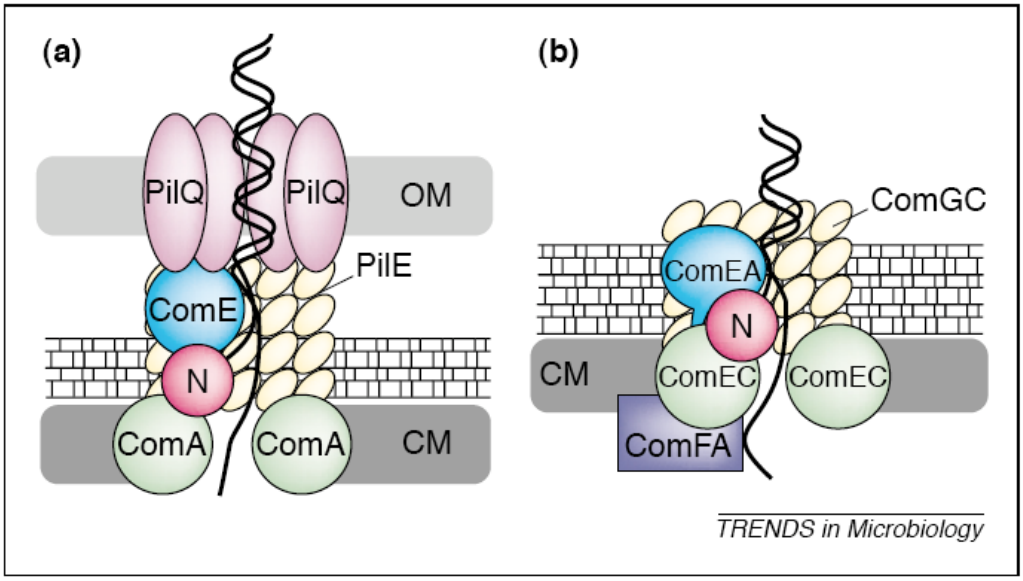

Figure 1.13. Natural competence and DNA entry into the cell of gram-positive vs. gram-negative species. a) *N. gonorrhea*, representative gram-negative bacteria. b) *B. subtilis*, representative gram-positive bacteria. This figure was reproduced from Claverys et al. (2003) TRENDS in Microbiology, 11 (4), 161-165[41].

Previous Efforts to Develop RecA Inhibitors

RecA-mediated activities, including the induction of the SOS response and homologous recombination through strand exchange, have been implicated in the spread of horizontally transferred antibiotic resistance genes and de novo antibiotic resistance development, therefore making RecA a unique target for inhibiting the spread of antibiotic resistance. However, few inhibitors of RecA had been revealed as of a few years ago. Since then, other members of the Singleton laboratory have had the opportunity to unearth inhibitors of RecA, including metal complexes, ATP analogs, peptides and small molecules.

In collaboration with the Kohn laboratory at the UNC Eshelman School of Pharnamcy, Dr. Andrew Lee had the opportunity to test Zn^{2+}, Hg^{2+}, Cu^{2+}, Ca^{2+},

Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ag^{+}, Cd^{2+} and Bi^{3+} metals for inhibition of the RecA protein. The reason for choosing metal complexes was due to the discovery of Kohn's laboratory in that the Rho protein was inhibited by Be^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+} and Bi^{3+} metal cations complexed with dithiols[46-48] and due to the fact that Rho is functionally and structurally homologous to RecA[49]. Metal complexes that he was able to show had inhibitory effects on RecA were Zn^{2+}, Hg^{2+}, Cu^{2+}, Ag^{+}, Cd^{2+} and Bi^{3+}, which in a light-scattering assay[46-48] displayed aggregation of RecA in vitro. After extensive investigation into bismuth-dithiols, Bi_3BAL in particular, it was shown that Bi_3BAL was able to irreversibly inactivate the RecA protein, but did not do so through competing with ATP or ssDNA (results submitted for publication). Thus, it is a promising lead compound for future inhibitors.

Another means of inhibiting RecA activity explored in the Singleton laboratory is the use of small peptide inhibitors that are rationally designed to interrupt the monomer-monomer interface of two RecA molecules, which would essentially disrupt the assembly of the RecA-DNA filaments[50]. Based on the N-terminal domain of RecA, the peptides INPEP and INPEP-SH (INPEP with a salt bridge) were designed to bind more tightly than another RecA monomer[50]. The IC_{50} value of ATP hydrolysis was lowered to 35 μM with INPEP and 30 μM, a 20-fold decrease as compared to the N-30 peptide modeling the N-terminus[]. By conjugating a cysteine residue on INPEP-SH with 2-thiopyridine to yield INPEP-STP, the IC_{50} was decreased to 3 μM, thus resulting in a very potent 29mer peptide inhibitor of RecA filament assembly and therefore attenuating RecA-mediated ATP hydrolysis and DNA binding [50].

Since the activity of RecA is dependent upon the formation of the nucleoprotein filament, a process that requires the hydrolysis of ATP to tightly bind ssDNA[51,53,54], seeking analogs of ATP that could potentially bind the active conformation of RecA to competitively inhibit hydrolysis or bind the inactive conformation of RecA to promote the dissociation of ssDNA[42] could hinder the recombinational capabilities of RecA. In a study done by Wigle, Lee and Singleton, it was shown that out of twenty-eight potential ligands of RecA consisting of general nucleotide triphosphates (NTPs) and nineteen synthetic analogs previously untested, six analogs were able to attenuate the DNA-dependent NTPase activity of RecA[52]. Substitutions that prevented RecA from using the analogs as substrates, and therefore attenuating NTP hydrolysis, were summarized as follows: (1) substitution of groups larger than a hydroxyl group (OMe, for example) on the C2' position of the ribose ring prevented ATP and UTP from acting as substrates; (2) adding a methyl or propynyl group on the C5 position of the pyrimidine NTPs; (3) substitution of aromatic groups larger than a benzyl ring on the N^6-amino group on the adenine ring[52]. Others were shown to be modest substrates of RecA.

Although nucleotide analogs are able to inhibit ATP hydrolysis of RecA, they were not suitable for cellular assays and therefore, other means of inhibition were sought out, which included screening of small molecules of a focused set of commercially available compounds[55] and high-throughput screening of a 35,780 compound library at the Biomanufacturing and Research Institiute and Technology Enterprise located at North Carolina Central University (unpublished

work). From the focused set of commercially available compounds, ATP hydrolysis by RecA was monitored with a fluorescent ATP assay involving the oxidation of horseradish peroxidase to resorufin as A_{595}[]. Five groups of compounds were tested: (1) vanillin, cinnamaldehyde, curcumin, genistin and genistein, which were shown to be active in other biological assays;[56-59] (2) adenosine nucleotide-like compounds[60,61]: PMPA, 5'-ASBA and methotrexate; (3) compounds that inhibit the gyrase-Hsp-90-like family of ATPases[62,63]: radicicol, novobiocin and coumermycin; (4) adenine-like inhibitors known to inhibit protein kinases[64,65]: PP2 and PP3; (5) inhibitors of purine nucleotide receptors that are non-nucleotides[66]: PPADS, Bis-ANS, suramin, Congo Red and ANS. The only compounds from this study that were able to inhibit RecA ATPase activity were three suramin-like agents from group 5: Congo Red, suramin and Bis-ANS[55]. Suramin was also shown to inhibit DNA three strand exchange at 100 μM[55].

Lastly, the results of they high-throughput performed at the BRITE center utilized a PMB ATPase fluorescence assay to measure the hydrolysis of ATP by RecA by measuring the interaction of inorganic phosphate with molybdate to form a phosphomolybdate blue complex, which can be measured at A_{650} (unpublished data). Of the 35,780 compounds screened, seventy-three were reported as hits and were classified into five compound classes. From the first compound class, one compound, termed A1, has proven to be a potent inhibitor of RecA in vitro (IC_{50} of 8 ± 1 μM), attenuated SOS induction in a GFP-

fluorescence cell based assay in conjunction with ciprofloxacin and inhibited the RecA-mediated DNA three strand reaction (see Wigle dissertation for results).

Specific Aims of This Thesis

As demonstrated by Dr. Tim Wigle, the compound A1 has proven to be a favorable potential inhibitor of RecA, and therefore a means of attenuating the transfer of antibiotic resistant genes. Therefore, the specific aims of this thesis are as follows: (1) demonstrate that the addition of A1 to cultures grown in sub-lethal dosages of chloramphenicol prevents or slows the development of resistance of *Escherichia coli* MG1655 wild type cells; (2) apply the Griffith experiment to *Escherichia coli* MG1655 cells to observe the transfer of antibiotic resistant genes from heat-killed resistant cells to wild type cells; (3) demonstrate that inhibitor compound A1 attenuates the process of transferring antibiotic resistant genes in LB medium from heat-killed resistant cells to wild type *Escherichia coli* MG1655 cells; (4) utilize molecular modeling methods of screening to find other potential RecA inhibitors based on inhibitory concentration data obtained from various high throughput screens.

A1 was shown to have a half maximal inhibitory concentration of 8 ±1 μM against the RecA protein in *in vitro* experiments. Therefore, utilizing a one-flask resistance assay, monitoring the cultures of MG1655 cells should reveal if A1 is able to penetrate into the cells and effectively inhibit RecA. This should theoretically attenuate the onset of resistance to antibiotics. However, if such resistance mechanisms of efflux and reduced membrane permeability are utilized, as often seen with chloramphenicol, then inhibiting RecA may have no effect on

the resistance development to chloramphenicol, since mutations are not always needed and the SOS response is not normally induced because chloramphenicol functions by inhibiting protein synthesis. RecA knockout cells will serve as a negative control, which should not develop resistance if RecA is necessary for the onset of resistance and may develop resistance if RecA is not necessary.

According to the Griffith experiment, DNA can be horizontally transferred from heat killed cells of a virulent strain of *Streptococcus pneumoniae* to an avirulent strain of *S. pneumoniae*, which results in a virulent strain that is able to kill mice. Recent studies have shown *E. coli* to be naturally competent at a much lower rate than other species of bacteria, such as *S. pneumoniae*. Wild type *E. coli* MG1655 cells, if able to develop competence in liquid medium, would be able to uptake and incorporate antibiotic resistant genes when inoculated with the supernatant of heat killed resistant cells, therefore resulting in resistant cultures more quickly than cells that are grown without the supernatant of heat-killed resistant cells in the presence of a sub-lethal dosage of chloramphenicol. RecA knockout cultures would not produce the onset of resistance or a difference in the rates of resistance development would not be observed.

RecA would be required to incorporate the resistant genes into the genome of MG1655 cells. Since A1 has inhibitory effects on RecA, adding it to cultures with heat-killed chloramphenicol resistant cells would attenuate the recombination of the resistant genes into the genome by inhibiting RecA. Resistance development to a sub-lethal dosage of chloramphenicol would be

deterred if A1 does serve as a RecA inhibitor. Again, *DrecA* cells will serve as a negative control.

Since A1 has been shown to be a promising candidate for inhibiting RecA and therefore attenuating the spread of antibiotic resistance, finding other compounds that can serve as inhibitors of RecA is fruitful. Also, utilizing a method that is cost and time effective is favorable. Molecular modeling seemed to be a practical approach to finding potential compounds by minimizing time spent in the laboratory and the cost of experiments. By utilizing a program entitles Molecular Operating Environment (MOE), common scaffolds can be abstracted from molecules shown to have inhibitory effects against the RecA protein. The molecules chosen to screen were seventy-three hit compounds obtained from the high-throughput screen performed by Dr. Tim Wigle at the BRITE center where A1 was discovered. These scaffolds can be input into a search in SciFinder Scholar to obtain similar compounds. Hypothetically, similar compounds will most likely have similar chemical and biological properties. Based on this assumption, new potential inhibitor compounds can be obtained and tested for inhibitory effects on RecA.

CHAPTER II

MATERIALS AND METHODS

One-flask resistance assay reagents

LB broth, purchased from Fisher Scientific, was prepared by adding 20 g of LB per liter of MilliQ de-ionized water. LB was distributed into 250 mL Erlenmeyer flasks, either 30 mL for starter cultures or 50 mL for assay cultures, and autoclaved for 50 min. LB-agar plates were prepared by adding 20 g of LB and 15 g of agar (Fisher Scientific) per liter of MilliQ de-ionized water, autoclaving for 20-30 min and pouring into culture plates purchased from Fisher Scientific.

Chloramphenicol was purchased from Sigma-Aldrich (St. Lois, MO) and was prepared in 34 ng/mL 1 mL stocks in absolute EtOH. Starting materials for A1 were purchased from Sigma-Aldrich and A1 was synthesized by Dr. Anna Gromova or myself according to protocol. Chloramphenicol E-tests strips (0.016 – 250 μg/mL) were purchased from AB-Biodisk (Piscataway, NJ). Dr. Jim Collins (Boston University, Boston, MA) graciously donated MG1655 wild type and Δ*recA* cells.

Growing Chloramphenicol resistant cells

Chloramphenicol resistant cells (Cam^r) were made by growing a 5 mL overnight MG1655 wt culture in LB at 37°C, inoculating 30 mL of LB with 2 mL of this culture and growing this culture to log phase in the 37°C shaker. Fifty mL of LB were then inoculated with the MG1655 wt starter culture to an OD_{600} of 0.05. This culture was then grown in the presence of 5 μg/mL Cam until cells reached saturation (OD_{600} close to 2). These cells were streaked and an E-test (AB-Biodisk, Piscataway, NJ) was performed to ensure resistance developed (MIC greater than 32 μg/mL). Cells were stored in 1 mL quantities in the -80°C freezer. For each assay, one or two 5 mL Cam^r cultures were grown at 37°C overnight from the freezer stock by stabbing the frozen stock and ejecting the tip into 5 mL of LB. To obtain heat killed supernatant, the overnight cultures were autoclaved for 15-30 min and were cooled at room temperature until needed.

One-flask resistance assay

Culture plates were prepared by T-streaking cells from a freezer stock and growing them overnight at 37°C. Overnight cultures were grown by picking individual colonies on the culture plate, harvesting them with a pipette tip and ejecting them into 5 mL of LB broth in glass culture tubes (Fisher Scientific), then placing them in the 37°C shaker. Two mL of the overnight cultures were added to 30 mL of LB and were grown to log phase (OD_{600} = 0.5-0.8) in the 37°C shaker. OD measurements were taken in a Perkin Elmer Spectrophotometer in 1 mL disposable cuvettes with a 1 cm path length and 600 nm wavelength.

While the starter cultures were growing, twelve flasks of 50 mL of LB were prepared per cell line (wt and Δ*recA*) with the following conditions: (1) Cam only with 0, 5 and 10 μg/mL concentrations of Cam; (2) Cam and A1 with 0, 5 and 10 μg/mL concentrations of Cam and 50 μM of A1 from a 50 mM stock in DMSO; (3) Cam and Cam^r with 0, 5 and 10 μg/mL concentrations of Cam and 1 mL of Cam^r heat killed cell supernatant; and (4) Cam with Cam^r and A1 containing 0, 5 and 10 μg/mL concentrations of Cam, 1 mL of Cam^r heat killed cell supernatant and 50 μM of A1 from a 50 mM stock in DMSO. When the starter cultures reached log phase, the assay cultures were inoculated with the starter cultures in each flask to an OD_{600} of 0.05 (about 3-5 mL of starter culture per flask). The assay cultures were grown in 37°C shaker and the OD_{600} was measured periodically for 120 hrs by taking 1 mL of culture out of the flasks utilizing sterile technique and taking measurements in the UV-Vis spectrophotometer.

At OD_{600} of 0.6, 1 and 2, E-tests were performed on LB/agar plates. This is done by diluting the cells in 1 mL Epindorf tubes in LB to a final volume of 1 mL to bring the concentration of the cells to an OD_{600} of 0.5. Cells are streaked with a sterile cotton swab in one direction across the plate, then are streaked in a 90° rotation across the plate. The E-tests are laid down in the center of the plate with flame-sterilized forceps and are read after 24 hrs of incubation at 37°C.

Molecular modeling screen

Figure 2.1 demonstrates the general workflow for this project. Firstly, the original library, consisting of the 35,780 compounds from the screen done at the BRITE center, was narrowed down to seventy-three hit compounds using the PMB assay (described in detail above) termed the selected molecules. These molecules were grouped into a structure-data file (SDF), which is required for input into modeling programs. Utilizing the Molecular Operating Environment (MOE) program, the common scaffolds were extracted. There were 48 unique compounds from the 73 hit compounds (eliminating duplicates and isomers), which contained 7 scaffolds. Two of these scaffolds were deemed to be unique. Three scaffolds were used to search the literature, one unique structure and two structures encompassing the other unique structure, which differed in one aromatic ring substituent and the presence of an oxygen group on another aromatic substituent. These three scaffolds were searched in SciFinder Scholar under the substructure search feature with the conditions of organics and all others, single components and one or more references available in CAPLUS or MEDLIN. This search yielded 356 compounds (297 for the first scaffold, 33 for the second scaffold and 26 for the third, different scaffold).

To represent all of the compounds searched, 92 of these compounds were randomly selected from the 356 compounds pulled from the literature. These representative compounds were converted into simplified molecular input line entry specification (SMILES) format, which describes chemical structures in short American Standard Code for Information Interchange (ASCII) strings, in CS

ChemDraw Ultra (Cambridge Software, Cambridge, MA) from the chemical name of the compounds. These SMILES strings were compiled into a text document, along with their registry numbers for easy identification, and were converted to a new SDF consisting of two sets of compounds: the experimental set (48 unique compounds) and the literature set (92 representative compounds). This new SDF was used to explore structure and activity space in MOE, breaking down each compound into its individual ligand fingerprint of bits/chemical fragments utilizing Tanimoto Similarity Coefficients. The Tanimoto similarity score is calculated with the following formula (bits are set to one when the chemical fragment is present in the molecules and are assigned zero when the chemical fragment is not present):

$$T = \frac{N_c}{N_a + N_b - N_c}$$

where T is the Tanimoto score, N_a is the number of bits set to one in the fingerprint of ligand a, N_b is the number of bits set to one in the fingerprint of ligand b and N_c is total number of bits set to one found in the fingerprints of both ligand a and b. A Tanimoto score is designated a value between zero and one with zero indicating no similarity and one indicating 100% similarity. A Tanimoto score $\geq$ 0.7 is indicative of two molecules having high structural similarity, which is indicative of similar biological activity. When clustered into a heat map with the compounds aligned along the Y-axis and chemical fragments along the X-axis, compounds that are similar will be clustered around specific chemical fragments Black spots indicate a Tanimoto score close to one and red indicates a

Tanimoto score close to zero. The right hand column will give the names of the input structures. Seeking areas with many black spots will indicate compounds that are similar in structure.

Lastly, using AutoQSAR in MOE can predict activities based on similarity structures. Using the initial IC_{50} values of the hit compounds, the IC_{50} values of the literature compounds can be predicted based on the Tanimoto score. In order to perform clustering, the activities were assumed to be 0; in AutoQSAR, the program predicts the most probably IC_{50} (activity) value.

This process can be repeated as hits are tested in the laboratory or as more data is compiled from other screens. The process can continually narrow down the selection as the procedure is repeated.

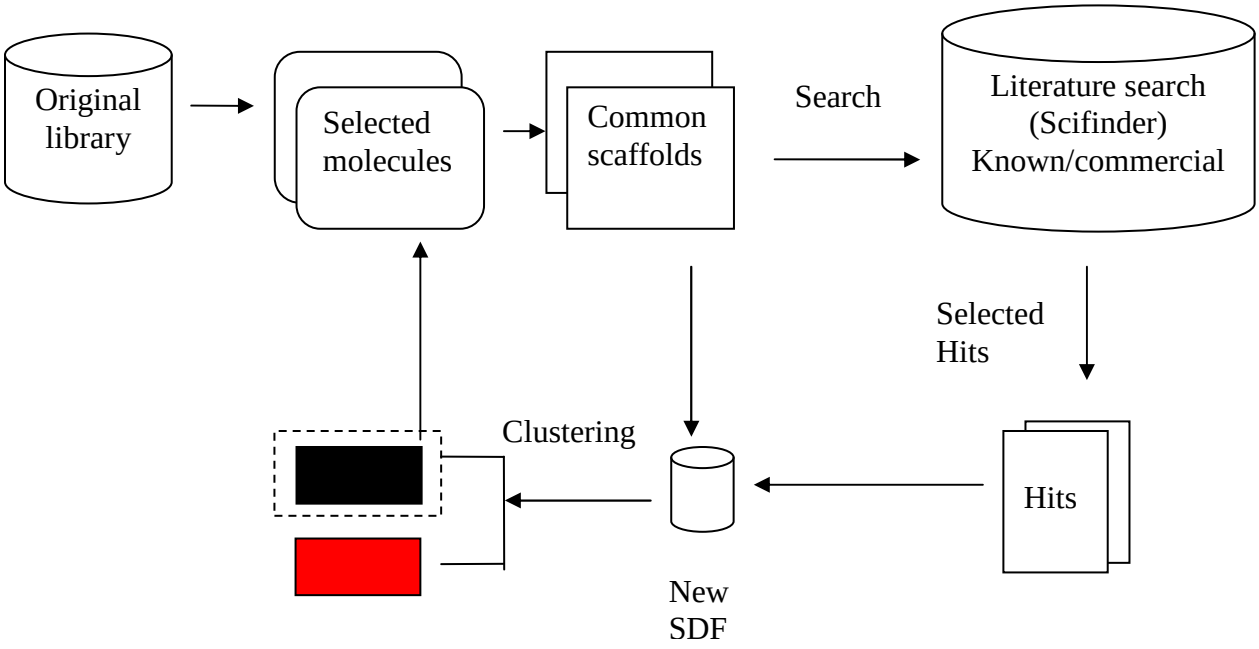

Figure 2.1. General workflow.
From the original library of 35,780, 73 hit molecules were selected. Seven common scaffolds were abstracted and three scaffolds (one unique and two similar) were used to search the literature compounds for known or commercially available compounds. Ninety-two compounds were randomly selected as hits and were used in conjunction with the experimental set (48 unique structures from 73 hits) to cluster the compounds in activity space.

PMB ATPase assay, hit validation (work done by Dr. Anna Gromova)

The same reagents are used in this assay as the PMB ATPase assay screen (see above). Each of the 7 compounds purchased were suspended in DMSO to a final concentration of 10 mM. In a 96-well round bottom plate (for dilutions only), each compound had a designated column, starting with concentrations of 2 mM and 1.5 mM in DMSO and volume of 200 μL, and were serial diluted in DMSO to obtain 2 mM, 1.5 mM, 1 mM, 0.75 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0.0625 mM. In a separate 96-well flat bottom microtitter plate (Evergreen

Scientific, Los Angeles, CA), 5 μL of each dilution was added from the dilution plate using a multichannel pipetter to give final concentrations of 100 μM, 75 μM, 50 μM, 37.5 μM, 25 μM, 12.5 μM, 6.25 μM and 3.125 μM of each inhibitor (columns 3-9). To columns 1 and 2, 5 μL of DMSO was added to each well to serve as positive and negative controls. Using a multichannel pipetter, to columns 2-9 of the assay plate, 85 μL of a cocktail solution was added, which consisted of RecA, poly(dT) ssDNA, $MgOAc_2$, and Tris-glycerol buffer (pH of 7.5) with final concentrations in each well corresponding to 0.5 μM RecA, 5 uM nts poly(dT), 10 mM $MgOAc_2$, 25 mM Tris·HOAc and 5 % v/v glycerol. Column 1, designated as the negative control column, contained 85 μL of an identical solution of this cocktail without the addition of poly(dT) and was added with a multichannel pipetter.

Once all of the reagents were added to the plate, the plate was shaken at 800 rmp for 30 sec and then placed in a 37°C incubator for 20 min. To each well of the assay plates, 10 μL of 7.5 mM stock of ATP (prepared in H_2O) was added to give a final concentration of 0.375 mM of ATP. The plate was shaken at 800 rpm for 30 sec and incubated at 37°C for 35 min, thus allowing the ATPase reaction to proceed. After this incubation time, 100 μL of the 1X PMB dye was added to all of the wells with a multichannel pipetter and the plate was shaken at 600 rpm and incubated at room temperature for 5 min. Using a POLARstar OPTIMA plate reader (BMG Labtech, Durham, NC), the plates were scanned for absorbance at 650 nm. Subsequent screening data informatics were processed using KaleidaGraph (Synergy Software, Reading, PA) and Microsoft Excel 2003

(Microsoft, Redmond, WA). For analysis, the percent inhibition of RecA ATPase activity was determined. This was done on a plate-to-plate basis, which compared the A_{650} value per compound well with the plate-averaged control wells, as per the following relationship:

$$\%\,inhibition = \left(1 - \left(\frac{A_{650} - \mu_{\min}}{\mu_{\max} - \mu_{\min}}\right)\right) \times 100$$

where A_{650} was the absorbance value at 650 nm in the presence of an inhibitor, μ_{min} was the value of the plate average minimum A_{650} signal control (i.e. average negative control) and μ_{max} was the value of the plate average maximum A_{650} signal control (i.e. average positive control).

CHAPTER III

THE DEVELOPMENT OF CHLORAMPHENICOL RESISTANT MAY BE ATTENUATED BY THE POTENTIAL INHIBITOR COMPOUND A1

Chloramphenicol is a broad-range bacteriostatic chemical agent used in the laboratory and in clinical practice to treat various gram-positive and gram-negative bacterial infections. It is able slow bacterial growth by inhibiting peptidyl transferase by binding to the 50S ribosomal RNA and therefore prevents peptide bond formation, which in return slows protein synthesis[11]. This drug was introduced in the late 1940's and resistance to this drug was first reported a mere ten years later[5]. Cells develop resistance to chloramphenicol in various ways, including an active efflux pump, reduced membrane permeability, alteration of the 50S ribosome through mutations in the 23S rRNA (G2057A), and full-fledged resistance that allows the cell to synthesize chloramphenicol acetyl transferase that renders the chloramphenicol ineffective by altering its structure to 1,3-diacetoxychloramphenicol, which is unable to bind the hydrophobic pocket of the 50S ribosome[7,9-18].

RecA may or may not be involved in the resistance development of bacterial cells to chloramphenicol. This is due to the fact that chloramphenicol is bacteriostatic as opposed to bactericidal[36]. With bactericidal agents, DNA damage is typical, which would inevitably lead to DNA repair and possibly the

induction of the SOS response[26,27,36]. RecA would of course be activated in this process. But with bacteriostatic agents that are not present in excessive concentrations (which would act as a bactericidal agent), SOS is not induced[36]. Therefore, the question arises if RecA would be turned on or not. And if chloramphenicol works by inhibiting protein synthesis, would RecA necessarily be synthesized in excess? Theoretically, if RecA is involved and is synthesized, then an inhibitor compound designed to hinder RecA's ability within the cell would impede or delay the resistance development of cells to chloramphenicol. If RecA is not involved or is not able to be synthesized, then resistance development to chloramphenicol would proceed as expected. Also, if RecA is knocked out, then an inhibitor compound designed to inhibit RecA would have no effect on the resistance development assuming RecA is involved in the resistance development process; therefore cells grown in its presence or not would be expected to not grow or develop resistance. However, if RecA is not implicated in this process, then resistance would develop normally, whether the RecA inhibitor compound is present or not.

Small molecules are attractive candidates for inhibitor compounds against the RecA protein, due to their ability to easily penetrate the cell. One such compound was discovered by Dr. Tim Wigle of the Singleton laboratory, in collaboration with Director Li-An Yeh and Professor Jonathon Sexton, while screening a chemical library of 35,780 small molecules for RecA inhibitors at the Biomanufacturing and Research Institute and Technology Enterprise located at North Carolina Central University (work to be published in the Journal of

Biomolecular Screening). From this screen came seventy-three hit compounds, which yielded five promising chemotype clades. One of these chemotype clades, designated A1, proved to be the most promising candidate with an IC_{50} value of 8 ± 1 μM while tested *in vitro* with RecA. It also demonstrated inhibition of strand exchange and SOS induction in other studies (work done by Dr. Tim Wigle). Bacterial culture studies would confirm the ability of A1 to hinder resistance development if cells, assuming RecA is involved in the process.

Bacterial Culture Studies of the Effects of Chloramphenicol on Wild Type *E. coli* MG1655 cells

Reported resistance to chloramphenicol in hospitals usually refers to the production of chloramphenicol acetyl transferase, which is utilized by cells to alter chloramphenicol in the cell, rendering it ineffective. This reaction occurs in two steps: (1) chloramphenicol is converted to 3-acetoxychloramphenicol by chloramphenicol acetyl transferase acetylating the hydroxyl group on the third position on the side chain of chloramphenicol; (2) 3-acetoxychloramphenicol is converted to 1,3-diacetoxychloramphenicol by a molecular rearrangement of the acetyl group from the third position to the first hydroxyl position, then chloramphenicol acetyl transferase acetylates the hydroxyl group on the third position again on the side chain of chloramphenicol. The acetyl groups are donated from acetyl coenzyme A, which is converted to coenzyme A as a byproduct.

However, in laboratory experiments, this type of resistance is most likely not observed because this is an advanced stage of resistance. In bacterial culture

experiments observing the first 100 hours of cell growth, one is more likely to observe initial stages of resistance, including a combination of active efflux pumps and reduced membrane permeability. There is a possibility that RecA would not be involved in the process of low level resistance to chloramphenicol, since DNA damage is not occurring and protein synthesis is slowed or attenuated (meaning the production of excess RecA would be attenuated).

To observe how chloramphenicol would affect wild type MG1655 cells in the initial stages of resistance development, a one-flask resistance assay was utilized. Briefly, this assay observed the growth of 50 mL cultures of MG1655 wt. cells in the presence of 0, 5 and 10 μg/mL of chloramphenicol for 100 hours by periodically monitoring the optical density of the cultures at a wavelength of 600 nm. When no chloramphenicol was added, cultures grew normally to saturation, or OD_{600} of 2, within 10 hours (Figure 3.1A, bottom panel, gray dotted line with open triangles) and had an MIC of about 8 μg/mL of chloramphenicol. When 10 μg/mL of chloramphenicol was added, cultures were unable to grow past an OD_{600} of 0.4 (Figure 3.1A, bottom panel, gray dotted line with open inverted triangles), thus indicating that cells were unable to develop resistance to chloramphenicol at this concentration. Lastly, resistance to chloramphenicol was observed in cultures grown in the presence of 5 μg/mL at about 40-60 hours, as shown by the increase of OD_{600} over time (Figure 3.1A, bottom panel, gray dotted line with closed circles) and the increase of the minimum inhibitory concentration (MIC) from approximately 8 μg/mL of chloramphenicol to 32-48 μg/mL (Figure 3.1A, top panel, gray dotted line with solid circles), as determined by E-tests.

However, whether or not RecA is involved in the resistance process is not clear from this data.

These results were shown to be reproducible (Figures 3.1B-D). The MICs for all experiments begin around 6-12 μg/mL and reach an MIC of 24-96 μg/mL. However, for the culture with 5 μg/mL of chloramphenicol in the third experiment (Figure 3.1C), possible resistance development to chloramphenicol was not observed until over 100 hrs when the OD_{600} approached 0.5, but did not reach 0.6 or above. Because the culture did not grow above OD_{600} of 0.6, the MIC was not determined.

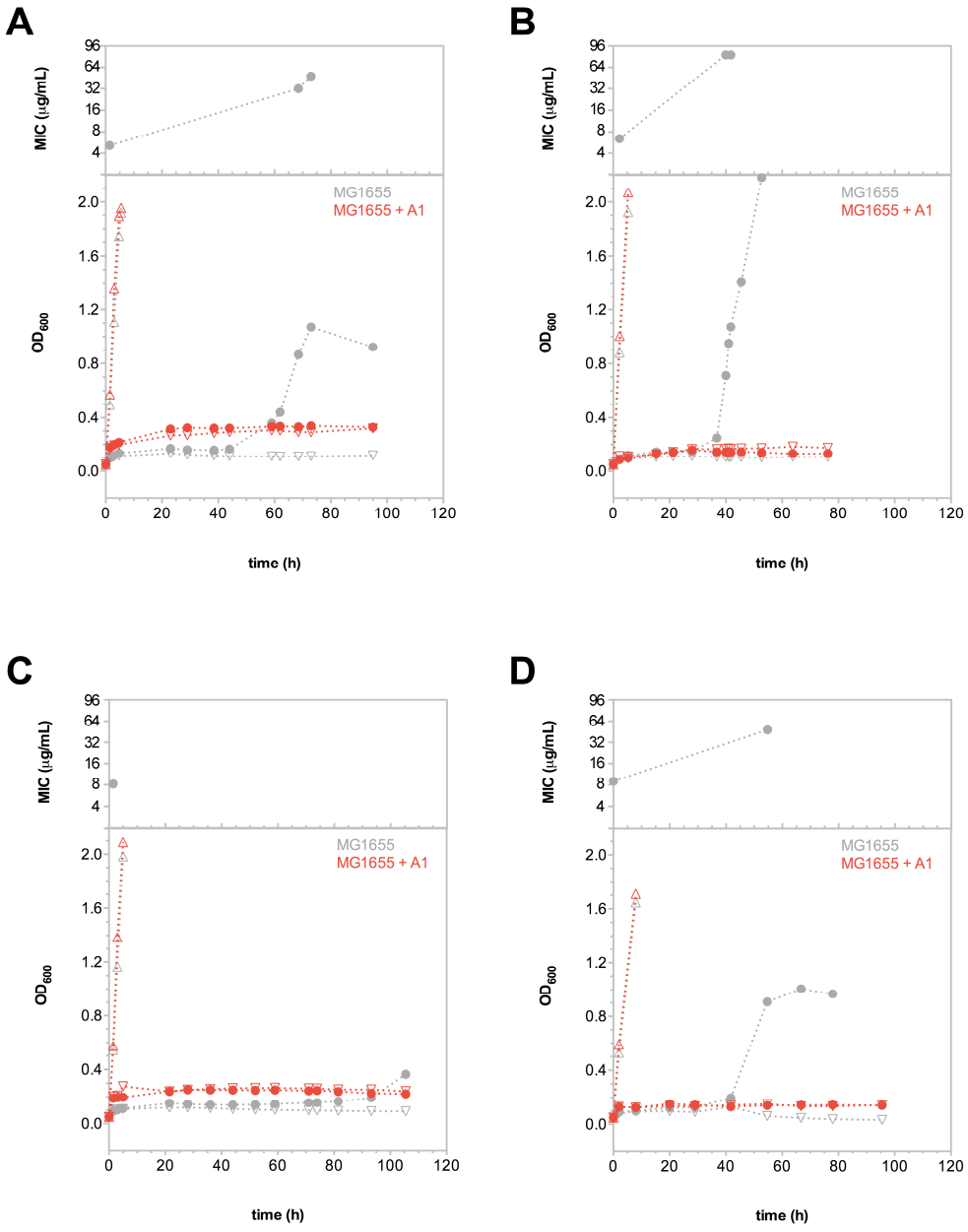

Figure 3.1 Resistance Development of MG1655 wild type cells in the presence of Cam and A1.

A-D show four separate experiments. Bottom panels show the OD_{600} vs time: gray dotted line with open triangles, 0 μg/mL Cam only; gray dotted line with closed circles, 5 μg/mL Cam only; gray dotted line with open inverted triangles, 10 μg/mL Cam only; red dotted line with open triangles, 0 μg/mL Cam and 50 μM A1; red dotted line with closed circles, 5 μg/mL Cam and 50 μM A1; red dotted line with open inverted triangles, 10 μg/mL Cam and 50 μM A1. Top panel shows the MIC vs time: dotted gray line with closed circles, 5 μg/mL Cam only.

Bacterial Culture Studies of the Effects of Chloramphenicol on Δ*recA E. coli* MG1655 cells

As stated in the previous section, low level resistance development to chloramphenicol would include reduced membrane permeability and an active efflux pump to hinder chloramphenicol from entering the cell and if it gets in the cell, then the efflux pump would eject it promptly from the cell. However, this kind of resistance development would most likely be RecA independent due to lack of DNA damage and SOS induction. Therefore, a cell line that is incapable of expressing *recA* would be able to demonstrate the involvement or lack of involvement of RecA If RecA is implicated in the resistance development of MG1655 *E. coli* cells, than resistance to chloramphenicol in a cell culture with a cell line deficient in *recA* would not develop resistance over time However, if the pathway of chloramphenicol resistance development is RecA independent, than resistant development in cultures of MG1655 Δ*recA E. coli* cells would ensue as observed in the previous section with wild type cells.

To observe how chloramphenicol would affect MG1655 Δ*recA* cells in the initial stages of resistance development, a one-flask resistance assay was again utilized. Briefly, this assay observed the growth of 50 mL cultures of MG1655 Δ*recA* cells in the presence of 0, 5 and 10 μg/mL of chloramphenicol for 100 hours by periodically monitoring the optical density of the cultures at a wavelength of 600 nm. Upon observation of the MG1655 Δ*recA* culture over approximately 100 hours of incubation in the absence of chloramphenicol, cells grew to saturation between 10-15 hrs (Figure 3.2A, bottom panel, blue dotted line,

open triangles). Using an E-test to determine the MIC of this culture, cells were shown to have an MIC of about 8 μg/mL of chloramphenicol. When in the presence of 10 μg/mL, cells did not grow above an optical density of 0.3 as seen in Figure 3.2A with the blue dotted line and open inverted triangles in the bottom panel. Interestingly, a culture grown in the presence of 5 μg/mL did grow after 45 hours, reaching an OD_{600} of 0.6 between 55-60 hours (Figure 3.2A, bottom panel, blue dotted line with closed squares). The E-test confirmed resistance development to chloramphenicol, reaching an MIC of about 48 μg/mL.

After reproducing this assay (Figures 3.2B-C), it is apparent that the MICs for all experiments were between 6-8 μg/mL of chloramphenicol when no chloramphenicol was added to the cultures and between 24-48 μg/mL in the presence of 5 μg/mL of chloramphenicol in the cultures. Times when growth was observed was also consistent between experiments, 10-15 hours without chloramphenicol and 40-50 hours when 5 μg/mL was present. These results suggest RecA is not involved in the resistance development to chloramphenicol, due to the fact that the results of this assay involving Δ*recA* cells are consistent with the results with wild type cells. Regardless of whether RecA is present or not seems to have no bearing on the development of resistance to chloramphenicol when 5 μg/mL is added to the cultures. This is most likely due to the nature of chloramphenicol resistance development, which does not rely on RecA to induce the SOS response. Rather, the process involves the expression of efflux pumps and reduced expression of membrane porins. Therefore, cells devoid of RecA would inherently be able to develop low-level resistance to chloramphenicol.

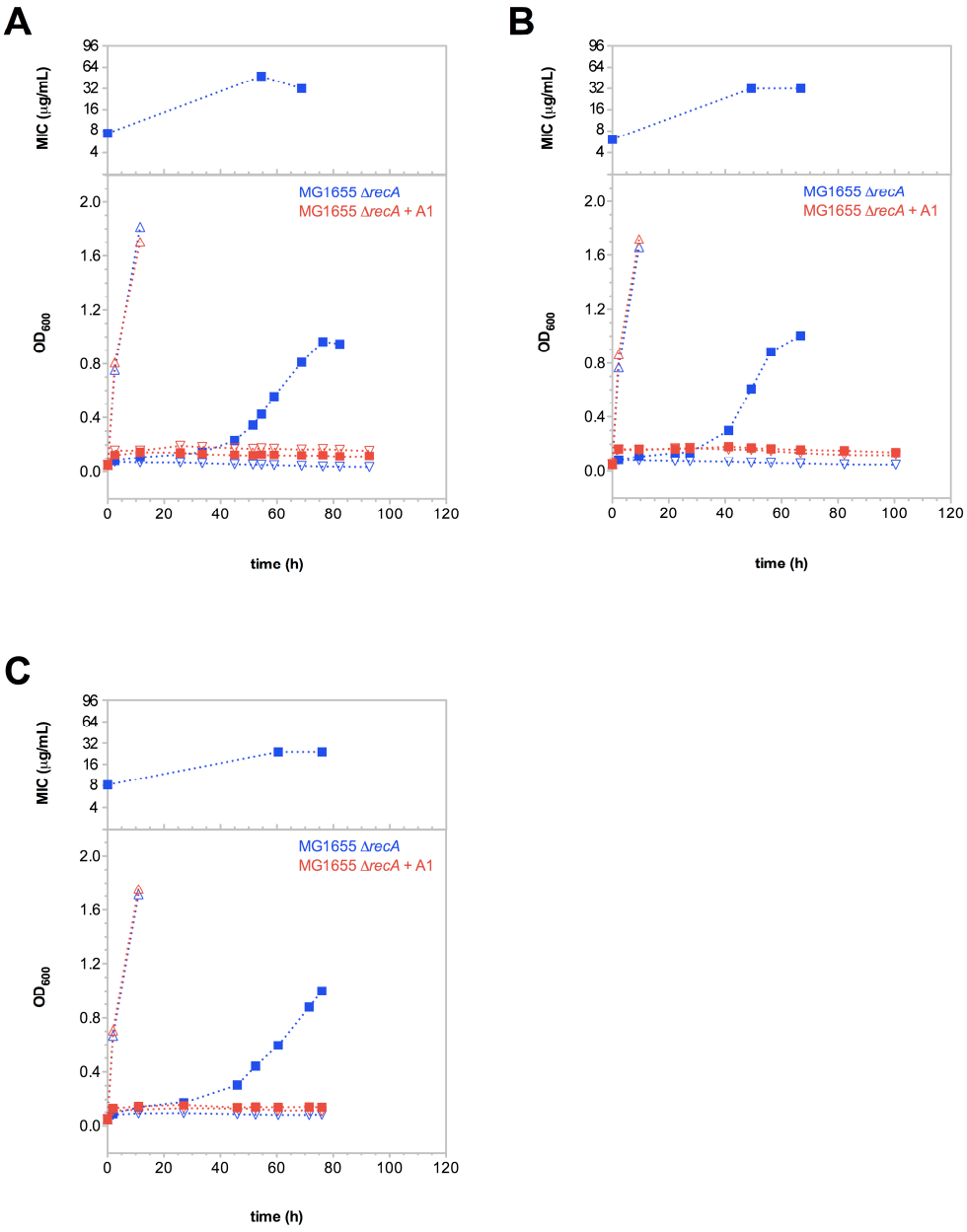

Figure 3.2 Resistance Development of MG1655 wild type cells in the presence of Cam and A1.

A-C show three separate experiments. Bottom panels show the OD_{600} vs time: blue dotted line with open triangles, 0 μg/mL Cam only; blue dotted line with closed squares, 5 μg/mL Cam only; blue dotted line with open inverted triangles, 10 μg/mL Cam only; red dotted line with open triangles, 0 μg/mL Cam and 50 μM A1; red dotted line with closed circles, 5 μg/mL Cam and 50 μM A1; red dotted line with open inverted triangles, 10 μg/mL Cam and 50 μM A1. Top panel shows the MIC vs time: dotted blue line with closed squares, 5 μg/mL Cam only.

Bacterial Culture Studies of the Effects of A1 on Wild Type *E. coli* MG1655 cells

One of the main goals of this laboratory is to find compounds that inhibit the RecA protein in hopes that DNA repair mechanisms and SOS induction can be avoided in order to eliminate bacterial infections, either as a stand-alone agent or in conjunction with other current antibiotics, to prevent the spreading and incorporation of antibiotic resistant genes. During a HTS performed by Dr. Tim Wigle of the Singleton laboratory, 73 such potential compounds were discovered, including the compound A1.

The compound designated A1 for investigational purposes was shown to have a low IC_{50} value when screened in vitro in RecA ATPase assays, thus indicating it may have inhibitory effects against RecA. It also demonstrated the ability to inhibit strand exchange between cssDNA and dsDNA in vitro and SOS induction within cells in conjunction with ciprofloxacin, a bactericidal agent (see Dr. Tim Wigle dissertation for results). Therefore, the question of whether or not this compound could demonstrate effectiveness in delaying or preventing the resistance development of bacterial cells to antibiotics arose. Chloramphenicol was chosen to start because RecA is typically not implicated in the resistance development of cells to chloramphenicol, seeing as chloramphenicol is a bacteriostatic agent and does not induce the SOS response. Therefore, adding an inhibitor of RecA should have no effect on the resistance development to chloramphenicol.

Again, utilizing the one-flask resistance assay, 50 μM of A1 was added to the cultures of MG1655 wild type *E. coli* cells with 0, 5 and 10 μg/mL of chloramphenicol. As expected, cells grown without chloramphenicol and in the presence of only 50 μM of A1 grew to saturation within ten hours, as would cultures without A1 (Figure 3.1A, bottom panel, red dotted line with open triangles). This is because A1 is not capable of killing cell cultures on its own; it is not a bactericidal agent. Cells grown in the presence of 50 μM of A1 and a lethal dosage of chloramphenicol, 10 μg/mL, are unable to grow above an OD_{600} of 0.4 (Figure 3.1A, bottom panel, red dotted line with open interved triangles). This is an indication that the cells were not able to develop resistance to chloramphenicol. Lastly, when a sub-lethal dosage of 5 μg/mL of chloramphenicol was added to cultures in conjunction with 50 μM of A1, cells did not grow above an OD_{600} of 0.4, as opposed to the observation as was seen in cultures when A1 was not present (Figure 3.1A, bottom panel, dotted red line with closed circles). Cells do not always reach saturation of $OD_{600} = 2$ or even reach an $OD_{600} = 0.6$, therefore MIC values were not determined. As seen in Figures 3.1B-D, these results are reproducible. These results indicate that A1 is able to deter the cell's ability to develop resistance to chloramphenicol. However, if this truly is the case, then A1 is not necessarily an inhibitor of RecA as previously believed. If it is truly deterring the resistance development to chloramphenicol, then it is able to hinder resistance development through a mechanism possibly independent of RecA, since the presence of chloramphenicol does not usually induce SOS.

Bacterial Culture Studies of the Effects of A1 on Δ*recA E. coli* MG1655 cells

Technically speaking, if RecA is necessary for the development of resistance of MG1655 *E. coli* cells to chloramphenicol, then having cells with RecA knocked out would not develop resistance in the presence of a sub-lethal dosage of antibiotic. However, as seen in Figures 3.2A-C, it was observed that resistance had indeed developed in cultures with the concentration of chloramphenicol of 5 μg/mL, thus indicating that RecA is not implicated in the development of resistance to chloramphenicol. Therefore, the addition of a RecA inhibitor should have no effect on the development of resistance if RecA is not implicated in the process because the process either does not require the presence of RecA and resistance will develop. If resistance is not observed, then the compound being added is not necessarily an inhibitor of RecA because it still is able to deter resistance development when RecA, its supposed target, is absent. Therefore, to really test the compound A1 as an inhibitor of RecA, it was used in the one-flask resistance assay.

As expected, when no chloramphenicol is added to cultures, cells grow to saturation within 10-15 hours (Figure 3.2A, bottom panel, red dashed line with open triangels) and are not resistance to chloramphenicol, as indicated by the low MIC value of 6-8 μg/mL. A concentration of 10 μg/mL of chloramphenicol is capable of stopping cultures from growing above and OD_{600} of 0.3 and therefore not allowing for resistance development (figure 3.2A, red dashed lines with open inverted triangles). Surprisingly, the addition of A1 to the cultures at a concentration of 50 mM and in the presence of 5 μg/mL of chloramphenicol

attenuates resistance development of MG1655 Δ*recA* cells to chloramphenicol (Figure 3.2A, bottom panel, dashed red lines with closed squares). Therefore, A1 is able to prevent resistance, but has no RecA effect. It is likely that RecA is not needed for resistance development to chloramphenicol and A1 may inhibit resistance through a RecA independent pathway.

These same results were seen in concurring experiments (Figures 3.2B-C). All experiments showed cultures growing to saturation in 10-15 hours with MIC values in the 6-8 μg/mL range when no chloramphenicol was added and did not grow in the presence of 10 μg/mL of chloramphenicol. Cultures with 5 μg/mL of chloramphenicol added were also consistent in that they all did not develop resistance in the presence of A1.

Conclusions

With antibiotic resistance development on the rise, finding novel means of combating bacteria to not only kill them, but to hinder their resistance developing mechanisms is in great demand. Making bacteria devoid of a mode of resistance development would reduce the spread of resistant genes by abrogating horizontally transferred resistant genes and mutations arising from the SOS response. Since recombinase protein A is implicated in the spreading of resistance genes through horizontal gene transfer and homologous recombination or through induction of the SOS mutagenesis pathway, it is a very promising target. Thus, inhibiting RecA in the cells would aid in the reduction of resistance development to antibiotics.

In this study, when introducing the antibiotic chloramphenicol to cultures of MG1655 *E. coli* wild type and Δ*recA* cells, cultures with a sub-lethal dosage of chloramphenicol (5 μg/mL) did develop resistance over time to the antibiotic. This would indicate that low level resistance development to chloramphenicol involving active efflux pumps and reduced membrane permeability would not include the RecA protein, as demonstrated by the one-flask resistance assay utilized in this study. Taking this one step further, the resistance observed was most likely not due to homologous recombination of horizontally transferred genes or SOS induction, since resistance was still observed in RecA knockout cells. Mutations necessary to slow or cease the production of porins on the cell surface (through which chloramphenicol would enter the cell) and activate efflux pumps would have been created through a RecA independent pathway.

The results of this type of resistance assay are confounding for an understandingn of the small molecule A1. Through screening and various experiments performed by Dr. Tim Wigle of the Singleton laboratory, the results of those experiments demonstrated the ability of A1 to hinder strand exchange between single stranded DNA and double stranded DNA in a strand exchange assay, the turnover of ATP in an ATPase assay and the induction of SOS as observed in a GFP SOS induction assay. All of these processes are controlled by RecA, which gives strong evidence to believe that A1 is a RecA inhibitor. However, the results of this study show that resistance development to chloramphenicol is inhibited in the presence of this compound. A1 obviously does not need RecA to be present to hinder resistance development, thus further

demonstrating that RecA is not necessary for chloramphenicol resistance development in MG1655 *E. coli* cells or that A1 has another mechanism of action within cells. More studies need to be completed to understand how A1 functions in the cell.

Lastly, the next step to understanding the involvement of RecA in bacterial culture studies would be to run the one-flask resistance assay with other antibiotics that are known to induce DNA damage, such as ciprofloxacin, and bactericidal agents such as ampicillin and kanamycin. Using these agents would induce DNA repair pathways by activating RecA and inactivating the repressor protein LexA, which allows for the expression of DNA repair proteins, including SOS mutagenic proteins. Knowing that RecA is needed for cell survival and resistance development to these antibiotics, this would serve to understand in more detail the ability of MG1655 *E. coli* cells to develop resistance to other antibiotics, as well as to clarify if A1 needs RecA to inhibit resistance development. Studies of the *recA* knockout strain might prove to have different results when cultured with or without A1 if A1 truly is a RecA inhibitor, meaning Δ*recA* cells with or without A1 should not develop resistance because resistant development is most likely dependent on RecA and the addition of an RecA inhibitor would make no difference (even if it does prevent resistance through a different pathway, there would still be no resistant development observed). The results for the wild type strain should remain the same if A1 is able to inhibit RecA. However, if A1 inhibits resistance in a RecA independent manor, then

resistance to these antibiotics might still ensue, depending on the means of resistance development for each individual antibiotic.

CHAPTER IV

APPLICATION OF THE GRIFFITH EXPERIMENT TO *ESCHERICHIA COLI* MG1655 CELLS TO OBSERVE THE POTENTIAL TRANSFER OF ANTIBIOTIC RESISTANCE GENES

During World War I, many soldiers were dying of pneumonia infections. In an attempt to aid soldiers fighting in this war, Frederick Griffith studied strains of *Streptococcus pneumoniae* in mice in attempts to uncover a vaccine against pneumonia. Using two strains of *S. pneumoniae*, an avirulent strain (R or rough strain) lacking a polysaccharide capsule and a virulent strain incorporating a polysaccharide capsule (S or smooth strain), he injected mice with these strains and observed if and when they died[37]. When injected with an R strain, the mice did not acquire pneumonia and lived. But mice injected with the S strain acquired pneumonia and died within a few days. The polysaccharide capsule is essential for virulence of *S. pneumoniae* in that it protects the bacteria from attack inflicted by the human immune system. The question arose of whether the avirulent strain could become virulent if in the presence of dead virulent cells, which would enable the avirulent strain to incorporate a capsule to protect itself. Therefore, when mice were injected with a mixture of living R and heat-killed S strains, the mice developed pneumonia and died[37]. As a control, mice were injected with heat-killed S cells and did not acquire pneumonia, therefore enabling them to live. Upon isolation and analysis of the strains extracted from the mice, Griffith

observed that all of the *S. pneumoniae* contained a polysaccharide capsule[37]. Somehow, the heat-killed S strain transferred information to the avirulent R strain, which allocated the capability of the R strain to develop a protective capsule. Thus, the transforming principle was born.

How this could happen remained elusive until the 1940's when Avery and colleagues observed that the addition of ribonucleases, chymotrypsin and trypsin (used for the breakdown of proteins and peptides) had no effect on *S. pneumoniae's* ability to transform the R strain to the S strain in the presence of the heat-killed S strain, thus disproving the common theory that proteins were responsible for this phenomenon[38]. But the addition deoxyribonucleopolymerase abrogated the transformation of the R strain to the S strain in the presence of the heat-killed S strain[38]. Finally, it was demonstrated that DNA was responsible for this transformation. The DNA from the heat-killed S strain was able to be taken up by the R strain and incorporated into its genome, therefore encoding the genetic information for the production of the polysaccharide capsule.

RecA is believed to be a part of this pathway by aiding the incorporation of the genes through homologous recombination and possibly with transport inside the cell by coating ssDNA upon entry into the cell, thus protecting it from nucleases[4,19,26,27]. Therefore, by inhibiting RecA, the transfer and incorporation of genes through transformation can be prevented. This can be an effective means of preventing the spreading of antibiotic resistant genes.

As mentioned, *Streptococcus pneumoniae* is able to uptake and incorporate DNA naturally, meaning it is naturally competent[4,40,41]. There are

other strains, both gram-positive and gram-negative, that are naturally competent, such as *Neisseria gonhorraea*, *Helicobacter pilori* and *Bacillus subtillis*. However, *Eschericia coli* is not thought to be naturally competent, despite some evidence to the contrary[39-45]. If *E. coli* is to be defined as naturally competent, then the transformation efficiency is very low compared to other strains.

MG1655 is a strain of *E. coli* that has very few genetic modifications, therefore it is a strain that is very similar to one that would be found in a patient[67]. Some of its genetic modifications include a knockout of the F factor, which disables the bacteria's ability to transfer genes through conjugation by hindering its ability to assemble a sex pilus; a knockout of λ, which prevents the degradation of foreign DNA upon entry into the cell; and rfb-50, which is an IS5 insertion sequence that results in the absence of O-antigen, thus resulting in a rough lipopolysaccharide (LPS) and increasing the permeability for hydrophobic antibiotics[67]. These genetic modifications are of interest when studying the possibility that *E. coli* could be naturally competent because conjugation can be ruled out as the means of gene transfer. Also, if genes are being taken up through naturally occurring mechanisms, then they will not be degraded upon entry into the cell. Also, having a rough LPS could also mean less barriers for DNA to battle when attempting to enter the cell due to less forces of repulsion between negatively charged LPS and negatively charged DNA.

Taking all of this into account, if *E. coli* is able to take up DNA naturally like *S. pneumoniae* is able, whether by a similar or unique pathway, then resistance to antibiotics would develop more quickly in the presence of heat-killed

cells that are resistant to that antibiotic than cells that are not in the presence of heat-killed resistant cells due to the uptake and incorporation of the resistant genes present in the supernatant of the heat-killed cells. Also, assuming RecA is involved in this process of DNA uptake and/or incorporation, an inhibitor of RecA would be able to deter this process. Therefore, the potential inhibitor compound A1 (discovered in a RecA ATPase HTS performed by Dr. Tim Wigle in collaboration with Dr. Jonathan Sexton and Director Li-ahn Yeh at the BRITE center at NCCU) is an ideal candidate to test this theory.

Bacterial Culture Studies of the Effects of the Addition of Chloramphenicol Resistant Cell Supernatant on Wild Type *E. coli* MG1655 cells

Back in 1929, Dr. Griffith observed a rather intriguing phenomenon. While experimenting on mice, he discovered the transfer of information between *Streptococcus pneumoniae*. While injecting mice with an avirulent, or rough, strain of *Streptococcus pneumoniae*, the mice did not develop pneumonia and lived. As expected, when injected with a living virulent, or smooth, strain, the mice did develop pneumonia and died. But when the mice were injected with a heat-killed virulent strain, the mice of course lived. However, when injected with a combination of the heat-killed virulent strain and the avirulent strain, the mice acquired pneumonia and died. Somehow, something from the heat-killed virulent strain was being transferred to the avirulent strain, informing it how to make itself virulent.

Scientists first predicted that proteins were responsible for this phenomenon, that proteins were carrying the information from the heat-killed virulent strain to the avirulent strain. It was not until 1944 when Avery and colleagues determined that DNA was responsible for this transfer of information. What actually happened was the DNA responsible for encoding the polysaccharide capsule that protects the bacteria from the human immune system was leaked from the heat-killed cells. The avirulent strain was able to take up and incorporate these genes into its own genome, therefore expressing the virulent factor, a process that is most commonly referred to as transformation. *Streptococcus pneumoniae* is able to perform transformation naturally through signaling pathways and the utilization of various proteins, including the RecA protein, which aids in homologous recombination of the newly acquired genes and possible through uptake by protecting the DNA from nucleases upon entry into the cell.

Since the transfer of antibiotic resistant genes through transformation is a major concern, being able to observe this in cultures is of interest. Applying the Griffith theory to *E. coli*, growing wild type cultures in the presence of heat-killed antibiotic resistant cell supernatant theoretically should develop resistance to that antibiotic faster than cells grown without the antibiotic resistant cell supernatant due to the uptake and incorporation of the resistant genes from the heat-killed resistant cells. This theory was tested using the one-flask resistance assay with chloramphenicol.

Briefly, the one-flask resistance assay is conducted by observing the growth of 50 mL cultures of MG1655 wt. cells in the presence of 0, 5 and 10 μg/mL of chloramphenicol and with or without 1 mL of heat-killed chloramphenicol resistant cell supernatant for 100 hours by periodically monitoring the optical density of the cultures at a wavelength of 600 nm. To confirm resistance development, an E-test is performed. In cultures without chloramphenicol, cells with and without chloramphenicol resistant cell supernatant grow to saturation, or an OD_{600} of 2, within 10 hours (Figure 4.1A, bottom panel: Cam only, dotted gray line with open triangles; Cam^r and Cam, solid green line with open triangles). In the presence of 10 μg/mL of chloramphenicol, cells did not grow above OD_{600} of 0.4, whether in the presence of Cam^r supernatant or not (Figure 4.1A, bottom panel: Cam only, dotted gray line with open inverted triangles; Cam^r and Cam, solid green line with open inverted triangles). This result indicates that whether or not resistant genes were transferred from the resistant cells to the wild type cells, the dose of chloramphenicol was high enough to prevent the development of resistance. However, in the presence of a sub-lethal dosage of 5 μg/mL of chloramphenicol, cells in the presence of chloramphenicol resistant cell supernatant developed resistance to chloramphenicol faster than cells without chloramphenicol resistant cell supernatant as shown by the increase in OD_{600} over time; resistance development was observed between 24-40 hours with chloramphenicol resistant cells as opposed to 40-60 hours without the heat-killed chloramphenicol resistant cell supernatant (Figure 4.1A, bottom panel: Cam only, dotted gray line with

closed circles; Cam^r and Cam, solid green line with closed circles). Also, under both conditions, the MIC increases from 6-12 μg/mL chloramphenicol to 24-96 μg/mL, thus confirming the development of resistance (Figure 4.1A, top panel: Cam only, dotted gray line with closed circles; Cam^r and Cam, solid green line with closed circles).

These results were shown to be reproducible (Figures 4.1B-D). The MICs for all experiments with or without heat-killed chloramphenicol resistant cell supernatant begin around 6-12 μg/mL and reach an MIC of 24-96 μg/mL. However, for the culture with 5 μg/mL of chloramphenicol in the third experiment (Figure 4.1C), possible resistance development to chloramphenicol was not observed until over 100 hrs when the OD_{600} approached 0.5, but did not reach 0.6 or above. Because the culture did not grow above OD_{600} of 0.6, the MIC was not determined. The culture with 5 μg/mL of chloramphenicol and heat-killed chloramphenicol resistant cell supernatant did develop resistance faster than chloramphenicol only, but did so more slowly than in the other experiments (Figures 4.1A, B and D). These results do indicate that chloramphenicol resistant genes from the heat-killed cells are being transferred to the wild type cells and are being incorporated and expressed.

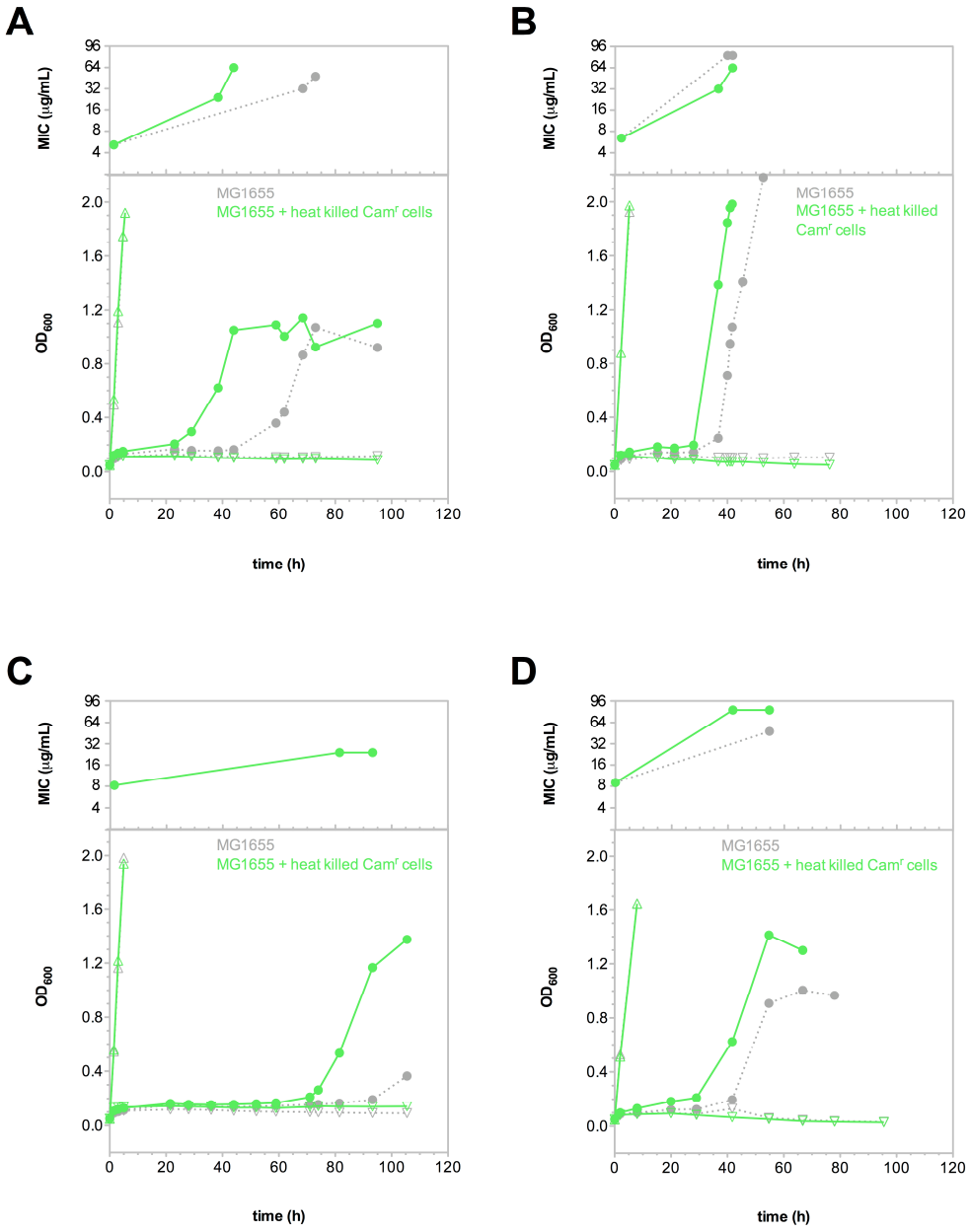

Figure 4.1 Resistance Development of MG1655 wild type cells in the presence of Cam and heat-killed Cam^r cells.

A-D are four experimental results for this assay. Bottom panels show the OD_{600} vs time: gray dotted line with open triangles, 0 μg/mL Cam only; gray dotted line with closed circles, 5 μg/mL Cam only; gray dotted line with open inverted triangles, 10 μg/mL Cam only; green solid line with open triangles, 0 μg/mL Cam and heat-killed Cam^r cells; green solid line with closed circles, 5 μg/mL Cam and heat-killed Cam^r cells; green solid line with open inverted triangles, 10 μg/mL Cam and heat-killed Cam^r cells. Top panels show the MIC vs time: dotted gray line with closed circles, 5 μg/mL Cam only; solid green line with closed circles, 5 μg/mL Cam and heat-killed Cam^r cells.

Bacterial Culture Studies of the Effects of the Addition of Chloramphenicol Resistant Cell Supernatant on Δ*recA E. coli* MG1655 cells

While attempting to apply the Griffith experiment to *E. coli* MG1655 cells, one would expect the wild type cells to have a means of incorporating the antibiotic resistant genes from the heat-killed resistant cells into the genome to enable the wild type cells to become resistant to the antibiotic, chloramphenicol in this particular study. RecA is suspected to be implicated in this process by utilizing homologous recombination of the DNA from the resistant cells and by possibly protecting the DNA from nucleases upon entry into the cell. In the previous chapter, it was demonstrated that RecA is not needed for the development of resistance to chloramphenicol, but the previous section of this chapter suggests that RecA can accelerate the development of resistance by exploiting eDNA from resistant cells. Therefore, performing the same experiments with a strain lacking RecA is essential to further elucidating this theory of the usage of resistance genes from external DNA.

Theoretically, if the cells are incorporating the resistant genes by uptaking external DNA from the media and making use of homologous recombination through a RecA dependent pathway, then removing RecA from the cells would attenuate this process. However, if resistance to chloramphenicol is a RecA independent process, as demonstrated in the previous chapter, then resistance can still develop, but will do so at about the same speed as cells without heat-killed chloramphenicol resistant cells present.

Upon analysis of MG1655 Δ*recA* cells in the one-flask resistance assay, it is apparent that in the presence of heat-killed chloramphenicol resistant cells, cells grown without any chloramphenicol grow to saturation in about 10-15 hours (Figure 4.2A, bottom panel, solid green line with open triangles). Upon the addition of 10 μg/mL of chloramphenicol and heat-killed chloramphenicol resistant cells, the *recA* knockout cells do not grow and are therefore assumed to have not developed resistance (Figure 4.2A, bottom panel, solid green line with open inverted triangles). The cultures grown in these conditions behave as expected. But when 5 μg/mL of chloramphenicol and heat-killed chloramphenicol resistant cells were present, the results that ensued are two fold: since the cells began to grow around 40 hours (Figure 4.2A, bottom panel, solid green line with closed circles) and had an MIC reaching 64 μg/mL (Figure 4.2A, top panel, solid green line with closed circles), the obvious conclusions are that RecA is needed to incorporate the resistant genes in order to accelerate the resistance development and the cells are still able to develop resistance to chloramphenicol at the same rate as the culture without heat-killed chloramphenicol resistant cells, therefore indicating a RecA independent pathway of resistance development as shown in the previous chapter.

Repeats of this experiment further supported the conclusions made in this experiment (Figures 4.2B-C) by confirming quick growth of cultures without chloramphenicol, no growth with 10 μg/mL and growth of cultures with 5 μg/mL and heat-killed chloramphenicol resistant cells between 40-50 hours with MICs

increasing to 32-64 μg/mL, which correlated with the cultures without heat-killed resistant cells.

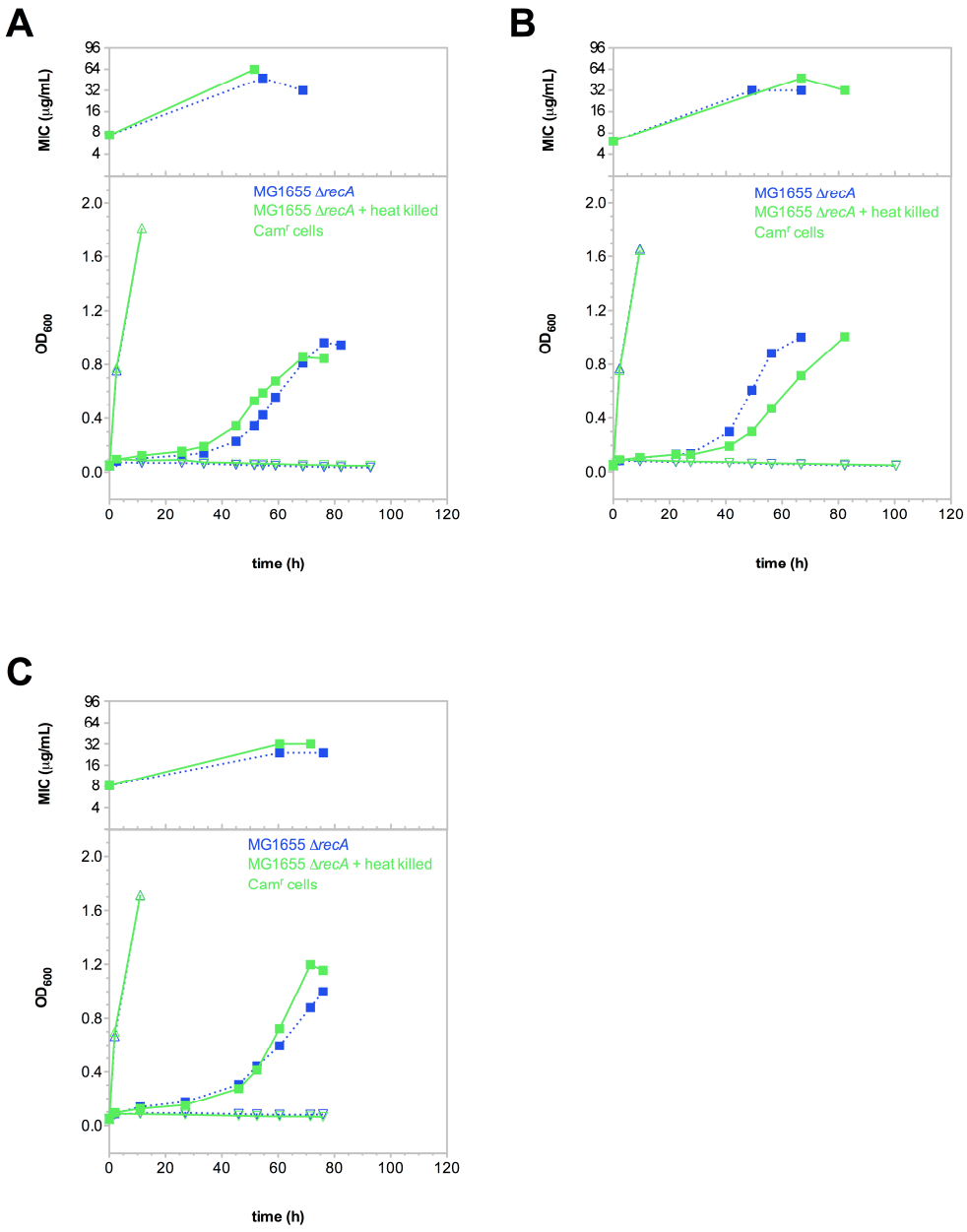

Figure 4.2 Resistance Development of MG1655 ΔrecA cells in the presence of Cam and heat-killed Cam^r cells.

A-C show three experimental results for this assay. Bottom panels show the OD_{600} vs time: blue dotted line with open triangles, 0 μg/mL Cam only; blue dotted line with closed squares, 5 μg/mL Cam only; blue dotted line with open inverted triangles, 10 μg/mL Cam only; green solid line with open triangles, 0 μg/mL Cam and heat-killed Cam^r cells; green solid line with closed squares, 5 μg/mL Cam and heat-killed Cam^r cells; green solid line withopen inverted triangles, 10 μg/mL Cam and heat-killed Cam^r cells. Top panels show the MIC vs time: dotted blue line with closed squares, 5 μg/mL Cam only; solid green line with closed squares, 5 μg/mL Cam and heat-killed Cam^r cells.

Bacterial Culture Studies of the Effects of the Addition of A1 and Chloramphenicol Resistant Cell Supernatant on Wild Type *E. coli* MG1655 cells

Bacteria have almost an unfathomable capability to survive compared to many organisms in existence. Especially when placed under stressful conditions, they have adopted various means of bypassing the stress in order to survive. Antibiotics, once thought to be a miracle when first introduced because of the many lives they saved by treating bacterial illnesses that were once life-threatening, hardly pose a threat to bacterial infections in recent years due to the transfer of antibiotic resistant genes, whether through vertical or horizontal gene transfer. Therefore, instead of attempting to develop new antibiotics, perhaps a more cunning approach to outsmart bacteria is to impair their resistance mechanisms, such as recombination, DNA repair mechanisms and SOS mutagenesis. RecA is an ideal target for this, since it is involved in all of these processes. RecA is also an ideal target to prevent the horizontal transfer of antibiotic resistance genes, since it aids in incorporation of the resistance genes into the genome of the recipient bacteria through homologous recombination and possibly aids in the uptake of exogenous DNA into the cell by coating it to protect it from nucleases.

Compound A1 has proven to be a highly potential inhibitor of RecA by attenuating the resistance development of MG1655 wt. *E. coli* cells to chloramphenicol (see chapter II, section Bacterial Culture Studies of the Effects of A1 on Wild Type *E. coli* MG1655 cells). MG1655 wt. *E. coli* cells were also shown to develop resistance to chloramphenicol more quickly in the presence of

heat-killed chloramphenicol resistant cell supernatant, thus indicating that genes are being horizontally transferred from the dead cells to the live wt. cells (see Chapter IV, section Bacterial Culture Studies of the Effects of the Addition of Chloramphenicol Resistant Cell Supernatant on Wild Type *E. coli* MG1655 cells). Since RecA would be responsible for incorporating those genes into the genome of the wt. cells, inhibiting RecA would more or less attenuate the process of transferring the chloramphenicol resistant genes. Therefore, investigating if A1 could also deter the resistance development of MG1655 wt. *E. coli* cells in the presence of heat-killed chloramphenicol resistant cell supernatant was worth observing.

Using the one-flask resistance assay, the growth of 50 mL cultures of MG1655 wt. cells in the presence of 0, 5 and 10 μg/mL of chloramphenicol and 50 μM of A1 with or without 1 mL of heat-killed chloramphenicol resistant cell supernatant was monitored for 100 hours periodically by recording the optical density of the cultures at a wavelength of 600 nm. To confirm resistance development, an E-test was performed. Within ten hours, cells grown without chloramphenicol grew to saturation, or an OD_{600} of 2 (Figure 4.3A, bottom panel, all lines with open triangles). There was no differentiation between cultures with or without heat-killed chloramphenicol resistant cells or A1. After 100 hours, it was apparent that cultures grown in the presence of 10 μg/mL of chloramphenicol did not grow above OD_{600} of 0.4. Again, there was no differentiation between cultures with or without heat-killed chloramphenicol resistant cells or A1 (Figure 4.3A, bottom panel, all lines with open inverted triangles). As demonstrated

previously, the addition of heat-killed chloramphenicol resistant cells accelerated the resistance development to 5 μg/mL of chloramphenicol of MG1655 wt. cells (Figure 4.3A, bottom panel, solid green line with closed circles). But with the addition of 50 μM of A1, resistance development to chloramphenicol was not observed in the presence of 5 μg/mL of chloramphenicol, whether heat-killed chloramphenicol resistant cells were added or not (Figure 4.3A, bottom panel, dotted red lines with closed circles contains no resistant cells and magenta lines with closed circles contains resistant cells). See Figure 4.3B-D for reproducibility of this assay. Assuming A1 is truly an inhibitor of RecA, then the results of this assay show that A1 is able to hinder RecA's ability to aid in the incorporation of chloramphenicol resistant genes into the MG155 wt. cells. If it is not a RecA inhibitor, then it is able to attenuate resistance development in a RecA independent manor.

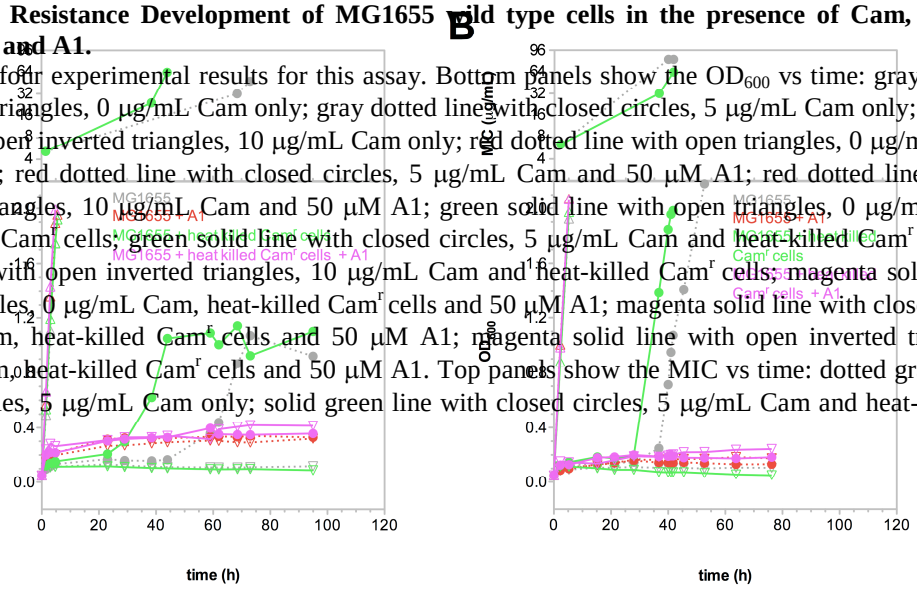

Figure 4.3 Resistance Development of MG1655 wild type cells in the presence of Cam, heat-killed Cam^r cells and A1.

A-D show four experimental results for this assay. Bottom panels show the OD_{600} vs time: gray dotted line with open triangles, 0 μg/mL Cam only; gray dotted line with closed circles, 5 μg/mL Cam only; gray dotted line with open inverted triangles, 10 μg/mL Cam only; red dotted line with open triangles, 0 μg/mL Cam and 50 μM A1; red dotted line with closed circles, 5 μg/mL Cam and 50 μM A1; red dotted line with open inverted triangles, 10 μg/mL Cam and 50 μM A1; green solid line with open triangles, 0 μg/mL Cam and heat-killed Cam^r cells; green solid line with closed circles, 5 μg/mL Cam and heat-killed Cam^r cells; green solid line with open inverted triangles, 10 μg/mL Cam and heat-killed Cam^r cells; magenta solid line with open triangles, 0 μg/mL Cam, heat-killed Cam^r cells and 50 μM A1; magenta solid line with closed circles, 5 μg/mL Cam, heat-killed Cam^r cells and 50 μM A1; magenta solid line with open inverted triangles, 10 μg/mL Cam, heat-killed Cam^r cells and 50 μM A1. Top panels show the MIC vs time: dotted gray line with closed circles, 5 μg/mL Cam only; solid green line with closed circles, 5 μg/mL Cam and heat-killed Cam^r cells.

Bacterial Culture Studies of the Effects of the Addition of A1 and Chloramphenicol Resistant Cell Supernatant on Δ*recA E. coli* MG1655 cells

In MG1655 wild type cells, experiments in this study thus far have shown that cultures in the presence of a sub-lethal dosage of chloramphenicol, cells are able to develop resistance to chloramphenicol most likely in a RecA independent manner. This was further supported when the assay results with Δ*recA* cells were still able to develop resistance to chloramphenicol at 5 μg/mL. However, when introduced to heat-killed chloramphenicol resistant cells, wild type cells were able to develop resistance more quickly than cells without the heat-killed cells while *recA* knockouts were not, which indicates that the uptake and incorporation of the exogenous DNA through homologous recombination through a RecA dependent pathway is operational. But interestingly, A1, a compound thought to be an inhibitor of RecA, was shown to prevent resistance development to chloramphenicol in all of these conditions thus far tested. This is perplexing, since this would indicate that A1 is not an inhibitor of RecA because the presence of RecA is not necessary for prevention of resistance development. Therefore, the last piece of the puzzle is necessary to elucidate: can A1 prevent the resistance development of MG1655 Δ*recA* cells to chloramphenicol when heat-killed chloramphenicol resistant cells are present.

After conducting the one-flask resistance assay with the conditions of 0, 5 and 10 μg/mL of chloramphenicol and heat-killed chloramphenicol resistant cells, the answer becomes clear: the answer is yes, A1 does appear to prevent the development of resistance to chloramphenicol in cultures grown with heat-killed

chloramphenicol resistant cells and 5 μg/mL of chloramphenicol as indicated by the lack of increase in OD_{600} (Figure 4.4A, bottom panel, solid magenta line with closed squares). The results of the control with no chloramphenicol were as expected: quick growth to saturation within 10-15 hours and therefore no resistance to chloramphenicol (MIC of about 8 μg/mL) (Figure 4.4A, bottom panel, solid magenta line with open triangles). Also as expected, the culture with 10 μg/ml of chloramphenicol and heat-killed chloramphenicol resistant cells does not grow (Figure 4.4A, bottom panel, solid magenta line with open inverted triangles). These results are twofold in that they indicate (1) A1 can hinder resistance development to chlorapmphenicol in a RecA independent manor, as already suspected from other results in this study; and (2) RecA would still be needed to incorporate the DNA encoding the resistant genes present in the media, since resistance development still did not ensue when the heat-killed resistant cells were present.

Replication of this assay yielded the same results (Figures 4.4B-C): (1) the quick growth of cultures with no chloramphenicol, 50 μM of A1 and heat-killed chloramphenicol resistant cells; (2) no growth of cultures with 10 μg/mL of chloramphenicol, 50 μM of A1 and heat-killed chloramphenicol resistant cells; and (3) cultures with 5 μg/mL of chloramphenicol, 50 μM of A1 and heat-killed chloramphenicol resistant cells did not develop resistance to chloramphenicol as indicated by their lack of growth.

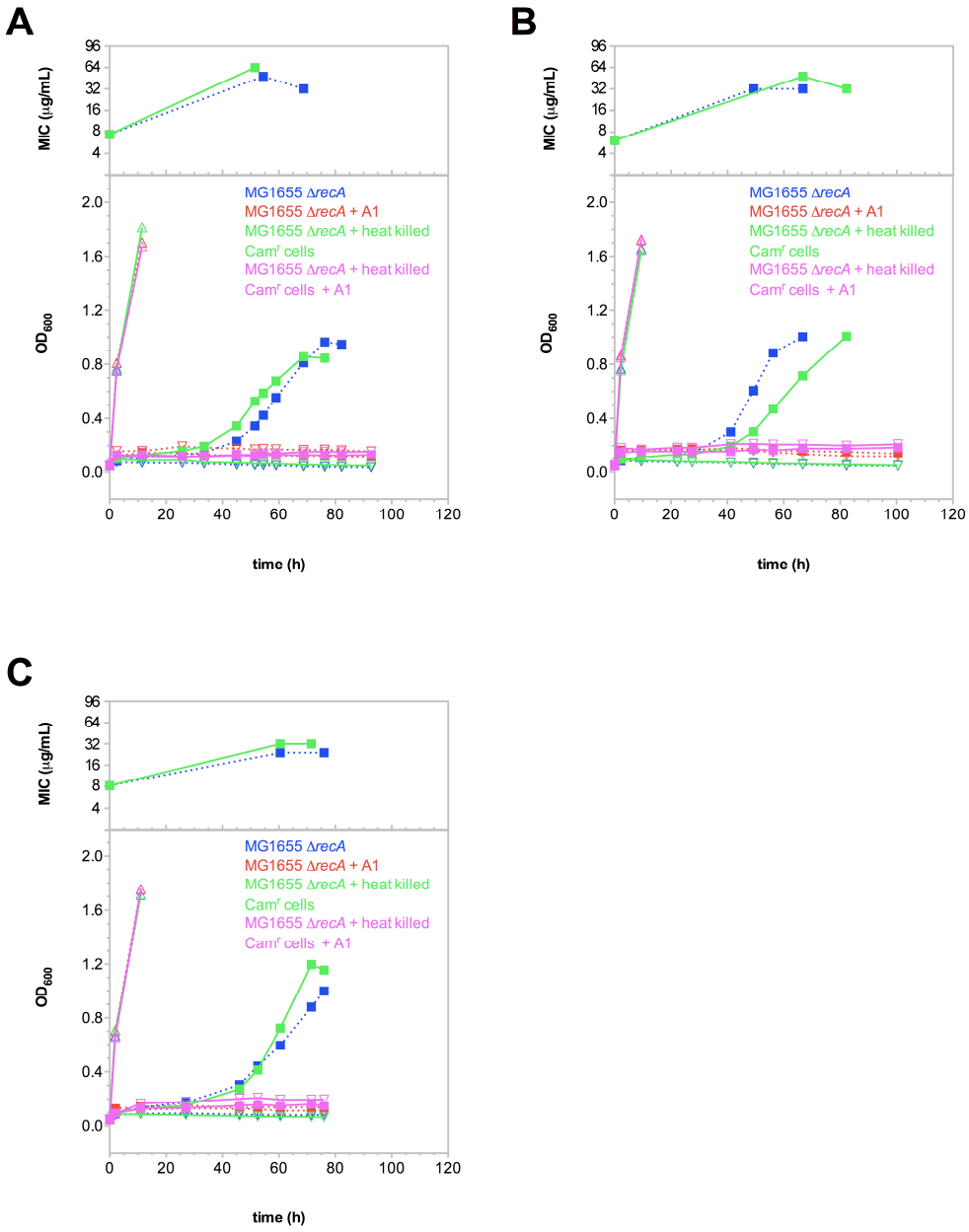

Figure 4.4 Resistance Development of MG1655 Δ*recA* cells in the presence of Cam, heat-killed Cam^r cells and A1.

A-C show three experimental results for this assay. Bottom panels show the OD_{600} vs time: blue dotted line with open triangles, 0 μg/mL Cam only; blue dotted line with closed squares, 5 μg/mL Cam only; blue dotted line with open inverted triangles, 10 μg/mL Cam only; red dotted line with open triangles, 0 μg/mL Cam and 50 μM A1; red dotted line with closed squares, 5 μg/mL Cam and 50 μM A1; red dotted line with open inverted triangles, 10 μg/mL Cam and 50 μM A1; green solid line with open triangles, 0 μg/mL Cam and heat-killed Cam^r cells; green solid line with closed squares, 5 μg/mL Cam and heat-killed Cam^r cells; green solid line with open inverted triangles, 10 μg/mL Cam and heat-killed Cam^r cells; magenta solid line with open triangles, 0 μg/mL Cam, heat-killed Cam^r cells and 50 μM A1; magenta solid line with closed squares, 5 μg/mL Cam, heat-killed Cam^r cells and 50 μM A1; magenta solid line with open inverted triangles, 10 μg/mL Cam, heat-killed Cam^r cells and 50 μM A1. Top panels show the MIC vs time: dotted blue line with closed squares, 5 μg/mL Cam only; solid green line with closed squares, 5 μg/mL Cam and heat-killed Cam^r cells.

Conclusions

Griffith and Avery in their time had some rather interesting discoveries: that bacteria cells could transfer information to one another via DNA. In *S. pneumoniae*, the virulence factor of a polysaccharide capsule was transferred from heat-killed virulent cells to avirulent cells, which were then able to incorporate these genes and protect themselves from the host's immune response.

This study used this concept to attempt to answer the question of whether antibiotic resistant genes could be transferred in *E. coli* cells in the same manor: could heat-killed chloramphenicol resistant cells excrete the DNA encoding for chloramphenicol resistance, which could be utilized by non-resistant cultures to develop resistance? When wild type MG1655 *E. coli* cells were introduced to heat-killed chloramphenicol resistant cells, resistance development was seen only at a concentration of chloramphenicol of 5 μg/mL, as seen by the increased optical density at 600 nm over time and the increased MIC from 6-12 μg/mL chloramphenicol to 24-96 μg/mL. The rate was also faster than wild type cells without the introduction of heat-killed chloramphenicol resistant cells by about 10-20 hours. These results supported theory that chloramphenicol resistant genes could be transferred from heat-killed cells to non-resistant cells, but Δ*recA* cells would give a clearer picture, since it is needed for the incorporation of genes from exogenous DNA into the genome of the recipient cells. Upon their analysis, it was demonstrated that only cultures with 5 μg/mL of chloramphenicol were able to develop resistance. However, there was no differentiation between cultures with or without heat-killed chloramphenicol resistant cells added. Having compiled

results from both strains, the conclusions are that exogenous DNA encoding chloramphenicol resistance can be uptaken and incorporated by MG1655 *E. coli* cells, but RecA is required for this process and resistance to chloramphenicol can ensue in a RecA independent manor, since Δ*recA* cells were still able to develop resistance to chloramphenicol.

Various in vitro studies with A1 strongly indicated that it is an inhibitor of RecA (see the dissertation of Dr. Tim Wigle). Therefore, testing this compound in bacterial cultures in the one-flask resistance assay seemed to be a reasonable next step to ensure this compound was able to function in live cell cultures. In the previous chapter of this study, it was demonstrated that A1 was able to attenuate resistance development of MG1655 wild type and Δ*recA E. coli* cells to chloramphenicol. If only wild type cells did not develop resistance, then one could conclude that RecA is necessary for the resistance development to chloramphenicol and A1 is definitely an inhibitor of RecA. Since this is not the case, resistance to chloramphenicol seems to be RecA independent and A1 has no preference for RecA when inhibiting resistance development. This is further supported when heat-killed chloramphenicol resistant cells were added to cultures. Even though wild type cells developed resistance faster with the heat-killed resistant cells and the Δ*recA* cells did not, again, A1 was still able to attenuate this process, thus confirming that it is able to deter resistance development in a RecA independent manor. Interestingly, even though wild type cells possessed the ability to incorporate the resistant genes from the heat-killed cells, because A1was present and did not require the inhibition of RecA to hinder

resistance, the incorporation of these genes was inconsequential when A1 was added to the cultures.

Understanding the effects of adding heat-killed resistant cells and A1 would be better understood when performing this study with other antibiotics that are bactericidal in nature, such as ciprofloxacin, kanomycin and ampicillin, which would more likely induce the SOS response in cells, thus depending on the involvement of RecA. If A1 attenuates resistance development in a RecA independent manor, then the results of studying these other antibiotics might show resistance development when A1 is present or might not, depending on the mechanism of action of each antibiotic. But the picture would be much clearer upon compilation of this data. However, the results of the addition of heat-killed resistant bacteria to wild type cells would most likely remain unchanged, since the incorporation of resistant genes accelerates the resistance development process. The addition to Δ*recA* cells could theoretically change, since RecA would most likely be necessary for the development of resistance with bactericidal agents, and would not develop resistance at sub-lethal dosages.

CHAPTER V

UTILIZATION OF MOLECULAR MODELING METHODS OF SCREENING TO FIND OTHER POTENTIAL INHIBITORS OF RECA

As the battle against the ever-evolving bacteria rages on, more and more antibiotic resistant bacteria emerge and therefore current antibiotics are rendered ineffective against them. A high demand has been established to discover chemical agents that can decimate these superbugs. Recent evidence has supported RecA as a novel target for battling these antibiotic resistant bacteria due to its involvement in SOS mutagenesis, DNA repair and recombinational-dependent horizontal gene transfer[19-26], which have been implicated in many bacteria's ability to develop tolerance to treatment with chemical agents and inevitably will lead to fully developed resistance[26,27]. Hindering RecA's ability to function displays tremendous potential to battle these superbugs by sensitizing them to current antibiotics and prohibiting them to recover from antibiotic attack by inhibiting the resistance pathways.

Small molecules that have the ability to penetrated the cell and inhibit RecA function are attractive candidates for drugable compounds. Theoretically, if these small molecules interact with the RecA protein and inhibit its function, synergistic effects can be observed with the use of current antibiotics, enabling

them to be more potent against bacteria and would decrease the spreading of bacterial strains already resistant to the current antibiotics.

Dr. Tim Wigle of the Singleton laboratory, in collaboration with Director Li-An Yeh and Professor Jonathon Sexton, has had the opportunity to screen for such small molecules at the Biomanufacturing and Research Institute and Technology Enterprise (BRITE) located at North Carolina Central University (NCCU). He was able to screen a small chemical library of 35,780 small molecules against the RecA protein. From this screen came seventy-three hit compounds that were utilized in a molecular modeling project in collaboration with Dr. M. Karthikeyan in the Dr. Alexander Trohsha laboratory.

In regards to drug discover, molecular modeling, the technique of mimicking the behavior of molecules, can be implemented by exploring structural and activity space in attempts to uncover molecules that have the desirable effects (in our case, inhibitors of the RecA protein) without having to spend many hours and much money screening libraries[68,69]. The hits from an already screened small molecule library at the BRITE center provided a basis to perform cost-effective and low budget screening. Implementing the molecular operating environment (MOE) program, similarity searching can be performed, which would uncover molecules with similar structures in the literature and that could potentially be commercially available. These molecules would theoretically have similar activities under the assumption that there is a strong correlation between structure and activity[68,69]. Compounds extracted from this search that already have a high potential of being active due to their structurally similarities to previously

established hit compounds can be tested in the laboratory to confirm or invalidate them as hit compounds against the RecA protein. Such compounds were extracted and explored as potential inhibitors of RecA.

Various Prediction Methods and the Theory Behind Similarity Searching

The theoretical basis for molecular modeling screening, in regards to medicinal chemistry applications, is molecules or compounds with similar structures will have similar biological activities[68,69]. Therefore, compounds with similar structures are predicted to have similar effects on the target or disease state of interest. This is one approach to finding new potential hit compounds: probing libraries of compounds to locate similar structures to the drug, ligand or inhibitor being investigated. Two other approaches that can predict inhibitors or ligands are analyzing the target or analyzing the disease state. Similarly, proteins that have similar sequences and/or function could have ligands that fit the target of interest. For example, ligands that work as inhibitors to the Rad51 protein may also inhibit the RecA protein due to the fact that they have similar functions and partial homology. Likewise, drugs available on the market that treat a specific disease state similar to the one of interest may also have medicinal effects to the disease of interest. Using this information to find target information is an indirect method due to off target effects and promiscuity of drugs. Disease states can have multiple enzymes, proteins and receptors involved, therefore attempting to find new drugs based on the disease state through the target can be tricky. See figure

5.1 to summarize the prediction methods. The method of interest in this project is the first direct approach: probing the potential inhibitors to find new inhibitors.

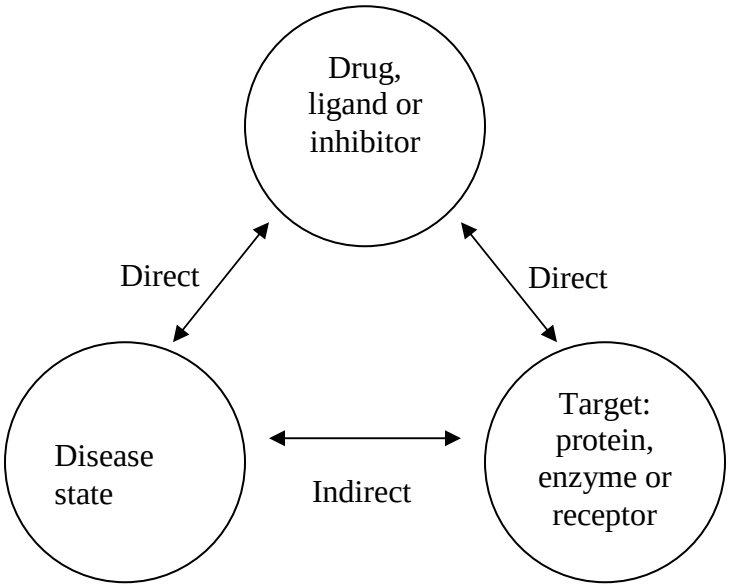

Figure 5.1. Prediction methods.
The drug/ligand/inhibitor compounds can be used to directly probe the target and disease state. The target (protein, enzyme, receptor) can be used to directly probe the drug/ligand/inhibitor and indirectly probe the disease state. The disease state can be used to directly probe the drug/ligand/inhibitors and indirectly probe the target.

Abstraction of Common Scaffolds from Seventy-three Hit Compounds from Biogen Library

In an attempt to identify compounds that are active against the RecA protein, Dr. Tim Wigle was able to work in collaboration with Director Li-An Yeh and Professor Jonathon Sexton of the Biomanufacturing and Research Institute and Technology Enterprise located at North Carolina Central University to screen a small chemical library of 35,780 small molecules against the RecA protein. He utilized a phosphomolybdate blue ATPase assay that measures ssDNA-dependent ATP hydrolysis as an indicator of activity of RecA by measuring the turnover of inorganic phosphates. Compounds that prevented the

production of inorganic phosphates from ATP would be considered active. From this compound library of 35,780 compounds, seventy-three hit compounds screened against the RecA protein were discovered. Seven common scaffolds from these seventy-three compounds were derived using Molecular Operating Environment (MOE), two of the scaffolds being unique. The seventy-three hits were condensed down to forty-eight unique structures, eliminating duplicates or isomers.

Again, theoretically, compounds with similar structures should have similar activities. Therefore, exploring the literature for compounds that are already known and have structural similarity to the unique scaffolds could yield new potential inhibitors of RecA. Figure 2.1 demonstrates the general workflow of this project.

Screening Literature for Similar Compounds Using SciFinder Scholar

In order to obtain known or commercially available similar compounds, three scaffolds were used as query structures in ScFinder Scholar. As stated, there were two unique scaffolds were abstracted using MOE. Two of the scaffolds used to search SciFinder Scholar were very similar, differing only by an aromatic ring on one position of the main ring and an oxygen substituent on another ring on only one scaffold. The third scaffold had a different composition. The search criteria included returning structures as organics and all others, single components and one or more reference available.

This process yielded three hundred and fifty-six compounds (two hundred and ninety-seven for the first scaffold, thirty-three for the second scaffold and twenty-six for the third, different scaffold). Ninety-two of these compounds were randomly selected to represent all of the compounds to condense the amount of compounds needed for clustering. These representative compounds were converted into simplified molecular input line entry specification (SMILES) format, which describes chemical structures in short American Standard Code for Information Interchange (ASCII) strings.

Two subsets of compounds now exist: the experimental set consisting of the forty-eight unique structures from the hit compounds (assigned an activity of one for modeling purposes) and the literature set consisting of the ninety-two compounds selected from the literature (assigned an activity of zero for modeling purposes).

Tanimoto Similarity Coefficients Allow for Clustering in Activity Space

There are many ways to describe compounds. These include physico-chemical descriptors; which encompass logP (hydrophobicity), Van der Waals interactions, solubility, ionization constant, molecular weight, electronic properties (electronegativity and polarizability) and atom, bond, ring, functional group counts; and pharmacological descriptors, which comprise of toxicity, binding interactions, biological target, anti-pathogenic capabilities and activity (IC_{50}, EC_{50}, etc.). The focus of this project was atom, bond, ring, and functional

group counts, which allows for ligand fingerprinting using Tanimoto similarity coefficients[69,70].

Ligand fingerprinting is a method of representing chemical data of a molecule in numerical form. Each compound in this project (forty-eight experimental compounds and ninety-two literature compounds) was broken down into chemical fragments or bits, which are atoms, functional groups, covalent bonds, rings, etc. The calculated value of similarity between two ligand fingerprints is termed a Tanimoto similarity score, which is calculated with the following formula[70] (bits are set to one when the chemical fragment is present in the molecules and are assigned zero when the chemical fragment is not present):

$$T = \frac{N_c}{N_a + N_b - N_c}$$

where T is the Tanimoto score, N_a is the number of bits set to one in the fingerprint of ligand a, N_b is the number of bits set to one in the fingerprint of ligand b and N_c is total number of bits set to one found in the fingerprints of both ligand a and b. A Tanimoto score is designated a value between zero and one with zero indicating no similarity and one indicating 100% similarity. A Tanimoto score $\geq$ 0.7 is indicative of two molecules having high structural similarity, which is indicative of similar biological activity.

As explained, each inhibitor compound is broken down into chemical fragments. When the fragment is present, the compound is assigned a value of one. If it absent, it is assigned a value of zero. All of these numbers are averaged, yielding a similarity score between zero and one. Compounds were characterized

according to similarity within their structures, forming various clusters in activity space (each cluster varying in numbers of experimental and literature structures). The fragments are aligned on the top axis of a cluster map. Each compound extracted from the literature is analyzed for these fragments. Each compound (experimental and literature) is aligned on the y-axis and compared to the chemical fragments. When these clusters were mapped employing Tanimoto similarity coefficients in comparison to the experimental set, black spots indicated a strong correlation between structures; red spots indicated a weak correlation and varying levels of correlation existed in between (Figure 5.2).

Upon analysis of the cluster map, many of the compounds extracted from the literature were similar to each other, meaning they have many of the same chemical fragments (Figure 5.3). Further examination uncovered four structures that were similar to the forty-eight hit compounds (Figure 5.3). Compound identification numbers are blocked due to conflictions with patents.

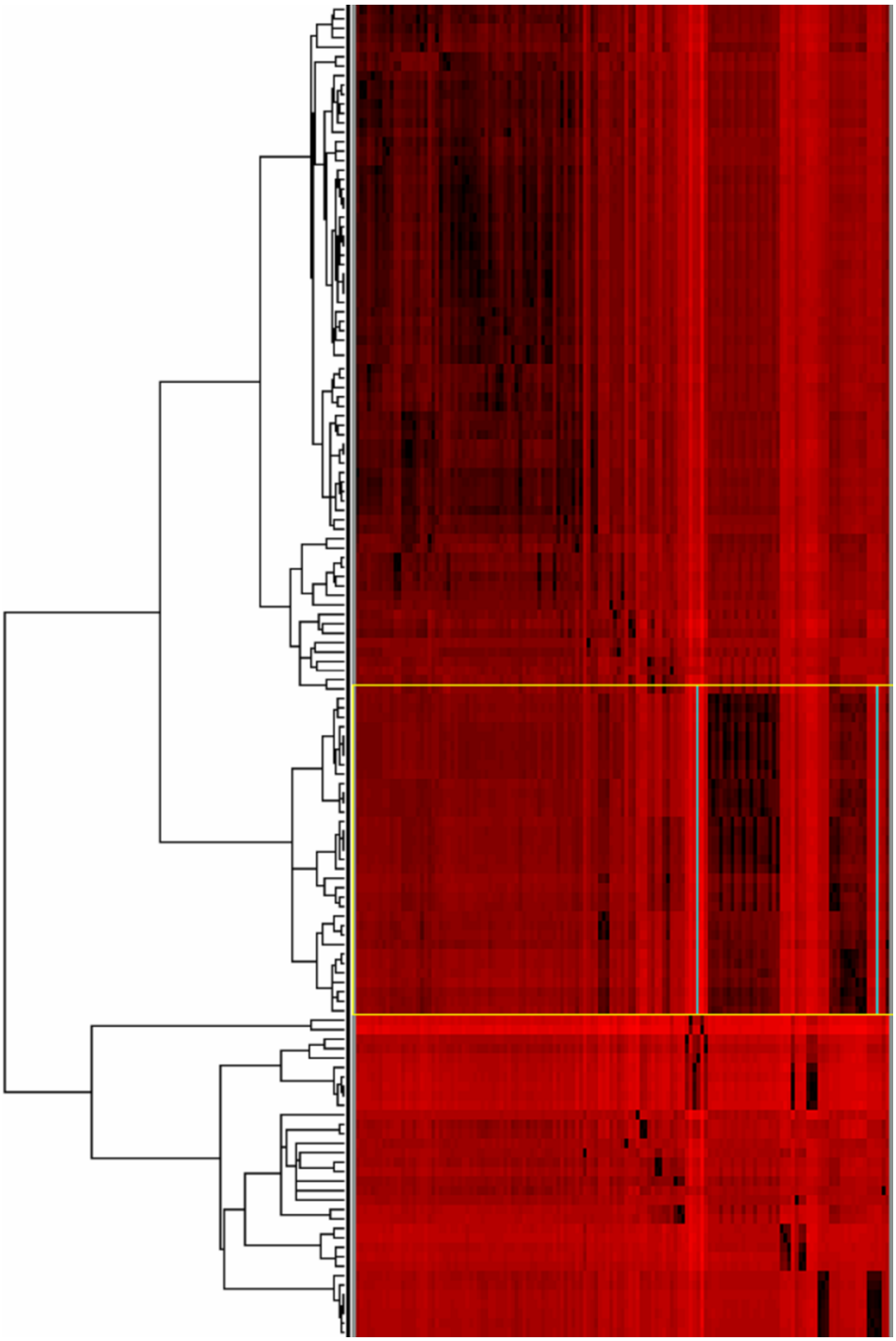

Figure 5.2. Clustering the molecules in activity space utilizing Tanimoto similarity coefficients. The experimental and literature compounds are aligned along the Y-axis and the chemical fragments or bits are aligned the X-axis. Black squares represent active compounds (similar structures to tested compounds). Red squares represent mostly literature compounds and are assumed 'inactive'.

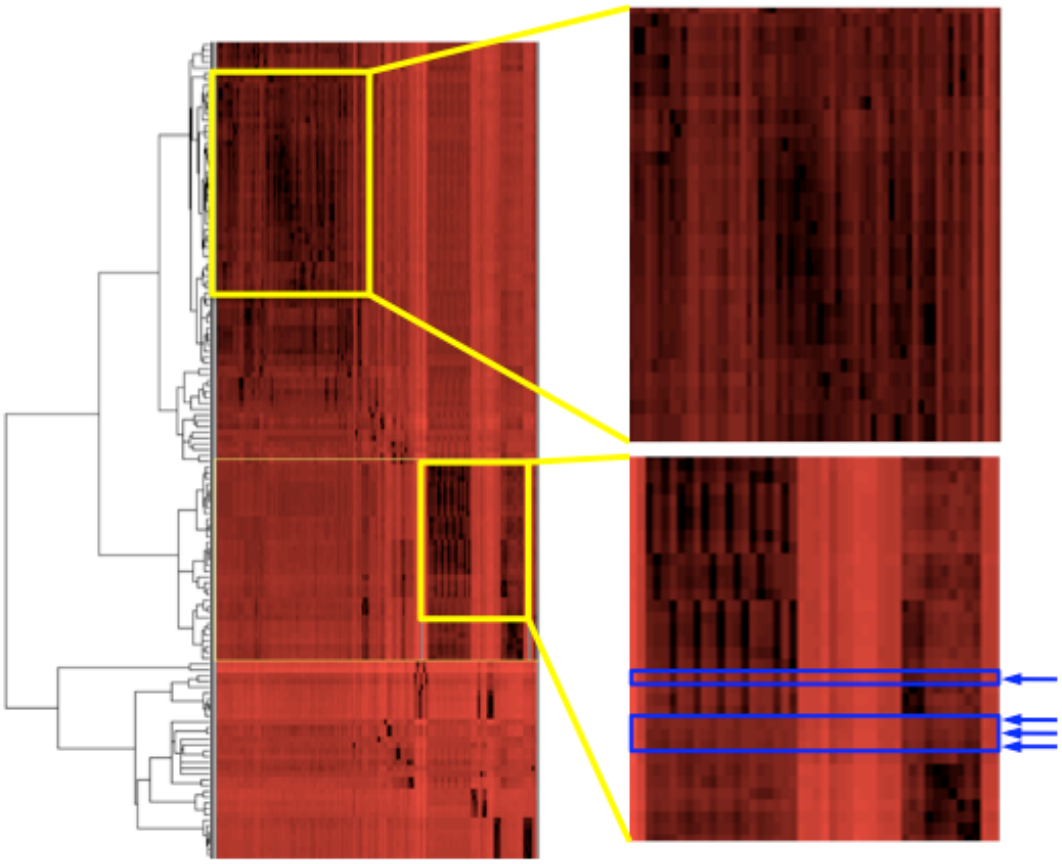

Figure 5.3. **Sections of the cluster map.**

All of the compounds (experimental and literature) are aligned along the Y-axis of the cluster map. The chemical fragments (or bits) are aligned along the X-axis. Closer observations of the upper left section with many black spots reveals the similarity amongst the literature compounds. The middle right section with many black spots reveals four structures from the literature that have high similarity to the experimental hits, which are indicated with blue arrows and boxes.

AutoQSAR Analysis Predicts Activity Based on Structure Similarity

Quantitative structure-activity relationship (QSAR) analysis predicts biological activity based on structure similarity, assuming there is a correlation between molecular structure and biological activity[68,69]. Therefore, a correlation is made between molecular properties or descriptors, including type of atoms, type of bonds, ring structures, volume of the molecule, electronic and steric factors and topology of the molecules. For this type of analysis to work, biological activity needs to be available. IC_{50} values from the hit compounds were used as a basis for this prediction method, again using the MOE program.

Compounds from the literature that were intentionally kept inactive for mapping purposes are designated projected activities based on descriptor and activity space (i.e. chemical fragments). The predicted activities can be ranked and prioritized; the most likely inhibitor candidates have a higher calculated value. The four compounds shown in the cluster map to be similar to the hit compounds had calculated activity values higher than 0.5 (0.5 would correlate to a percent inhibition of 50%). Twelve other compounds also were shown to have predicted activities greater than 0.5 (table 5.1). Due to the fact that these compounds were not commercially available, they were disregarded for the time being.

Molecule Identifying #	Assigned Activity	Calculated Activity
84	0	0.5043
85	0	0.5449
86	0	0.5500
87	0	0.5500
88	0	0.5500
90	0	0.5649
91	0	0.5735
92	0	0.5735
93	0	0.5821
95	0	0.6097
102	0	0.6524
103	0	0.6524
108	0	0.6537
124	0	0.6856
125	0	0.6868
130	0	0.7346

Table 5.1 . AutoQSAR (PLS) results.

Molecules extracted from the literature 'intentionally' kept inactive show projected activity based on chemical descriptor/activity space. The higher the calculated value, the more promising is the candidate.

Screening for and testing of Commercially Available Compounds

The goal of this project was to attempt to find compounds that were already easily obtainable, preferably commercially available. The SciFinder search and AutoQSAR produced some new, interesting potential hits, but obviously did not entirely correspond with our demands. Therefore, I took the three scaffolds in our original search and ran the search again, but this time searching under the following conditions: returning structures as organics and all others, single components, one or more reference available and commercially

available compounds only. This process yielded a total of thirteen compounds: one from scaffold one, three from scaffold two and ten from scaffold three. Two were purchased from scaffold two and five were purchased from scaffold three. For some compounds, the exact compound was not purchased due to cost effectiveness, but one very similar in its place was purchased.

The PMB screening assay was utilized to determine the IC_{50} values of the purchased compounds against the RecA protein. Of the seven compounds tested, none were found to be active, meaning all had IC_{50} values well above 250 μM.

Conclusions

Thus far, this collaborative project has not generated any new potential hits against the RecA protein. One can assume the reason for this is the lack of negative hits within the search. The only data available to us during at the time was the structures of the seventy-three hit compounds. The structures of the other three thousand five hundred and three compounds were unobtainable. Molecular modeling is only truly effective when accurate models can be created, which requires negative hits to distinguish between subtleties within structures that may alter the effectiveness of the ligand to the target.

Currently, we have more data for lead compounds screened against RecA, as well as structures of negative hits (including the seven screened here and from other screens). Other libraries and compounds that have been screened include the LOPAC library (1281 compounds, 17 hits, 0.7% hit rate), the NCI library (2104

compounds, 3 hits, 0.14% hit rate), the Asinex library (hits only, 30 hits, 0.07% hit rate), the Harvard ICCB library (55 compounds, 6 hits, 16% hit rate) and natural products (13 compounds, 6 hits, 46% hit rate). Structural activity relationship studies were also done on two of the common scaffolds in the seventy-three hits from the library from the BRITE center (also the unique scaffolds extracted with MOE). This study composed of sixteen compounds synthesized by Dr. Anna Gromova in our laboratory, which produced seven hits at a 44% hit rate.

With all of this new data compiled, we can create a new database of our own consisting of 3565 compounds, 123 hits and 3442 negatives. This would be an excellent foundation to begin this project at step one. New scaffolds can be abstracted and searched in SciFinder, then the hits can be selected for translation in to SMILES format, which can then be clustered in activity space in search of new potential hits. MOE can give initial hits, but perhaps another method of model generation, such as *kNN* (k-nearest neighbor algorithm), can be implemented to really narrow down hits before they are tested in the laboratory in order to maximize time and cost effectiveness.

This project has immense probability to unearth new potential inhibitors of RecA. The time and cost saved will also be invaluable. Hopefully in the not too distant future, the collaborative efforts between the Singleton laboratory and the Tropsha laboratory will be exhibit fruitfulness in our efforts to discover RecA inhibitors and to the fights against bacterial resistance mechanisms.

CHAPTER VI

CONCLUSIONS

The introduction of antibiotics was by far one of the most crucial medical advancements made in the twentieth century. Upon their launch into society, the lives of many were spared, especially those who fought in World War II. However, when penicillin was finally given to patients, Fleming himself warned about the implications of underdosage that so easily leads to resistance. Over the past fifty years or so, many antibiotics have been misused or over-prescribed to patients thus leading to the rise in antibiotic resistant strains of infectious bacteria. Regretfully, at the close of the twentieth and opening of the twenty-first century, antibiotic resistance has risen to exorbitant proportions and those infectious diseases that claimed the lives of so many in the early twentieth century are once again an immense concern. Funding for antibacterial research is dwindling due to excessive expense of research, the short-comings of target based design and the short shelf life of antibiotics[projan]. As demonstrated with the rise of resistance, bacteria are highly adaptable and have a remarkable capability of eliminating their susceptibility to antibiotics. Therefore, merely killing bacteria with new chemical agents is no longer the answer. New strategies need to be implemented.

Theoretically speaking, if the mechanisms by which bacteria become resistant, inhibiting these mechanisms may just be the answer to this crisis.

Acquired resistance, occurring in only select isolates of a particular species, can be spread to other bacteria through mobile genetic elements or can be developed through *de novo* mutations. Since the RecA protein has been implicated in both of these processes (by aiding homologous recombination of resistant genes into the host genome and through SOS induction leading to mutagenesis)[4,5,7, 19-35], it is a novel target for combating antibiotic resistance. Hindering RecA's involvement in DNA repair, horizontal gene transfer and stress-induced SOS mutagenesis could potentially lead to a drastic reduction in the spreading of resistant genes and increased efficacy of current antibiotics that normally would have lost their therapeutic effects. Dr. Tim Wigle was able to test various classes of compounds for inhibitory effects against the RecA protein, which included metal cations, nucleotide analogs, and small drug-like molecules[Wigle]. One of the most promising candidates, designated A1, came from a Biogen Indec compound library. A1 was shown to inhibit the RecA-mediated SOS induction when administered to live bacteria in conjunction with ciprofloxacin and increased the toxicity of ciprofloxacin when added to cultures, thus rendering the bacteria unable to survive by inactivating RecA and therefore DNA repair pathways and SOS mutagenesis.

Since this compound proved to be a promising candidate to inhibit RecA and therefore resistance mechanisms, I decided to study this compound in a prolonged assay that would observe not just the viability of cells with A1 (meaning the first eight hours of culture), but for one-hundred hours, which would give more time to observe resistance onset. I chose chloramphenicol to start, since

experiments have been well characterized with resistance assays in our laboratory and it serves as a vital control experiment, since chloramphenicol resistance development does not necessarily utilize RecA. After conducting the one-flask resistance assay with chloramphenicol, upon administration of sub-lethal dosages of chloramphenicol, resistance to this antibiotic was observed in *E. coli* MG1655 wild type and Δ*recA* cells. This result does clearly indicate that resistance to chloramphenicol is RecA independent. However, when A1 was added to the cells, resistance development was not observed in wild type or Δ*recA* cells. This result is rather perplexing, since A1 was clearly demonstrated to have inhibitory effects on the RecA protein, yet is able to prevent resistance development to an antibiotic that does not require the utilization of RecA within the cell. Thus, it may be conjectured that A1 is effective in preventing antibiotic resistance without a RecA effect.

To further study A1 and possibly shed more light onto this somewhat contradictory data, the classic Griffith experiment was applied to *E. coli* cells. Assuming that *E. coli* is able to uptake exogenous DNA (as demonstrated by various research groups[39-45]), adding the supernatant of heat-killed chloramphenicol resistant cells would theoretically contain DNA encoding chloramphenicol resistance which would be absorbed in to the cell and incorporated into the host's genome by a RecA dependent process. If this is able to occur, then resistance development to chloramphenicol would be observed at a faster rate than cells not in the presence of the heat-killed resistant cells. This proved to be true when the one-flask resistance assay was conducted. As a

negative control, Δ*recA* cells were used. This result supported the hypothesis that RecA would be needed to incorporate the resistant genes, since Δ*recA* cells developed resistance to a sub-lethal dosage of chloramphenicol with the heat-killed resistant cells at the same rate as the cells without the heat-killed resistant cells. However, this still does show that RecA is not needed to develop resistance to chloramphenicol in general. The results of A1 added to the cultures further supports this conclusion. Again, A1 was able to attenuate the resistance development of MG1655 wild type and Δ*recA* cells to chloramphenicol, whether in the presence of heat-killed resistant cells or not. A1 is potent in regards to hindering resistant development, however, it is disappointing to find that it may not be a RecA inhibitor as originally hypothesized.

Lastly, the final project of this thesis involved the mining of compounds found in the literature for potential inhibitors of RecA. Collaborating with Dr. Alexander Tropsha and Dr. M. Karthikayan in the UNC Eshelman School of Pharmacy, this was done by taking the hit compounds from the screen of the Biogen Indec library and extracting the common scaffolds which were input into SciFinder to obtain similar compounds, assuming that a positive correlation exists between chemical structure and biological activity[68,69]. From the structures obtained from the literature, similarity calculations were performed, as well as predictions based on the crude IC_{50} values from the hit compounds to predict possible activity of the compounds. Disappointingly, the compounds predicted to have the best activity were not commercially available. However, upon more searching, commercially available compounds were purchased and tested.

Unfortunately, the compounds were shown to be inactive against the RecA protein. Despite this fact, more libraries have been screened and can be utilized in the search. The project may still prove to be successful in finding inhibitors of RecA with the addition of the compounds from other libraries.

The studies in this work may be taken further by applying other antibiotics to the resistance assays. Since ciprofloxacin was shown to have its toxicity greatly enhanced by the presence of A1 in viability assays, perhaps in resistance assays, the development of resistance will still be attenuated. Perhaps it will not if A1 inhibits RecA independent mechanisms of resistance only. Other antibiotics would be interesting to study as well. Further elucidation of A1's actions within the cells would be valuable in understanding its mode of action and therefore perhaps a new means of inhibiting antibiotic resistance, whether through RecA inhibition or not. A1 is a significant breakthrough in the battle against infectious diseases and should be further scrutinized.

REFERENCES

1. Fleming, A. (1929) On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzæ*. *British Journal of Expirimental Pathology 10 (31*), 226–36.

2. Chain, E., Florey, H. W., Gardner, A.D., et al. (1940) Penicillin as a chemotherapeutic agent. *Lancet Aug 24*, 226-231.

3. Alexander Fleming's Nobel Laureate speech, December 11th, 1945 [From *Nobel Lectures, Physiology or Medicine 1942-1962*, Elsevier Publishing Company, Amsterdam, 1964]

4. Cirz, R. T., Gingles, N. and Romesberg, F. E. (2006) Side effects may include evolution, *Nature Medicine 12*, 890-1.

5. Clatworthy, A. E., Pierson, E. and Hung, D. T. (2007) Targeting virulence: a new paradigm for antimicrobial therapy, *Nature Chemical Biology 3*, 541-548.

6. Projan, S. J. (2003) Why is big Pharma getting out of antibacterial drug discovery?, *Current Opinion in Microbiology* 6, 427-30

7. McDermott, P. F., et al. (2003) Antimicrobials: modes of action and mechanisms of resistance, *International Journal of Toxicology 22*, 135-143.

8. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2002) *Molecular Biology of the Cell, 4th ed*. Published by Garland Science, New York, NY.

9. Nikaido, H. (1989) Outer membrane barrier as a mechanism of antimicrobial resistance, *Antimicrobial Agents and Chemotherapy 33 (11),* 1831-1836.

10. Ehrlich, J., Barts, Q. R., Smith, R. M., Joslyn, D. A. and Burkholder, P. R., (1947) Chloromycetin, a new antibiotic from a soil actinomycete, *Science 106*, 417.

11. Brock, T. D. (1961) Chloramphenicol, *Bacteriological Reviews 25(1),* 32-48.

12. Pages, J., James, C. and Winterhalter, M. (2008) The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria, *Nature Reviews in Microbiology 6*, 893-903.

13. Li, X. and Nikaido, H. (2004) Efflux-mediated drug resistance in bacteria, *Drugs 64(2),* 159-204.

14. Elkins, C. A. and Nikaido, H. (2002) Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominately by two large periplasmic loops, *Journal of Bacteriology 184(23),* 6490-6498.

15. Poole, K. (2005) Efflux-mediated antimicrobial resistance, *Journal of Antimicrobial Chemotherapy 56,* 20-51.

16. Ettayebi, M., Prasad, S. M. and Morgan, E. A (1985) Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*, *Journal of Bacteriology 162(2)*, 551-557.

17. Trieu-Cuot, P., de Cespedes, G., Bentorcha, F., Delbos, F., Gaspar, E. and Horaud, T. (1993) Study of heterogeneity of chloramphenicol acetyltransferase (CAT) genes in Streptococci and Enterococci by Polymerase Chain Reaction: characterization of a new CAT determinant, *Antimicrobial Agents and Chemotherapy 37(2)*, 2593-2598.

18. Schwarz, S., Kehrenberg, C., Doublet, B. and Cloeckaert, A. (2004) Molecular basis of bacterial resistance to chloramphenicol and florfenicol, *FEMS Microbiology Reviews 28(5)*, 519-542.

19. Roca, A. I., and Cox, M. M. (1997) RecA protein: structure, function, and role in recombinational DNA repair, *Progress in Nucleic Acid Research and Molecular Biology 56*, 129-223.

20. Kowalczykowski, S. C. and Eggleston, A. K. (1994) Homologous pairing and DNA strand-exchange proteins, *Annual Review of Biochemistry 63*, 991-1043.

21. Bjedov, I., Tenaillon, O., Gerard, B., Souza, V., Denamur, E., Radman, M., Taddei, F. and Matic, I. (2003) Stress-induced mutagenesis in bacteria, *Science 300*, 1404-9.

22. Schlacher, K., Pham, P., Cox, M. M. and Goodman, M. F. (2006) Roles of DNA polymerase V and RecA protein in SOS damage-induced mutation, *Chemical Reviews 106*, 406-19.

23. Lusetti, S. L. and Cox, M. M. (2002) The bacterial RecA protein and the recombinational DNA repair of stalled replication forks, *Annual Review of Biochemistry 71*, 71-100.

24. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. and Rehrauer, W. M. (1994) Biochemistry of homologous recombination in Escherichia coli, *Microbiological Reviews 58*, 401-65.

25. Donaldson, J. R., Courcelle, C. T. and Courcelle, J. (2006) RuvABC is required to resolve holliday junctions that accumulate following replication on damaged templates in *Escherichia coli*, *Journal of Biological Chemistry 281*, 28811-21.

26. Hastings, P. J., Rosenberg, S. M. and Slack, A. (2004) Antibiotic-induced lateral transfer of antibiotic resistance, *Trends in Microbiology 12*, 401-4.

27. Furuya, E. Y. and Lowy, F. D. (2006) Antimicrobial-resistant bacteria in the community setting, *Nature Reviews Microbiology 4*, 36-45.

28. Giese, K. C., Michalowski, C. B. and Little, J. W. (2007) RecA-dependent cleavage of LexA dimers, *Journal of Molecular Biology 377*, 148-161.

29. Little, J. W. (1991) Mechanism of specific LexaA cleavage - autodigestion and the role of RecA coprotease, *Biochemie 73(4)*, 411-422.

30. Little, J. W. (1982) Control of the SOS regulatory system by the level of RecA protease, *Biochimie 64*, 585-9.

31. Crowley, D. J. and Hanawalt, P. C. (1998) Induction of the SOS response increases the efficiency of global nucleotide excision repair of cyclobutane pyrimidine dimers, but not 6-4 photoproducts, in UV-irradiated *Escherichia coli*, *Journal of Bacteriology 180*, 3345–3352.

32. Sassanfar, M. and Roberts, J. W. (1990) Nature of the SOS-inducing signal in *Escherichia coli*. The involvement of DNA replication, *Journal of Molecular Biology 212*, 79–96.

33. Michel, B. (2005) After 30 years of study, the bacterial SOS response still surprises us, *PLoS Biology 3*, e255.

34. Dajkovic, A., Mukherjee, A. and Lutkenhaus, J. (2008) Investigation of regulation of FtsZ assembly by SulA and development of a model for FtsZ polymerization, *Journal of Bacteriology 190 (7)*, 2513-2526.

35. Fuchs, R. P., Fujii, S. and Wagner, J. (2004) Properties and functions of Escherichia coli: Pol IV and Pol V, *DNA Repair and Replication 67*, 229-264.

36. Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. and Collins, J. J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics, *Cell 130*, 797-810.

37. Griffith, F. (1981) The significance of pneumococcal types. *Reviews of Infectious Diseases, 3(2)*, 372-395. [Reprinted from (1928) *The Journal of Hygiene 27*, 113-159]

38. Avery, O. T., MacLeod C. M. and McCarty M. (1979) Studies on the chemical nature of the substance inducing transformation of pneumococcal types-induction of transformation by a deoxyribonucleic-acid fraction isolated from pneumococcus type-III, *Journal of Experimental Medicine 149 (2)*, 297-319. [Reprinted from (1944) *The Hospital of the Rockefeller Institute for Medical Research*, New York, NY, 137-158].

39. Panja S., Aich, P., Jana, B. and Basu, T. (2008) How does plasmid DNA penetrate cell membranes in artificial transformation process of Escherichia coli?, *Molecular Membrane Biology 25(5)*, 411 422.

40. Dreiseikelmann, B. (1994) Translocation of DNA across bacterial membranes, *Microbiological Reviews 58*, 293-316.

41. Claverys, J. and Martin, B. (2003) Bacterial 'competence' genes: signatures of active transformation, or only remnants?, *TRENDS in Microbiology 11(4)*, 161-165.

42. Maeda, S., Sawamura, A. and Matsuda, A. (2004) Transformation of colonial *Escherichia coli* on solid media, *FEMS Microbiology Letters 236*, 61-64.

43. Maeda, S., Ito, M., et al. (2006) Horizontal transfer of nonconjugative plasmids in a colony biofilm of *Escherichia coli*, *FEMS Microbiology Letters 255*, 115-120.

44. Baur, B., Hanselmann, K. and Jenni, B. (1996) Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence, *Applied and Environmental Microbiology 62(10)*, 3673-3678.

45. Sun, D., Zhang, Y., et al. (2006) *Escherichia coli* is naturally transformable in a novel transformation system, *FEMS Microbiology Letters 265*, 249-255.

46. Weber, T. P., Widger, W. R. and Kohn, H. (2003) Metal dependency for transcription factor rho activation, *Biochemistry 42*, 1652-9.

47. Weber, T. P., Widger, W. R. and Kohn, H. (2003) Metal-1,4-dithio-2,3-dihydroxybutane chelates: novel inhibitors of the Rho transcription termination factor, *Biochemistry 42*, 9121-6.

48. Brogan, A. P., Verghese, J., Widger, W. R. and Kohn, H. (2005) Bismuth-dithiol inhibition of the *Escherichia coli* rho transcription termination factor, *Journal of Inorganic Biochemistry 99*, 841-51.

49. Yu, X., Horiguchi, T., Shigesada, K. and Egelman, E. H. (2000) Three-dimensional reconstruction of transcription termination factor rho: orientation of the N-terminal domain and visualization of an RNA-binding site, *Journal of Molecular Biology 299*, 1279-87.

50. Cline, D. J., Holt, S. L. and Singleton, S. F. (2007) Inhibition of *Escherichia coli* RecA by rationally redesigned N-terminal helix, *Organic and Biomolecular Chemistry 5 (10)*, 1525-1528.

51. Lee, J. W. and Cox, M. M. (1990) Inhibition of RecA protein promoted ATP hydrolysis. 2. Longitudinal assembly and disassembly of RecA protein filaments mediated by ATP and ADP, *Biochemistry 29*, 7677-83.

52. Wigle, T. J., Lee, A. M. and Singleton, S. F. (2006) Conformationally selective binding of nucleotide analogues to *Escherichia coli* RecA: a ligand-based analysis of the RecA ATP binding site, *Biochemistry 45*, 4502-13.

53. Yamazaki, J., Horii, T., Sekiguchi, M. and Takahashi, M. (2003) Regulation of RecA protein binding to DNA by opposing effects of ATP and ADP on inter-domain contacts: Analysis of urea-induced unfolding of wild-type and C-terminal truncated RecA, *Journal of Molecular Biology 329*, 363-70.

54. Ellouze, C., Selmane, T., Kim, H., Tuite, E., Norden, B., Mortensen, K. and Takahashi, M. (1999) Difference between active and inactive nucleotide cofactors in the effect on the DNA binding and the helical structure of RecA filament dissociation of RecA-DNA complex by inactive nucleotides, *European Journal of Biochemistry 262*, 88-94.

55. Wigle, T. J. and Singleton, S. F. (2007) Directed molecular screening for RecA ATPase inhibitors, *Bioorganic & Medicinal Chemistry Letters 17*, 3249-3253.

56. Ohta, T., Watanabe, M., Shirasu, Y. and Inoue, T. (1988) Post-Replication Repair And Recombination In Uvra Umuc Strains Of Escherichia-Coli Are Enhanced By Vanillin, An Antimutagenic Compound, *Mutation Research 201*, 107.

57. Shaughnessy, D. T., Schaaper, R. M., Umbach, D. M. and Demarini, D. M. (2006) Inhibition of spontaneous mutagenesis by vanillin and cinnamaldehyde in *Escherichia coli*: Dependence on recombinational repair, *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 602*, 54.

58. Oda, Y. (1995) Inhibitory effect of curcumin on SOS functions induced by UV irradiation, *Mutation Research Letters 348*, 67.

59. Yang, Y. X. and Fix, D. (2006) Genetic analysis of the anti-mutagenic effect of genistein in *Escherichia coli*, *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 600* , 193.

60. Knight, K. L. and McEntee, K. (1985) Affinity labeling of a tyrosine residue in the ATP binding-site of the RecA protein from *Escherichia coli* with 5'-para-fluorosulfonyl-benzoyladenosine, *Journal of Biological Chemistry 260*, 10177-10184.

61. Scholz, G., Barritt, G. J. and Kwok, F. (1992) Affinity labeling of the active-site of brain phosphatidylinositol 4-kinase with 5'-fluorosulphonylbenzoyl-adenosine, *European Journal of Biochem*istry *210*, 461.

62. Burlison, J. A., Neckers, L., Smith, A. B., Maxwell, A. and Blagg, B. S. (2006) Novobiocin: redesigning a DNA gyrase inhibitor for selective inhibition of Hsp90, *Journal of the American Chemical Society 128*, 15529.

63. Corbett, K. D. and Berger, J. M. (2006) Structural basis for topoisomerase VI inhibition by the anti-Hsp90 drug radicicol, *Nucleic Acids Research 34*, 4269.

64. Wang, Y., Wagner, M. B., Kumar, R., Cheng, J. and Joyner, R. W. (2003) Inhibition of fast sodium current in rabbit ventricular myocytes by protein tyrosine kinase inhibitors, *Pflügers Archiv 446*, 485.

65. Lambrecht, G., Braun, K., Damer, M., Ganso, M., Hildebrandt, C., Ullmann, H., Kassack, M. U. and Nickel, P. (2002) Structure-activity relationships of suramin and pyridoxal-5 '-phosphate derivatives as P2 receptor antagonists, *Current Pharmaceutical Design 8*, 2371.

66. Jacobson, K. A., Costanzi, S., Ohno, M., Joshi, B. V., Besada, P., Xu, B., and Tchilibon, S. (2004*)* Molecular recognition at purine and pyrimidine nucleotide (P2) receptors, *Current Topics in Medicinal Chemistry 4*, 805.

67. Guyer, M. S., Reed, R. R., Steitz, J. A. and Low, K.B. (1980) Identification of a sex-factor-affinity site in *Escherichia coli* as gamma-delta, *Cold Spring Harbor Symposia on Quantitative Biology 45*, 135-140.

68. Johnson, M. A. and Maggiora, G. M. (1990) Concepts and applications of molecular similarity; John Wiley and Sons: New York, NY.

69. Haranczyk, M. and Holliday, J. (2008) Comparison of similarity coefficients for clustering and compound selection, *Journal of Chemical Information and Modeling 48(3),* 498-508.

70. Tetkk, I. V., Sushko, I., Pandey, A. K., Zhu, H. and Tropsha, A. (2008) Critical assessment of QSAR models of environmental toxicity against tetrahymena pyriformis: focusing on applicability domain and overfitting by variable selection, *Journal of Chemical Information and Modeling 48 (9),* 1733–1746.

71. Lee, A. M., Wigle, T. J. and Singelton S. F. (2007) A complementary pair of rapid molecular screening assays for RecA activities, *Analytical Biochemistry 367(2),* 247-258.

72. Singleton, S. F., Roca, A. I., Lee A. M. and Xiao, J. (2007) Probing the structure of RecA-DNA filaments. Advantages of a fluorescent guanine analog, *Tetrahedron 63(17),* 3553-3566.

73. Xiao, J., Lee, A. M. and Singleton, S. F. (2006) Direct evaluation of a kinetic model for RecA-mediated DNA-strand exchange: The importance of nucleic acid dynamics and entropy during homologous genetic recombination, *Chembiochem 7(8),* 1265-1278.

74. Lee, A. M. and Singeton, S. F. (2006) Intersubunit electrostatic complementarity in the RecA nucleoprotein filament regulates nucleotide substrate specificity and conformational activation, *Biochemistry 45(14),* 4514-4529.

75. Xiao, J., Lee, A. M. and Singleton, S. F. (2006) Construction and evaluation of a kinetic scheme for RecA-mediated DNA strand exchange, *Bipoymers 81(6),* 473-496.

76. Lee, A. M., Ross, C. T., Zeng, B. B. and Singleton, S. F. (2005) A molecular target for suppression of the evolution of antibiotic resistance: Inhibition of the Escherichia coli RecA protein by N-6-(1-naphthyl)-ADP, *Journal of Medicinal Chemistry 48(17),* 5408-5411.

77. Lee, A. M. and Singleton, S. F. (2004) Inhibition of the *Escherichia coli* RecA protein: zinc(II), copper(II) and mercury(II) trap RecA as inactive aggregates, *Journal of Inorganic Biochemistry 98(11),* 1981-1986.

78. Roca, A. I. and Singleton, S. F. (2003) Direct evaluation of a mechanism for activation of the RecA nucleoprotein filament, *Journal of the American Chemical Society 125(50),* 15366-15375.