SPECIFIC FUNCTIONS OF THE SOS RESPONSE PARTICIPATE IN SURVIVAL TO FLUOROQUINOLONES

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ABSTRACT OF DISSERTATION

Persisters are bacterial cells that are able to tolerate antibiotic treatment without acquiring heritable resistance. It has previously been shown that the SOS-regulated toxin TisB induces persister formation by forming a channel in the inner membrane, dissipating the proton motive force and reducing the concentration of intracellular ATP. However, this mechanism is wasteful because depletion of the proton motive force will activate futile respiration and exhaust the cell’s stored nutrients, leaving the cell unable to resuscitate. Previous studies have shown that DNA damage leads to the cessation of respiration, leading to the possibility that this phenomenon may complement the action of TisB. We are interested in discovering the cause of respiration shutdown, and identifying its role in survival to antibiotics. We have found that in both a recA deletion strain and lexA3 mutant strain DNA damage does not lead to cessation of respiration, indicating that this shutoff is dependent on an active SOS response. To identify the mechanism of respiration shutoff, two genetic screens were performed using an indicator of respiration to identify mutants lacking this shutoff phenotype. Analysis of the protein composition in respiring culture versus non-respiring cultures was performed using 2D gel electrophoresis. This information can be further used to increase our understanding on the mechanism behind this shutoff and its significance.

This work also looks at the role of repair in antibiotic tolerance. The processes outlined above will stop growth and prevent further damage, however in order for these responses to be generated there must be DNA damage present for the SOS response to be induced. However, without an active repair system these cells would not be able to
survive and resuscitate because of the incurred damage. Mutations resulting in deficiency in SOS induction and repair of DSBs either completely abolish or lead to extremely low persistence to FQs but do not affect persistence to other types of antibiotics, proving that repair is necessary even in TisB-dependent non-growing cells. We have analyzed knock-outs of all known SOS genes [1, 2] in order to identify other SOS functions crucial for persister formation. We have found that the absence of DinG, UvrD and RuvAB lead to a sharp decrease in the surviving fraction at high and low concentrations of the antibiotic, confirming that the repair processes these proteins catalyze are essential for persistence. In addition, the inactivation of recF leads to an increase in persistence, identifying the RecFOR recombination pathway as a poisoning mechanism. We have shown before that the persistence to FQs is a result of an induced, active process [3]. Here we confirm that all the cells get damaged and also show that the same repair mechanism is taking place in the entire population.
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LIST OF ABBREVIATIONS

2DE: 2-dimensional gel electrophoresis
2D-DIGE: 2-dimensional differential gel electrophoresis
ATP: adenosine triphosphate
Cat: chloramphenicol resistant cassette
Cip/Cipro: ciprofloxacin
CFU: colony forming units
DTT: dithiothreitol
dsDNA: double-stranded deoxyribonucleic acid
DSBs: double stranded breaks
EMS: ethyl methanesulfonate
ETC: electron transport chain
FACS: fluorescence-activated cell sorting
FQ: fluoroquinolone
GFP: green fluorescent protein
IEF: isoelectric focusing
IPG: immobilized pH gradient
Kan: kanamycin
KO: knockout
MIC: minimum inhibitory concentration
MMC: mitomycin C
NAD: nicotinamide adenine dinucleotide
OD: optical density
OE: overexpressing
ORF: open reading frame
PCR: polymerase chain reaction
PMF: proton motive force
Rif: rifampicin
RNA: ribonucleic acid
TA: toxin/antitoxin module
TCA: tricarboxylic acid cycle
UVR: ultra violet radiation
CHAPTER 1 INTRODUCTION

1.1 Persister Cells

In face of a potentially lethal stress, different phenotypes of genetically identical bacterial cells can make a difference between life and death. A clonal population of cells of a strain genetically susceptible to an antibiotic can harbor tolerant phenocopies which will survive the antibiotic challenge (Figure 1-1). These cells are termed persisters and are of special interest because of their ability to influence the outcome of antibiotic treatments [4-6]. Upon reinoculation into fresh medium, persister cells are able to grow and repopulate the culture. After retreatment, the culture exhibits a similar number of persister cells, demonstrating that persisters are phenotypic variants rather than resistant mutants. Persister cells have been observed in all bacterial species observed thus far [7], and have also been observed in Candida albicans biofilms [8].

Persister cells were first observed in 1944 by Joseph Bigger in a culture of Staphylococcus aureus treated with the beta lactam penicillin [9]. Bigger noticed that the culture was unable to be sterilized by the antibiotic, and established that the survivors were not resistant mutants but rather phenotypic variants of the wild type that were able to survive treatment. More recently, persister cells have been shown to be present in biofilms [8, 10-12]. Biofilms have been implicated in many chronic infections, such as wound injuries, infections of medical device implants, and in the cystic fibrosis lung. Persister cells are protected from the immune system within the biofilm matrix, and are able to repopulate the matrix once antibiotic therapy has been discontinued, causing a relapsing infection (Figure 1-2).
Figure 1-1. Persister cells differ from resistant cells upon addition of antibiotic.

Persister cells are a subpopulation that are able to survive in the presence of antibiotic and are able to reform the population after the antibiotic has been removed. Resistant cells are able to grow in the presence of the drug due to loss or mutation of the antibiotic target.

Indeed, when looking at clinical isolates, late isolates have been found to form more persisters than early isolates in yeast [13], *Pseudomonas aeruginosa* [5], *Escherichia coli* (unpublished) and *Mycobacterium tuberculosis* (unpublished).

1.2 Mechanisms of antibiotic tolerance

The first mechanism of persister formation was identified in the 1980s during a search for mutants of *E. coli* that exhibited high survival to ampicillin. The mutation was mapped to *hipA*, the toxin of the *hipBA* toxin-antitoxin pain [14]. Two
Figure 1-2. Model of biofilm tolerance to antibiotic treatment due to persister cells.

Treatment with antibiotics kills both planktonic and biofilm cells (colored blue). The immune system is able to kill planktonic persister cells (pink) that are able to survive the antibiotic treatment. The persister cells (pink) within the biofilm are able to evade the immune system and survive treatment. Once the treatment has been discontinued and the drug has been removed, the persister cells are able to repopulate the biofilm. Adapted from [4].

Point mutations in hipA (hipA7 allele) cause a 1,000-10,000 fold increase in persisters levels. However, a knockout of hipA or hipBA does not have a persister phenotype, leaving the role of the wild type hipA in persister formation unknown [15, 16].
Initially, it was thought that HipA increased survival via phosphorylation of the essential translation factor EF-Tu. This lead to stasis due to the inability of EF-Tu to bind aminoacyl-tRNA [17]. However, it has recently been shown that the target of HipA is glutamyl-tRNA synthetase (GltX) [18]. The activation of HipA phosphorylates Gltx, leading to an accumulation of uncharged tRNAGLU. This uncharged tRNAGLU will then enter the ribosomal A site and trigger the activation and release of RelA. The activation of RelA will lead to an increase in the concentration of (p)ppGpp, and will result in inhibition of transcription, translation and cell wall synthesis. The induction of the stringent response has been shown to lead to an increase in persisters [19].

There have been many other studies whose aim has been to identify persister genes. Shah et al. used a strain of E. coli which expressed unstable GFP under the control of a growth-rate dependent promoter [20]. Using FACS, they found that the dim population was enriched for ofloxacin-tolerant persister cells, thus showing that persisters may have a slower rate of translation. The RNA from isolated persister cells was analyzed using microarrays to detect differences in expression between the persister cells, exponentially growing cells, and stationary cells. Approximately 420 up-regulated genes were identified, while roughly the same number was down-regulated. Unexpectedly, the transcriptome of persister cells more closely resembled that of exponential cells, rather than stationary cells. Shah et al. identified a number of genes that were likely to contribute to the persister phenotype that were shown to be up-regulated in persister cells. This group included a number of toxin-antitoxin (TA) modules (chp, relE, dinJ/yafQ, yefM/lyoeB and ygiUT), stationary genes (bolA, hdeA, katE) and glycerol metabolism genes (glpD, glpQ). The authors delved further into the role of the TA modules, as
overexpression of HipA [21] and RelE [22] have been shown to produce an antibiotic tolerant state. However, Shah et al. found that no single knockout, or attempted combinations of deletions, was able to produce a strain with fewer persisters compared to the wild type. This has been a common result when studying the role of TA modules in persister formation, as strain with 8 TAs was not found to have a phenotype (unpublished). More recently however, a strain with 10 TAs deleted has shown a 10 fold decrease in persisters [19].

A number of screens have also been performed to identify genes involved in persister formation. Spoering et al. used an overexpression library to screen for strains with increased survival to ampicillin [23]. One of the genes identified was glpD, which encodes the aerobic glycerol-3-phosphate dehydrogenase. Overexpression of GlpD increased survival to ampicillin, while a glpD knockout reduced persister levels. However, in no deletion or combination of deletions did any one strain completely lack persisters. This study suggests that glycerol metabolism plays a part in the formation of persisters, although the exact role is unclear. Recently, glycerol metabolism has been found to also play a role in persister formation in M. tuberculosis (Heather Topley et al., unpublished).

In addition to the above overexpression screen, two screen of deletions libraries have been performed to identify genes necessary for persister formation. One of the screens, which used a P. aeruginosa transposon library, failed to identify any mutants that completely lacked persisters [24]. However, one of the hits with a moderate phenotype was the SOS helicase DinG. The second study performed by Hansen et al. screened an ordered knockout library of E. coli ORFs [15], and also failed to identify any
mutants that formed no persisters in response to ofloxacin. Interestingly, there were a number of SOS-dependent repaired genes identified in this screen, but these genes were not considered for further work in this study.

The lack of a mutant that is unable to form persisters brought to the surface an important question about the nature of persister cells. At the time, the prevailing view in the field was that persisters are multidrug tolerant cells that are constantly present in the population. These cells are formed by stochastic means [25-27]. However, different concentrations of the same antibiotic (Figure 1-3) or different antibiotics do not result in the same number of persisters (Figure 7-1). Dorr et al. set out to answer these questions by increasing our understanding of the mechanism of persister formation to the fluoroquinolone (FQ) ciprofloxacin [28]. To differentiate between preexisting persisters and those induced by the antibiotic, the number of persisters were measured in different backgrounds with an altered capacity for SOS induction or for double strand break repair. They found that the majority of persisters were induced by the antibiotic, and the formation of persisters was dependent on the SOS response. The importance of the SOS response and DNA repair has also been shown in uropathogenic isolates of E. coli [29] as well as in E. coli biofilms [30]. In a separate paper, Dorr et al. identified one mechanism important for the formation of persisters, the SOS-dependent, type I toxin TisB [31]. TisB has been found to form anion-selective pores in the membrane [32], depleting the proton motive force and leading to a decrease in intracellular ATP levels. This drop in energy causes the cell to stop growth and enter a dormant-state. However, a deletion in TisB does also not result in a complete lack of persisters, and in fact has no phenotype with low to moderate levels of SOS induction, indicating that there are additional
mechanisms to ensure survival to FQs. This discovery that the SOS response was responsible for persister formation to FQs led us to question what specific components of this stress response pathway were the most crucial to survival.

Figure 1-3. Persister level is dependent on the concentration of antibiotic used.

When exponentially growing cultures of *E. coli* are treated with varying concentrations of the FQ antibiotic ciprofloxacin, the level of persisters varies. When strains are treated with a higher dose of ciprofloxacin, there are fewer persister formed than when treated with a lower dose, even when all concentrations are above the minimum inhibitory concentration (MIC) and all strains are in the same growth phase. The MIC of *E. coli* to ciprofloxacin is 0.01 µg/ml.
1.3 Survival to Fluoroquinolones and the SOS Response

Bacteria have developed sophisticated responses allowing them to adapt to and survive a variety of environmental stresses. These stress responses involve damage sensing and ultimately the activation or repression of transcriptional regulons, either through alternative sigma factors subunit of RNA polymerase, or through DNA-binding two-component response regulators. The physiological state of the cell is adjusted to the one optimal for repair by rearranging the metabolic fluxes and the expression of repair proteins.

It follows that in a non-synchronized bacterial culture, which is not in the state of balanced growth, there will be two sources of phenotypic diversity: the number of target molecules and the state of the stress response dealing with a specific damage. Hence we expect a wide distribution of different states, from the most susceptible – high concentration of the target and no expression of the stress response, to the most resistant - very few target molecules and the high expression of the stress response. Therefore in contact with an antibiotic the entire population will not experience the same amount of damage nor will each cell be able to repair the damage to the same extent. When put in a specific time frame, it means that different subpopulations will be killed at different times and it is also possible that there will be a subpopulation which will survive. This is reflected in a typical biphasic killing curve which is observed following the survival of a bacterial population exposed to an antibiotic. The surviving population is the persister fraction.

In the case of FQ antibiotics, the targets are gyrase and topoisomerase IV [33]. Upon FQ binding these enzymes become endonucleases introducing DSBs into the
bacterial chromosome [34]. Cells can repair DSBs by largely relying on the DNA-damage inducible SOS gene network [35, 36]. The susceptibility to the FQ therefore depends on the cellular concentration of the active gyrase and topoisomerase molecules and the state of the induction of the SOS response. The SOS gene network is controlled by the LexA repressor, and LexA regulated genes exhibit heterogeneous expression in a culture not subject to an external SOS-inducing treatment [37]. The autocleavage of the LexA repressor is activated by the formation of ssDNA-RecA filaments, allowing LexA to disassociate from the DNA, inducing expression of the SOS response (reviewed in [38]. The heterogeneity of expression is a result of stochastic factors resulting from the binding affinity of LexA to different SOS boxes and intrinsic DNA damage [39, 40]. Combined heterogeneity of the expression of the target and the repair mechanism translates into a wide spectrum of phenotypic states and hence susceptibility to a FQ.

We have examined mutants in all known SOS-regulated genes to identify functions essential for tolerance in *Escherichia coli*. The absence of 4 repair proteins, DinG, UvrD, RuvA and RuvB were found to have a decrease in persister levels. Analysis of the respective mutants indicates that in addition to repair of double strand breaks, tolerance depends on the repair of collapsed replication forks and stalled transcription complexes. Taken together, these results show that active repair is necessary for long term survival.

1.4 The History of Respiration Shutoff

Although we have found that repair of the damage plays a key role in long term survival to FQs, [28], alternative damage prevention pathways exist, most likely to
protect cells from incurring more damage and allow the current damage to be repaired. One damage prevention pathway is dependent on the SOS regulated tisAB/istR toxin antitoxin locus, which has been shown to contribute to persister formation [31]. When overproduced, membrane peptide TisB decreases proton motive force and intracellular ATP levels [32]. During SOS induction, the expression of TisB may induce stasis and prevent killing by the antibiotic. However, TisB only influences persister formation at high ciprofloxacin concentration, therefore it is not the only mechanism of FQ tolerance. A mechanism that possibly coincides with the action of TisB is cessation of respiration after DNA damage. Disassociation of the proton motive force causes the rate of respiration to increase to compensate for the sudden drop in ATP. This is a wasteful mechanism because the cell’s internal store of nutrients is depleted, making resuscitation after the removal of drug difficult. We hypothesize that the combination of TisB and the unknown mechanism of respiration shutoff causes the cell to stop growing, allowing it to better survive DNA damaging agents by preventing further damage from occurring. By stopping growth, these mechanisms help prevent further damage and allow the cell to repair the breaks already acquired.

Cessation of respiration due to ultra violet radiation (UVR) was first documented in the early 1950s by Albert Kelner as he sought to identify the mechanism of photoreactivation [41]. It was not until the late 1960s that researchers began looking into the phenomenon [42-44]. The first experiments showed that UVR induced the stoppage of aerobic respiration by measuring presence of oxygen in the culture. (Figure 1-3).

Different experiments showed that protein translation was necessary for the cessation of respiration [42]. Cultures were treated before UVR with either
chloramphenicol, which prevents protein chain elongation by inhibiting the peptidyl transferase activity of the ribosome, or with 5-fluorouracil, which inhibits the cell’s ability to synthesize DNA. Pretreatment by both compounds allowed respiration to continue, illustrating that the mechanism of respiration shutoff is a protein whose expression is induced by the damage. [42].

The role of DNA repair in respiration shutoff was also studied. A strain able to repair the damage (WP2) was compared to a repair deficient strain, known as rec− at the time. This gene would later be named recA, and its importance in the induction of the SOS response and its role in homologous recombination would later be discovered. When experiments were performed using the rec− strain, Swenson and Schenley found that oxygen was still being consumed by the cell [45]. The SOS repair hypothesis was first proposed in 1975 by Miroslav Radman [35], but was not connected to respiration shutoff until 1983. Using RecA mutants, one study performed by Barbé et al. found that only the proteolytic function of RecA was required for respiration shutoff [46], and that induction of the SOS response was not required. However, this same group also found that mitomycin C did not induce the SOS response. There was no follow-up study. In 1985, it was found that RecBCD activity was also required for respiration shutoff. More specifically, it was the ATP-dependent double-strand exonuclease activity that was required for shutoff [47].
Figure 1-4. Original experiment showing the cessation of respiration after UV radiation. Roughly 80 minutes after a culture of *E. coli* is irradiated, oxygen is no longer being consumed. Adding cassamino acids to the culture reduces the length of the cessation. Adapted from [42].

This activity is most likely required in order to process the break so RecA can be loaded onto the DNA, thus inducing the SOS response. RecF was also shown to be required for the respiration phenotype [48], which also could be induced by mitomycin C [49]. Other factors that may play a role are cyclic adenosine monophosphate (cAMP) [50], whose addition increases the length of respiration shutoff, or the loss of nicotinamide adenine dinucleotides (NAD+) to the medium during respiration shutoff [51]. However, the details behind these potential mechanisms were never uncovered.
Figure 1-5. Respiration cessation is caused by varying doses of UVR in the wild type and a rec- mutant. Different doses of UVR have different effects on the length of respiration shutoff. Different *E. coli* strains show different phenotypes to each dose, however the phenotype is seen in both backgrounds. A *rec-* mutant does not fully stop consuming oxygen with any dose of UV. Adapted from [45].

In 1995, 10 years after the most recent respiration cessation paper had been published, another group published that the RecA-mediated 2-keto-4-hydroxyglutarate aldolase (KHG), encoded by *eda*, was required for recovery of respiration [52]. This group found that RecA and LexA, while not necessary for the cessation of respiration,
were necessary for the recovery of the cells. It was also shown that KHG was not the only protein required for recovery, and that additional proteins regulated by the SOS response were also required. Additional proteins necessary for recovery were never identified. For all the work that was done to understand this phenomenon, the mechanism of respiration shutoff was never identified. Indeed, there is also contradictory data for the requirements of respiration shutoff.

The role of aerobic respiration has never been connected to the formation of a tolerant state. Many studies have showed a link between different metabolic pathways and persister formation (reviewed in [53]). Although weak links have been established between energy metabolism and persister formation [54, 55], the role of aerobic respiration has not been studied. We reasoned that a drop in energy levels caused by respiration shutoff, such as those seen by the overexpression of TisB, would result in a drug tolerant state due to the lack of available ATP.

1.5 Dissertation Aims

The aim of this dissertation is to further examine the specific functions of the SOS response and understand how those functions contribute in the survival to FQs. The second chapter of this dissertation will focus on the role of repair in survival. More specifically, the role of DinG, UvrD and RuvAB will be examined. The remaining chapters will focus on understanding damage prevention through respiration shutoff. Chapter 3 will examine the basic requirements necessary for respiration shutoff, and show how this phenotype can protect against a different class of antibiotic. The
remaining chapters describe different attempts to identify and describe the mechanism of respiration shutoff.
CHAPTER 2 TOLERANCE OF ESCHERICHIA COLI TO FLUOROQUINOLONE ANTIBIOTICS DEPENDS ON SPECIFIC COMPONENTS OF THE SOS RESPONSE PATHWAY

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2.1 Abstract

Bacteria exposed to bactericidal fluoroquinolone (FQ) antibiotics can survive without becoming genetically resistant. Survival of these phenotypically resistant cells, commonly called persisters, depends on the SOS gene network. We have examined mutants in all known SOS-regulated genes to identify functions essential for tolerance in Escherichia coli. The absence of DinG and UvrD helicases and Holliday junction processing enzymes RuvA and RuvB lead to a decrease in survival. Analysis of the respective mutants indicates that in addition to repair of double strand breaks, tolerance depends on the repair of collapsed replication forks and stalled transcription complexes. Mutation in recF results in increased survival, which identifies RecAF recombination as a poisoning mechanism that has not previously been linked to FQ lethality. DinG acts upstream of SOS promoting its induction, whereas RuvAB participates in repair only. UvrD directly promotes all repair processes initiated by FQ-induced damage and prevents
RecAF-dependent misrepair making it one of the crucial SOS functions required for tolerance

2.2 Introduction

In the face of a potentially lethal stress, the presence of phenotypic variants of genetically identical bacterial cells can make a difference between survival of the lineage and extermination. A clonal population of cells of a strain genetically susceptible to an antibiotic can harbor tolerant phenocopies that will survive the antibiotic challenge. These cells are termed persisters and are of special interest because of their ability to influence the outcome of antibiotic treatments [5, 7]. Toxins from chromosomally encoded toxin-antitoxin (TA) modules have been shown to play a role in persister formation under certain condition [26, 31, 56] To date, screening of E. coli mutant libraries have yielded no single mutants that completely lack persisters [23, 57]. These studies have shown that there are many pathways that lead to persister formation [7]. We have shown that tolerance to fluoroquinolones (FQs) antibiotics depends on a functional SOS response [3]. The SOS regulated \( \text{tisAB/istR} \) toxin antitoxin locus contributes to tolerance to ciprofloxacin [31]. When overproduced, the membrane peptide TisB decreases proton motive force and intracellular ATP levels [32]. During SOS induction, \( \text{tisB} \) is overexpressed, which may induce stasis and prevent killing by the antibiotic. However, it only influences tolerance at high ciprofloxacin concentration (100X minimal inhibitory concentration, MIC), therefore it is not the only mechanism of FQ tolerance.
Fluoroquinolones target gyrase and topoisomerase IV [33]. Upon FQ binding these enzymes become endonucleases introducing DSBs into the bacterial chromosome [34]. Cells repair DSBs largely through the DNA-damage-inducible SOS gene network [35, 36]. The susceptibility to the FQ therefore depends on the cellular concentration of the active gyrase and topoisomerase molecules and the state of induction of the SOS response.

Gyrase and topoisomerase IV are essential during replication and transcription [58] and their maximal amount in a cell is expected during maximal growth rate. Indeed, \textit{gyrA} and \textit{gyrB}, structural genes of DNA gyrase, reach maximal expression in the early exponential growth phase and decrease during subsequent growth, attaining the lowest level in stationary phase [59, 60]. The susceptibility to FQs would be expected to parallel to this dynamic and experimental data confirm this; the bactericidal effect of FQs is the largest in early exponential phase and the lowest in stationary phase [3, 26].

The SOS gene network is controlled by the LexA repressor, and LexA regulated genes exhibit heterogeneous expression in a culture not subject to an external SOS-inducing treatment [37]. The heterogeneity of expression is a result of stochastic factors resulting from the binding affinity of LexA to different SOS boxes and intrinsic DNA damage [39, 40, 61]. Combined heterogeneity of the expression of the target and the repair mechanism translates into a wide spectrum of phenotypic states and hence varied susceptibility to a FQ.

In this study we have analyzed knock-outs of all known SOS genes [1, 2] in order to identify the SOS functions crucial for tolerance. We have found that the removal of DinG, UvrD and RuvAB lead to the formation of fewer persisters at high and low
concentrations of the antibiotic, confirming that the repair processes these proteins catalyze are essential for tolerance. In addition, the inactivation of recF leads to an increase in tolerance, identifying the RecFOR recombination pathway as a poisoning mechanism.

2.3 Results

We have previously shown that tolerance to FQs depends on SOS response [3]. At high FQ concentrations, the component of the SOS response responsible for tolerance is the tisB/lstR toxin/antitoxin (TA) module [31]. Recently it has been reported that deletion of the Lon protease, which regulates the antitoxins of 10 TA modules, leads to a decrease in tolerance to a FQ [62]. Given that SulA, a cell division inhibitor induced during the SOS response, is also a substrate of the Lon protease [63], we decided to reexamine this issue. SulA proteolysis is essential for resuming cell division after repression of the SOS response, which would suggest that decreased tolerance of a lon deletion strain might have resulted from unchecked production of SulA, rather than from the inability of these cells to degrade antitoxins. We measured tolerance to ciprofloxacin in a lon mutant and confirmed that it is indeed low. However, the deletion of sulA in a lon- background restored tolerance to the wild type level, showing that most of the loss of tolerance to a FQ in a lon- mutant results from SulA-blocked cell division upon SOS induction (Figure 2-1). This experiment also confirmed that the persister fraction depends on a functional SOS response. The level of persisters surviving treatment with a β-lactam antibiotic was unaffected in lon, sulA and a double mutant, consistent with the specificity of the SOS-dependent mechanism of tolerance to FQs (data not shown)
Figure 2-1. Survival of SulA and Lon mutants after ciprofloxacin challenge.

Strains were challenged with 0.1 μg/ml ciprofloxacin for 6 hours in exponential phase. Colony forming units (CFU) counts were determined by plating. The data are averages of 3 independent experiments and error bars indicate standard error.

To identify other functions of the SOS response besides tisB/lstR, important for FQ tolerance, we compared the survival of deletion mutants of SOS-regulated genes [1, 2] to that of the wild type following time-dependent killing in cultures treated with ciprofloxacin at 10X MIC (minimal inhibitory concentration) (Table 1, Figure 7-2).
### Table 2-1. Genes Regulated by the LexA Repressor

<table>
<thead>
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<tbody>
<tr>
<td>dinB</td>
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<td>uvrA</td>
<td>yceP</td>
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<td>uvrB</td>
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<td>ybfE</td>
<td>yfjE</td>
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<tr>
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<td>sulA</td>
<td>ybiA</td>
<td>yfjF</td>
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<tr>
<td>hlyE</td>
<td>symE</td>
<td>ybiB</td>
<td>yfjG</td>
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<tr>
<td>hokE</td>
<td>umuC</td>
<td>ycaJ</td>
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<td>lit</td>
<td>umuD</td>
<td>ybtE</td>
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Deletions of *dinG*, *uvrD*, and *ruvA* showed 13-, 434-, and 12-fold decrease in tolerance, respectively (Figure 2-2A-C). All of the deletion mutants have the same ciprofloxacin MIC as the wild type. This shows that the decrease in tolerance in these mutants is not due to an intrinsic increase in susceptibility to the antibiotic. The decrease in tolerance is also not growth phase dependent as all three mutants show low persister levels in both exponential and stationary phase (Figure 2-2D-F).
Figure 2-2. Survival of the wild type and mutant strains in exponential and stationary phase. (A-C) Exponentially growing cells were treated with 0.1 μg/ml ciprofloxacin for 6 hours. Survival was determined by dilution and plating to count CFU. The graph represents at least 20 independent experiments and error bars represent standard error. (D-F) Stationary phase cultures were treated with 1 μg/ml ciprofloxacin. Viable counts were determined by plating. The graphs represent three independent experiments. Standard error is indicated by the error bars. Wt, wild type

DinG and UvrD are both DNA helicases. DinG can remove R-loops and promote replication across transcription units [64, 65]. UvrD catalyzes unwinding of forked DNA structures [66] and the dismantling of RecA nucleoprotein filaments [67], allowing
various repair processes such as nucleotide excision repair [68], mismatch repair [69], and replication fork restart [70]. DinG and UvrD both participate in the removal of stalled RNA polymerase (RNAP) [65]. RuvA forms a complex with RuvB and RuvC, which resolves Holliday junctions (HJ) formed during the recombinational repair of damaged DNA [71]. All of these repair processes involve ssDNA intermediates. ssDNA is the SOS inducer so the activities of DinG, UvrD and RuvA can potentially influence SOS induction [72]. While the production of these proteins increases during SOS induction, they are all present in an uninduced cell. Therefore DinG, UvrD and RuvA can promote tolerance either through influencing SOS induction or through the repair functions they perform, or both. In order to distinguish among these possibilities, we measured the persister fraction of the respective deletion mutants in a strain where the SOS regulon is derepressed constitutively (lexA (Def)). In this genetic context, the dinG deletion had no effect on persister levels. When SOS functions are expressed prior to the addition of FQ, DinG is dispensable for tolerance (Figure 2-3A). This suggests that DinG promotes tolerance mainly through facilitating SOS induction upon FQ treatment. Single deletions of both uvrD and ruvA decreased the persister level even in a lexA(Def) background (Figure 2-3B-C). Loss of UvrD decreased tolerance 23-fold in lexA(Def), compared to the ~430 fold decrease in a lexA+ background. Therefore, UvrD appears to promote tolerance through stimulating SOS induction and by an additional route, likely participating in recombinational repair of DSBs and restarting of stalled replication forks. The loss of RuvA lowered tolerance in both backgrounds, 57-fold and 12-fold, suggesting that RuvA does not play a role in SOS induction during FQ treatment. These results suggest that RuvA promotes tolerance solely through its role in repair.
Survival of *dinG*, *uvrD* and *ruvAB* mutants in a strain constitutively expressing SOS. Exponentially growing cells were treated with 0.1 μg/ml ciprofloxacin. Mutants were made in a strain constitutively expressing SOS (lexA(Def)). Graphs represent 10 independent experiments and error bars represent standard error.

To quantify SOS induction directly we measured the induction of β-galactosidase under the control of the SOS inducible recA promoter using various concentrations of ciprofloxacin. A *dinG* mutant has a deficient SOS response compared to the wild type across a range of ciprofloxacin concentrations (Figure 2-4A). In contrast, the induction level in a *uvrD* mutant parallels the wild type (Figure 2-4B) except for the higher basal expression of the SOS response, which is consistent with previous studies [73]. These measurements report the average SOS induction of the entire population. We next looked into SOS induction in single cells using flow cytometry in order to probe for differences in the distribution of SOS induction levels across the population in different mutant backgrounds following exposure to ciprofloxacin. We measured the expression of green fluorescent protein (GFP) under the control of the *sulA* promoter, another SOS-inducible
gene. In the wild type, the majority of cells exhibited a strong SOS response after 120 minutes of ciprofloxacin treatment (Figure 2-5A), reflected in a narrow distribution of fluorescence values. This pattern changed little during the next 120 minutes. In the dinG knockout after initial 120 minutes of treatment there was a much wider distribution of induction states across the population with fewer highly induced cells compared to the wild type and with many cells showing little or no SOS induction (Figure 2-5B). The pattern did not change during an additional 120 minutes, suggesting that the defect in induction is not due to a slower response. These results confirmed that the removal of DinG leads to the impairment of the overall SOS induction, its effect ranging from delaying to abolishing induction.

The difference between the wild type and the uvrD knockout was less pronounced than between dinG and the wild type (Figure 2-5C). The basal level of SOS induction was higher, as expected [73]. At 120 and 240 minutes there were more medium to highly induced cells compared to the wild type. The ruvA mutant was undistinguishable from the wild type in both assays, further confirming the role of RuvABC complex in repair processes not influencing the SOS induction (Figure 7-3).
Figure 2-4. SOS induction during ciprofloxacin treatment. Induction of SOS response was measured by assaying for β-galactosidase activity in strains carrying lacZ under control of recA SOS-inducible promoter after 15 min of treatment with ciprofloxacin with the concentration indicated on x-axis (A) a dinG mutant and a (B) UvrD mutant. Graphs are an average of at least 3 experiments and error bars represent standard error.
Figure 2-5. Single cell analysis of SOS induction during ciprofloxacin treatment.

Induction of SOS response was measured using a flow cytometry by following GFP expression in strains carrying the gfp gene under control of sulA SOS-inducible promoter. Exponential phase cells were treated with 0.1 μg/ml ciprofloxacin. 10,000 cells were analyzed before ciprofloxacin treatment and again after 120 and 240 minutes for (A) wt, (B) dinG mutant and (C) an uvrD mutant.
RuvAB catalyzes the late step in recombination, branch migration [74], and together with RuvC, the resolution of HJ [75]. Therefore, a *ruvAB* mutant is expected to be as deficient in repair as RecA or RecBC mutants that catalyze the initial steps in recombination, which is not the case. In a *ruvAB* mutant, HJ can be resolved by an alternative pathway catalyzed by RecG, which is not part of the SOS regulon.

Figure 2-6. The role of Holliday junction processing in persistence to fluoroquinolones. Exponentially growing cultures of cells were exposed to 0.1 μg/ml ciprofloxacin. CFU counts were determined by serially dilution and then plating. Error bars represent standard error. Graphs represent ten independent experiments.
We combined *ruvAB* with a *recG* mutant, and the resulting strain is indeed as deficient in survival as a *recBC* mutant, confirming that complete recombinational repair is needed for tolerance (Figure 2-6). However it is not clear whether SOS-induced level of RuvAB is needed for tolerance or the basal level is sufficient.

Stalled gyrase and the resulting DSBs create replication and transcription blocks. Transcription blocks cause RNAP to stall and its removal leads to the formation of R-loops. In turn, R-loops can cause replication stalling [76]. The DSBs and stalled replication fork cannot be processed until the RNAP dissociates and the resulting R-loop has been removed. By removing RNAP (together with UvrD) and R-loops, DinG [65] could allow the processing of the break or stalled replication fork, formation of ssDNA and ultimately SOS induction. If that is the case, the role of DinG in tolerance should correlate with the overall transcription level in the cell. The more transcription complexes there are, the more survival should depend on DinG. To test this we measured the tolerance in cultures subjected to different growth regimes. The overall transcription level is proportional to the growth rate, as the bulk of transcription takes place at ribosomal operons [77]. We manipulated the growth rate and hence the transcription levels by growing cells in different media: rich MHB medium, MOPS minimal medium with glucose or glycerol as a sole carbon source, and at different temperatures: 37°C and 28°C. We measured the persister levels in wild type and a *dinG* deletion mutant. In rich medium, a *dinG* mutation decreased tolerance 22-fold, compared to 5.2- and 2.2-fold in minimal media with glucose and glycerol, respectively (Figure 2-7A). Slowing down growth by decreasing temperature from 37°C to 28°C obviates the need for DinG function in tolerance altogether (Figure 2-7B). In addition, the effect of a *dinG* mutation
Figure 2-7. Role of DinG in survival in different growth conditions. Exponentially growing cultures of dinG mutant and wild type were treated with 0.1 μg/ml of ciprofloxacin for six hours, then CFU counts were determined by serial dilution and plating. (A) Level of persistence in different minimal media; the medium type and generation time in minutes are indicated on the x-axis. (B) Different temperature regimes after six hours of treatment. Error bars represent standard error and graphs represent four independent experiments.

is less pronounced in stationary phase compared to exponential phase (Figure 2-2A, 2-2D). This is consistent with lower transcription levels in stationary phase. These results are consistent with the role of DinG in allowing DSB processing by RNAP and R-loop removal.

UvrD exerts quality control over the RecA-dependent recombination intermediates and also inhibits the formation of RecF-dependent RecA filaments [67].
RecF mutation increases survival, suggesting that RecF-dependent recombination is indeed happening during the processing of FQ-induced lesions and is toxic. As with the RecBCD pathway, the RecF pathway depends on RecA. To confirm this we combined a recA deletion with a recF deletion. The survival of the double mutant was as low as recA single mutant, confirming that the RecF effect is due to its interaction with RecA (Figure 8A). In an uvrD background, recF increases the survival, but not to the level of a single recF mutant, confirming that aside from preventing RecF-dependent toxic recombination, UvrD has an additional role in surviving FQ treatment (Figure 8B). Given the extent of the recF effect, this additional role is stronger than the action of UvrD as a RecF counteractor.

Because it participates at all stages of DSB repair, UvrD could indeed be the single most important SOS function for tolerance to FQs. In order to test this, we have cloned the uvrD gene onto a low copy vector under the control of an IPTG-inducible promoter (Figure 2-9). First, we introduced this plasmid into an uvrD deletion strain in order to show that it complements the uvrD defect. Next, we introduced it into a strain carrying the lexA3 mutation. This strain is unable to induce SOS and shows a low tolerance phenotype. In the resulting strain (lexA3 carrying the plasmid with IPTG-inducible uvrD gene) uvrD is the only SOS gene that is overexpressed upon the addition of IPTG into growth medium prior to ciprofloxacin treatment. In this strain the tolerance was increased to the level of the complemented strain, confirming that UvrD could indeed be the crucial component of the SOS gene network needed for tolerance to FQ antibiotics.
Figure 2-8. The role of RecF in survival to ciprofloxacin treatment. Cells were diluted and grown to late exponential phase and then treated with 0.1 μg/ml of ciprofloxacin. Data are from at least twenty independent experiments and error bars represent standard error.
Figure 2-9. UvrD overproduction suppresses SOS deficiency. Exponentially growing cultures were treated with 0.1 mM IPTG to induce UvrD vector expression 30 minutes prior to the addition of 0.1 μg/ml ciprofloxacin. Graphs are averages of at least five experiments and error bars represent standard error.

2.4 Discussion

Bacteria can survive antibiotic treatments by becoming resistant through a genetic change; mutation or gene acquisition. Mechanisms of resistance have been studied extensively and most cases are well understood. On the other hand, antibiotic tolerance, involving no genetic change, has only recently been recognized as a potentially important factor influencing the efficiency of existing antibiotic therapies [5]. The molecular mechanism(s) leading to this epigenetic phenomenon have become a subject of intense research.
In the case of fluoroquinolone antibiotics, tolerance largely depends on the SOS gene network [3], a cellular stress response induced by DNA damage. Mutations resulting in deficiency in SOS induction and repair of DSBs either completely abolish or lead to extremely low tolerance to FQs, but do not affect tolerance to other types of antibiotics. Type I TA module tisABlistR has a role in FQ tolerance, although only at very high antibiotic concentrations [31]. It is a part of the SOS gene network but its precise role has not been established. In this work we have identified additional SOS genes needed for FQ tolerance regardless of the antibiotic concentration.

Recently it was shown that the deletion of the gene encoding Lon protease, the regulator of the antitoxin component of 10 TA modules, results in a low tolerance phenotype, arguing for the major role of these modules in tolerance [62]. We confirmed that tolerance is reduced in the absence of Lon protease. However, inactivation of sulA completely suppresses lon tolerance deficiency during ciprofloxacin treatment (Figure 1). FQs are potent inducers of the SOS response, therefore during FQ treatment SulA is produced and blocks cell division during the repair process. The resumption of growth depends on SulA removal by proteolysis. In the absence of the Lon protease SulA is not degraded, leading to an irreversible cell-cycle checkpoint due to SulA-blocked cell division.

In *E. coli*, FQs target gyrase and topoisomerase IV [33]. Gyrase is essential for replication and transcription, relaxing super helical tension ahead of the progressing replication fork and transcription bubble. It cuts the DNA and the two ends are then twisted around each other and resealed to form supercoils. FQs block the resealing step and the gyrase is therefore stuck to the cut DNA strands. This reaction intermediate is
called the cleavage complex and is essentially a DNA adduct. DSBs can form by direct cleavage of this adduct by XseAB (exonuclease VII) [78] and also by a recombination nuclease initiating repair of the replication fork stalled by the cleavage complex. In both cases the free DNA ends are released. The multifunctional enzymatic complex RecBCD, essential for the repair of DSBs, initiates the repair process. It loads onto the DNA ends, unwinds the dsDNA and degrades it until it encounters the regulatory sequence (Chi site), after which it degrades one of the strands and loads RecA [79]. RecA is the main recombinase that catalyzes a DNA synapsis reaction between ssDNA and a complementary region of double stranded DNA. In the RecA-ssDNA nucleoprotein filament, RecA becomes activated, and this activated form stimulates the autocatalytic cleavage of the LexA repressor, activating the SOS response. The essential nature of these early steps in survival of FQ treatment is reflected by extremely low or totally suppressed survival of RecB and RecA mutants as well as the LexA mutant unable to undergo self-cleavage and induce SOS [3]. RecBCD is constitutively produced and RecA is under SOS control with a basal level high enough to carry out recombination. The dependence of tolerance on SOS implies that there are other functions that are needed in addition to the repair of DSBs. We have screened the deletion mutants of all known SOS genes (Table 2-1) and found that tolerance most depended on dinG, ruvAB and uvrD.

The analysis of the respective mutants and their known roles suggest that FQ treatment leads to replication fork collapse, stalling of transcription complexes and formation of DSBs. Therefore, survival ultimately depends on the interplay between the repair of DNA breaks, clearing of the transcription complexes and restarting of the replication fork. These processes depend and/or influence each other and also share some
proteins, revealing a complex cascade of events initiated by the action of a FQ.

DinG is a DNA helicase that can remove D- and R-loops in vitro [64]. In vivo studies demonstrated its role in clearing stalled transcription complexes and promoting replication across active transcription units [65].

We found that in cells with SOS genes derepressed constitutively, DinG is dispensable for tolerance, which means that it acts upstream of SOS induction (Figure 3A). This is confirmed by measuring SOS induction directly and finding that it is severely impaired in a dinG mutant upon exposure to FQ (Figures 4A, 5B). DinG stimulates SOS induction specifically during FQ treatment and has no influence on the induction resulting from other inducers such as mitomycin C (Figure 7-4). This means that it promotes the formation of the SOS inducing signal, ssDNA during processing of DSBs and has no influence on the induction resulting from the processing of single-stranded gaps. Following DSBs, ssDNA is formed by RecBCD and therefore DinG must directly or indirectly facilitate its action. Given the known roles in vitro and in vivo of both RecBCD and DinG, it is unlikely that DinG directly influences loading of RecBCD onto DNA ends or its helicase and nuclease activities.

DinG clears the stalled replication complexes in vivo by displacing RNAP and removing R-loops. In doing so it helps in the resolution of the replication/transcription collisions. By clearing the stalled transcription complexes during FQ treatment, DinG may enable RecBCD to process DSBs, release the ssDNA and hence trigger the induction of the SOS response. This is consistent with the finding that reducing overall transcription level decreases the role of DinG in tolerance (Figure 2-7).

UvrD is a multifunctional enzyme: it edits the RecA-formed recombination
intermediates, dismantling intermediates of insufficient length and also prevents the formation of RecF-dependent RecA filaments [67]. RecF helps the loading of RecA onto single stranded gaps and stabilizes the RecA-DNA filaments that form. Even though the RecA-DNA filament is the initial intermediate in recombination that is essential for repair of many types of DNA lesions, out-of-control RecA filamentation can, depending on the substrate, prevent the repair and become a poisoning mechanism. RecF/A filaments can form at stalled replication forks, where by initiating pairing of single-strand gaps they prevent the replication restart and the repair of DSBs. UvrD’s ability to prevent this toxic recombination makes it a major player in both restarting collapsed replication forks and DSB repair. The absence of UvrD decreases tolerance to FQ almost as much as RecA suggesting that quality control of recombination that UvrD exerts is crucial for survival (Figure 2-8).

We found that inactivation of RecF results in increased tolerance and that this effect is dependent on RecA (Figure 2-8A). This confirms that RecF/RecA filaments form during FQ treatment and that their formation contributes to the toxicity. recF mutation increases the survival of the uvrD mutant, showing that part of UvrD’s role in promoting survival is the suppression of RecF toxicity. Single strand gaps, which are substrates for RecF/RecA, are not introduced directly by FQ-blocked gyrase, therefore they must result from a different process. Their most likely source is the collapsed replication fork. Active gyrase is located ahead of replisome. Moving at 800 bp/sec, replication forks must run into FQ-blocked gyrase shortly after the formation of the cleavage complex, which leads to their collapse. In a different system Cirz et al. [80] showed that tolerance to FQ depends on PriA, a protein essential for replication fork restart, which is strong evidence
that replication forks do disintegrate during FQ treatment and that their repair/restart is vital for survival.

RuvA and RuvB form a complex with RuvC that then mediates branch migration and resolution of the Holliday junction, the final steps of recombination. SOS induction is not impaired in ruvA and ruvB mutants but the survival is decreased. It means that RuvAB does not participate in formation of the SOS inducing signal, but acts downstream of SOS induction. This is consistent with its role in recombinational repair either of gyrase induced DSBs, collapsed replication fork or both. Even though resolution of HJ is essential for completing repair ruvAB mutants survive better than recBC mutants that are unable to initiate recombination. In the absence of RuvAB, RecG can catalyze the resolution of HJ. The double mutant ruvA recG (as well as ruvB recG) is as sensitive as a recBC mutant, confirming that all steps of recombinational repair are needed for tolerance [3].

Taken together our findings confirm that tolerance to FQs primarily depends on the capacity of the cell to repair the DNA damage resulting from FQ action. RecA is the central protein and it has two roles: sensing the damage allowing for induction of repair proteins through the induction of the SOS network and carrying out recombination. Besides halting cell division while repair takes place, the SOS response increases the capacity for damage repair and/or tolerance. The capacity to carry out recombination is increased mainly through the elevated levels of RecA and RuvAB. However it is not clear whether SOS-induced levels of RecA and RuvAB are indeed needed for FQ tolerance or elevated levels of some other SOS functions. Both DSBs and collapsed replication forks are ultimately repaired through recombination, but during the processing
of these lesions there is a considerable potential for improper pairing, which can impede the repair and lead to cell death. The UvrD helicase exerts quality control over recombination and is therefore an essential part of the repair process. We show that increasing the amount of UvrD by overproducing it artificially is sufficient to ensure a wild type level of survival in the absence of SOS induction (Figure 2-9). This raises the possibility that UvrD is the crucial SOS function needed for tolerance to FQs, but it is not excluded that overexpression of other SOS genes or combination of genes can have the same effect.

Mutation in all gene functions which promote and suppress survival affect the bulk as well as the persister fraction. The initial rate of killing is increased in recA, recB, ruvAB, dinG and uvrD mutants and is slowed down in a recF mutant. When repair is completely suppressed (such as in recBC, ruvA recG, priA backgrounds) there are no survivors. We have shown before that the tolerance to FQs is a result of an induced, active process [3]. Here we confirm that all the cells get damaged and also show that the same repair mechanism is taking place in the entire population. In studies of survival of γ-irradiated cells, it was estimated that E. coli K-12 can successfully repair up to 4 simultaneous DSBs [81]. Therefore, the persister fraction likely consists of the cells that received between 1 and 4 DSBs and managed to repair them. Restoration of the collapsed replication fork is an essential part of successful repair indicating that at the time of the FQ treatment future persister cells are actively replicating. Cells growing exponentially with the minimal generation time (20 min in rich LB medium), have 4-8 chromosomes [82] and very high transcription activity, the bulk of it at ribosomal RNA operons [77]. Those cells have 4-8 replication forks and multiple transcription bubbles, all relying on
gyrase and topoisomerase IV to relieve the negative supercoil accumulation. They collapse when they collide with FQ induced DNA-gyrase or DNA-topoisomerase IV adducts. The amount of DSBs formed due to these collisions is beyond the cell’s repair capacity. On the other hand cells that slow down and have fewer replication forks and reduced transcription activity experience a few breaks and are able to repair them and survive, forming a persister fraction.

The question remains as to what happens after the completion of the repair. The medium is not exhausted and can support growth and the antibiotic is still present, therefore the surviving cells could resume growth and become vulnerable again, yet there is no significant loss of viability for an extended period of time. The induction of the SOS-controlled TisB toxin dissipates the proton motive force [32] and depletes the cellular ATP pool [83], possibly preventing further damage by effectively shutting down the cell after the repair of the original lesions. However, this mechanism would only operate in a fraction of cases as the survival is TisB-dependent only at high concentrations of FQ. Upon SOS induction, an uncharacterized SOS-controlled mechanism halts respiration [49, 50], which would be another way of slowing down all cellular processes and preventing further damage.

Even in the absence of this type of damage avoidance by SOS-induced slowdown or complete dormancy, the repair can probably be completed during the SOS-imposed division block. Broken chromosomes lose superhelicity in the large domains around the break so there will be a time window during the repair process and shortly after its completion, during which gyrase and topoisomerase IV are not active, thus rendering cells insensitive to FQ. When the repair is completed, SOS is shut off and superhelicity is
restored, the cells would become susceptible again. However, the damage would be repairable because it would occur before multiple replication forks and transcription bubbles form, leading to a few lesions only, not exceeding the repair capacity. In this scenario the persistent state would consist of successive rounds of low amounts of damage followed by repair.

Beside FQ antibiotics, ionizing irradiation and starvation for the DNA precursor dTTP (leading to thymineless death) also result in multiple DSBs and in both cases survival depends on RecABCD/RuvAB repairing the breaks and UvrD counteracting RecAF toxic recombination, revealing a common survival mechanism following chromosome fragmentation [84-87].

The data presented identify the main functions controlled by the SOS response in tolerance to FQ antibiotics (Figure 2-10). Upon exposure to a FQ, gyrase and topoisomerase IV are blocked and form DNA-adducts that lead to the collapse of the replication forks and stalling of the transcription complexes. Processing of these lesions leads to the formation of DSBs, which in turn results in the induction of the SOS gene network and hence the elevated levels of the proteins needed for repair. The repair consists of clearing the stalled transcription complexes, restoring replication forks and repairing the DSBs. Recombination plays a central role in the repair process but because of the type of lesions some recombination functions can interfere with each other and exacerbate cell death. The quality control of the recombination process is therefore one of the main contributors to survival. The intrinsic property of FQ induced damage, its sensing, the induction of the SOS response and the ensuing repair process make cells refractory to the FQs, rendering them tolerant without genetic change.
Figure 2-10. Model of cellular events following exposure to fluoroquinolones.

Tolerance to fluoroquinolones through the SOS response pathway involves two main pathways: damage repair and damage prevention.

2.5 Materials and Methods

Tolerance Assays

All killing experiments were conducted at 37°C in cation adjusted Mueller Hinton Broth (MHB, Teknova) buffered with 0.1 M HEPES pH 7.2 unless otherwise noted. Cultures were treated with 0.1 μg/ml ciprofloxacin, unless otherwise noted. Tolerance was tested by diluting an overnight culture of 1:100 in 3 mL of fresh MHB in 17- by 100-
mm polypropylene tubes placed at an angle on a rotating platform and growing for 1.5 hours with shaking (200 rpm), until there were approximately $2 \times 10^8$ colony forming units (CFUs) per ml. CFU counts were measured by washing cells with 1% NaCl to remove the antibiotic, serially diluting and plating on Luria-Bertani (LB) agar supplemented with 20 mM MgSO$_4$ to neutralize ciprofloxacin carry over. The colonies were counted after 40 hours incubation at 37°C. For UvrD complementation experiments, 0.1 mM IPTG was added to the diluted culture 30 minutes prior to ciprofloxacin treatment. All antibiotics and chemicals were purchased from Sigma (St. Louis, MO). For the minimal media persister assays, cultures were grown to stationary phase in minimal medium and diluted into the same medium. Cells were diluted 1:100 into MOPS minimal medium [88] with 0.2% glucose as a carbon source and grown for 2 hours at 37°C with shaking. Cells were diluted 1:50 into MOPS medium with 0.3% glycerol as a carbon source and grown for 3.5 hours under the same conditions. For experiments done at 28°C, cells were diluted 1:100 in buffered MHB and incubated for 12 hours with shaking.

**Strain Construction**

*E. coli* K-12 MG1655 was used as the parental strain (wild type) for all strain construction. Bacterial strains are listed in Table 2-2. P1 transduction was used to move different alleles from the KEIO collection [89] into MG1655. The kanamycin cassette was cured using pCP20 when needed. Precise deletions were made using the methods described in [90] into the MG1655 background. MG1655 pZS*34uvrD* was constructed by cloning the ORF of *uvrD* into the KpnI/ClaI (in bold) cloning site of pZS*34* [91] using the primers uvrDkpn
(ATTTTAAGGTACCCAGGAGGCAGCTAATGGACGTTTCTTACCTGCTCGACA
GCC) and uvrDClaI (TATTAATCGATTACCCGACTCCAGCCGGGCCTATG).

Table 2-2 Bacterial Strains

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<td>n/a b</td>
<td></td>
</tr>
<tr>
<td>MV1603</td>
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<td>AB1157 λ d(recA::lacZ)</td>
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\( cl(\text{Ind}^{-})\) AmpR

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<sup>a</sup> Transduced allele is indicated in bold.

<sup>b</sup> Not applicable

<sup>c</sup> Precise deletions were constructed using the methods described in [90].

<sup>d</sup> Transductants were selected on MOPS/maltose minimal medium, checked for UV-sensitivity and confirmed by sequencing.

<sup>e</sup> Strain was constructed by transducing the kanamycin resistant allele from the Keio Collection into MG1655 before curing the kan<sup>R</sup> cassette by expressing the flp recombinase as described in [90].

<sup>f</sup> kan<sup>R</sup> cassette was cured by expressing the flp recombinase as described in [90].

<sup>g</sup> Transductants were selected for resistance to trimethoprim.

<sup>h</sup> Plasmids were introduced via electroporation and then selected for resistance to chloramphenicol.

### Measurement of SOS Induction

To measure SOS induction by measuring β-galactosidase production of strains carrying recA::lacZ reporter fusion [96], overnight cultures were diluted 1:100 in 3 ml MHB and grown for 1.5 hours in 17- by 100-mm polypropylene tubes with shaking. An 800 μL aliquot was removed from the culture and the optical density (OD) 600 was measured. Cultures were treated with 0.1 μg/ml ciprofloxacin or 4 μg/ml mitomycin C at 37°C for ~20 minutes. Both the treated and untreated culture were measured for
*recA::lacZ* expression using the methods described in [97] with the exception that β-mercaptoethanol was not added to the Z-buffer stock, and was instead added directly to the reaction tube. Miller units were calculated as described in [97].

Flow Cytometry Analysis

An *E. coli* strain with GFP under the *sulA* promoter [37] was used to measure the induction of the SOS response by flow cytometry. Cells were grown as described above and treated with 0.1 μg/ml ciprofloxacin. Prior to the addition of antibiotic, and every 15 minutes after, 10,000 cells were analyzed using the BD Aria II SORP flow cytometer to measure GFP. Data were analyzed using Flowjo software (Treestar, Inc., San Carlos, CA).
CHAPTER 3 FLUOROQUINOLONES INDUCED RESPIRATION SHUTOFF FACILITATES TOLERANCE TO AMINOGLYCOSIDES

Alyssa Theodore, Brian Conlon and Kim Lewis

Manuscript in Preparation for Publication

3.1 Abstract

Persisters are bacterial cells that are capable of tolerating antibiotic treatment in the absence of heritable resistance. These persister cells are able to survive treatment in part by inducing stress response pathways that allow the cells to repair the damage and enter a dormant-like state to prevent the accumulation of damage. Previous work has shown that ultraviolet radiation (UVR) leads to the cessation of respiration in growing cultures of Escherichia coli. UVR leads to the induction of lesions in DNA, which induces the SOS DNA damage repair pathway. When E. coli is treated with fluoroquinolone (FQs) antibiotics, which results in double-stranded DNA breaks, persister formation is dependent on active induction of the SOS response. We reasoned that treatment with FQs would also lead to shutoff of aerobic respiration, depriving the cell of energy and causing it to shut down. Here we show the FQs do cause shutoff of aerobic respiration, and that this phenomenon is dependent on a functional SOS response. Our findings also show that shutoff of respiration can protect the cells from aminoglycoside antibiotics, indicating that persister cells formed using this mechanism are tolerant to other classes of antimicrobials.
3.2 Introduction

Bacterial populations form persister cells, phenotypic variants of the wild type that are tolerant to treatment by antibiotics [4, 26, 27, 98]. A clonal population of cells that are susceptible to an antibiotic will have a subpopulation that is able to survive treatment without gaining heritable resistance. These persister cells are of interest because of their role in the recalcitrance of chronic or biofilm infections. The transient nature of persister cells makes them difficult to study, thus many of the mechanisms of persister formation are poorly understood. To date, screening of two different *Escherichia coli* libraries [15, 23] and a *Pseudomonas aeruginosa* transposon library [24] failed to yield any single mutant that entirely lack persisters, indicating that there are multiple, redundant mechanisms of persister formation. Persisters have also been shown to form stochastically, and can be pre-existing in the environment before the addition of an antibiotic [25, 26].

In the case of fluoroquinolone (FQ) antibiotics, persister formation is induced by the addition of the drug and is dependent on the SOS DNA damage response pathway [28]. The SOS response is a gene network composed of over 40 genes whose transcription is regulated by the LexA repressor and induced by expression of activated RecA. FQs induce double-stranded breaks (DSBs) in the DNA in cells with active gyrase or topoisomerase IV. This leads to induction of the SOS response, which in turn can induce persister formation. Two SOS-dependent mechanisms necessary for survival
have been identified. One pathway shows that tolerance depends on the repair of collapsed replication forks and stalled transcription complexes that occur as a result of FQ treatment [99]. The second mechanism prevents further damage by decreasing the amount of available ATP in the cell, and is dependent on the type I toxin TisB [31].

TisB is a type I toxin regulated by the LexA repressor. The expression of TisB is further controlled by its constitutive antitoxin, the IstR-1 RNA. TisB is localized to the inner membrane where it forms an anion channel, dissipating the proton motive force and decreasing intracellular ATP levels [31, 32, 83]. Overexpression of the TisB toxin has been shown to create a multi-drug tolerant state, while the deletion of tisB forms fewer persisters when treated with FQs. However, TisB alone as a persister mechanism is wasteful, since the rate of respiration will increase to compensate for the sudden drop in intracellular ATP concentrations. As the cells uses up its internal store of resources due to the effect of TisB, resuscitation from the persister state will be difficult, and the persister cells will be unable to grown and repopulate their environment. TisB may be a protection mechanism under extreme damage-causing conditions, as it plays a role at high concentrations of ciprofloxacin [31, 99]. We reasoned that there is another mechanism that coincides with the action TisB.

When a growing culture of E. coli is irradiated with ultra violet (UV) light, aerobic respiration ceases [41-44]. This shutoff requires protein translation [42], as well as a RecA and active DNA repair [45, 46]. Cessation of respiration is a reversible process, but prolonged respiratory failure can lead to cell death [100]. The mechanism of respiration shutoff is unknown. We reasoned that a different type of DNA damage, such as the DSBs that occur during FQ treatment, would also stop aerobic respiration.
Cessation of respiration would lead to cell shutdown and persister formation. Shutoff will also prevent the rate of respiration from needlessly increasing due to the action of TisB at higher FQ concentrations.

Here we show that FQ treatment causes aerobic respiration to cease and that this is dependent on an active SOS response. This shutoff of respiration protects the cells from treatment with aminoglycosides, as respiration cessation will lead to depolarization of the membrane, thus decreasing the proton motive force (PMF).

### 3.3 Results

**Cessation of Respiration depends on the SOS Response**

As previous studies show that ultra violet radiation (UVR) or treatment with mitomycin C (MMC) stop aerobic respiration, we decided to test whether this phenomenon would occur upon addition of the fluoroquinolone ciprofloxacin and the formation of DSBs. We found that respiration cessation does occur in *E. coli* that is treated with lethal doses of ciprofloxacin in exponential phase (Figure 3-1). The phenotype of respiration cessation is dependent on the growth rate of the culture. A culture with a shorter generation time (Figure 3-1A) stops respiring sooner when treated with the same concentration of ciprofloxacin as a culture with a longer generation time (Figure 3-1B). The generation time in MOPS minimal medium with glucose and cassamino acids is 38 minutes, while in MOPS with malate the generation time is approximately 120 minutes. The amount of time it takes respiration to fully stop is roughly half the generation time. As the rate of transcription plays a role in cessation of respiration, these results further show that the mechanism is induced upon DNA damage.
Figure 3-1. Cessation of respiration is dependent on the generation time. When wild type cultures of *E. coli* are grown in two different media, the phenotype of respiration shutoff varies. When grown in MOPS medium with glucose and cassamino acids (A), respiration shuts off faster than in a culture with a longer generation time (B, MOPS medium with malate).
We next wanted to confirm that this phenotype is dependent on an active SOS response. To do this, we used two mutants that are deficient in SOS induction. The first mutant, a recA deletion strain, is deficient in both SOS induction as well as in an essential part of DSB repair, homologous recombination. The second strain used carried a mutation (lexA3) that codes for a non-inducible repressor but is proficient in homologous recombination. We find that both a recA mutant (Figure 3-2A) and a lexA3 mutant (Figure 3-2B) do not exhibit respiration cessation after treatment with ciprofloxacin. These results confirm that an active SOS response is necessary for cessation to occur in order to induce expression of the shutoff mechanism.

To confirm this phenotype is independent of previous discovered persister mechanisms, we also measured oxygen consumption in a tisAB knockout to confirm respiration cessation is independent of TisB (Figure 3-3A). The absence of TisB does not have an effect on respiration, indicating that respiration cessation is an additional mechanism of persister formation. As expected, overexpression of TisB increases the rate of oxygen consumption (Figure 3-3B). Taken together, these results indicate that there is an additional mechanism dependent on the SOS response that gives persister cells the ability to survive FQ treatment.

**Ciprofloxacin pretreatments facilitates protection to aminoglycosides**

It was important to learn whether this state induced by ciprofloxacin would protect cells from different classes of antibiotics. We reasoned that due to the dissipation of the
Figure 3-2. The SOS response is required for respiration shutoff in *E. coli*. When a strain deficient in the SOS response is treated with 10X the MIC of ciprofloxacin for 2 hours, respiration continues until all the available oxygen is used up. This can be seen in a *recA* deletion strain (A) and an uncleavable *lexA3* mutant (B). Strains were grown in MOPS medium with 0.4 % malate.
proton motive force, the cells would be protected from aminoglycosides, as the uptake of this antibiotic is energy-dependent [101]. Although the exact mechanism of aminoglycoside uptake is unclear, it has been shown that uptake requires PMF generation [101]. Formation of the proton gradient is coupled with the movement of electrons through the ETC [102, 103]. Respiration cessation will decrease the PMF, leading to tolerance to aminoglycosides. To test this, we pretreated a growing culture of *E. coli* with low levels (2X and 4X MIC) of ciprofloxacin. These concentrations of ciprofloxacin were chosen so as not to induce the TisB toxin [31, 99], which has been shown to dissipate the PMF due to formation of anion channels [32] and create aminoglycoside tolerant persisters. We also used a defined medium with malate as the sole carbon source to prevent other metabolic pathways from being active. Pretreatment with 2X MIC of ciprofloxacin for 1 or two hours yielded a 2000-fold and 120 fold increase in survival compared to kanamycin alone (Figure 3-4A). When pretreated with 4X MIC of ciprofloxacin, 1 and 2 hours of pretreatment led to a 127-fold and 245 fold increase in survival (Figure 3-4B). These results are consistent with the effect cessation of respiration has on the membrane potential and PMF, which can be seen by the lack of killing by kanamycin.

We also tested the effect respiration shutoff would have on another class of antibiotics. Although the target of rifampicin is known to be RNA polymerase, the mode of killing is not yet understood [104]. As wild type *E. coli* is not susceptible to rifampicin, most likely due to poor penetration, we used a strain carrying a mutation that increased outer membrane permeability. When rifampicin was added to a culture that had been pretreated with ciprofloxacin, there was no addition killing (Figure 3-5).
Figure 3-3. TisB plays no role in induced respiration shutoff. When the SOS induced toxin TisB is deleted (A), respiration cessation occurs after 1 hour of ciprofloxacin treatment in MOPS 0.2 % glucose medium. When TisB is overexpressed (B), respiration increases as expected due to the action of TisB.
This was true using the same conditions that protection from aminoglycosides was seen to. Interestingly, the addition of rifampicin to the culture seemed to protect the cell from further killing by ciprofloxacin. This may be due to the reduced rate of transcription and cell stasis caused by the addition of rifampicin, which made the cells less susceptible to killing by fluoroquinolones.

**Oxygen Consumption of Inverted Membrane Vesicles**

As the mechanism of respiration shutoff is unknown, we next wanted to understand which component of respiration is impacted. We reasoned that the most logical way to shut off respiration would be to directly inhibit the electron transport chain (ETC) by an insertion into the inner membrane or by a tightly bound inhibitor of an ETC component. When oxygen is present, *E. coli* will use aerobic respiration for redox balancing and optimal energy production through the PMF and ATP synthase. When oxygen is the sole terminal electron acceptor, the crucial enzymes are the NADH dehydrogenases (Nuo and Ndh) and succinate dehydrogenase (Sdh), and the terminal oxidases in *E. coli* are cytochrome bd-1, bd-II and bo [105-111]. It is possible that the inhibitor of respiration inhibits one or more of these components.

We isolated membrane vesicles from a growing culture of *E. coli* as well as from a culture that had stopped respiring due to ciprofloxacin treatment. We found that when NADH was added to both the treated and untreated vesicles, oxygen was consumed until the NADH or the oxygen was completely used up (Figure 3-6). To determine if NADH was the limiting factor, additional NADH was added to the culture, and oxygen
Figure 3-4. Ciprofloxacin pretreatment protects from aminoglycoside treatment.

Pretreatment with 2x MIC (A) or 4X MIC (B) of ciprofloxacin protects cells against killing by the aminoglycoside kanamycin. Strains were exposed to ciprofloxacin in exponential phase. Each graph represents the average of at least 5 independent experiments. Error bars represent standard error.
Figure 3-5. Ciprofloxacin pretreatment results in some protection to rifampicin.

When a growing cultures of cells carrying the \textit{imp}4213 mutation is pretreated with 2X MIC (A) and 4X MIC (B) of ciprofloxacin for two hours (green lines). There is no additional killing when 100X MIC of rifampicin is added. Experiments represent 3 independent experiments. Error bars, standard error
Figure 3-6. Respiration of isolated inverted membrane vesicles. When supplied with 5mM NADH, inverted membrane vesicles will consume the available oxygen until either the NADH or the oxygen is used up. The phenotype is not dependent on the concentration of membrane vesicles, as both a higher concentration (A) and a lower concentration (B) will continue respiring as long as an electron donor is supplied.
consumption resumed (data not shown). The rate of respiration is dependent on the concentration of membrane vesicles. The higher the concentration, the quickly the oxygen is reduced. Respiration continues until the limiting factor has been depleted. These results indicated that the mechanism of respiration is not embedded into the inner membrane. Unexpectedly, we found that membrane vesicles isolated from a ciprofloxacin treated culture was able to consume oxygen at a faster rate than the untreated membrane vesicles, which was only seen in the diluted sampled (Figure 3-6B).

To further confirm that the mechanism of respiration shutoff is not an integral membrane protein, we measured the rate of oxygen consumption during all steps of the isolation procedure after the addition of an electron donor. We found that before cells were homogenized using a French press, ciprofloxacin treated cells were unable to respire. However, after the culture was homogenized this phenotype disappeared, indicating that the mechanism is loosely associated with the membrane (Figure 7-4). These results do not rule out the possibility that there are multiple components required for the shutoff of respiration, including a membrane component.

**Loss of NAD+ to the medium during respiration shutoff**

A loss of nicotinamide adenine dinucleotides (NAD+) from the cell into the medium could also account for the cessation of respiration [51]. We decided to further examine the role this loss may have in respiration shutoff. We measured the amount of NAD+ in the medium from growing cultures of *E. coli* that were treated with 10X MIC (0.1µg/ml) and 100X MIC (1.0 µg/ml) of ciprofloxacin (Table 3-1). We detected no NAD+ in the medium of the culture treated with a lower concentration of ciprofloxacin. The culture
treated with 100X MIC of ciprofloxacin experienced the loss of NAD+ into the medium. The loss of NAD+ is only measured at a higher concentration of ciprofloxacin, and thus more DNA damage and higher SOS induction. These results point to the possibility that more than one mechanism may contribute to DNA damage-induced respiration shutoff, and the induction of each mechanism is dependent on the level of DNA damage the cell is experiencing. These results also hint that ciprofloxacin may be causing changes to the cell membrane.

Table 3-1. NAD+ leakage into the medium during ciprofloxacin treatment.

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<tr>
<td>0.1 µg/ml</td>
<td>&lt;10 pM</td>
</tr>
<tr>
<td>1.0 µg/ml</td>
<td>0.3 µM</td>
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3.4 Discussion

Persister cells have proven difficult to study due to their low number and the redundancy of mechanisms. It has previously been shown that persister formation to fluoroquinolone antibiotics requires an active SOS response and DNA repair [3, 99]. The SOS-induced toxic peptide TisB has also been implicated in persister cell formation by decreasing intracellular ATP levels, leading to the formation of the persister state [31, 83]. TisB acts
by forming an anion-specific pore in the inner membrane, dissipating the PMF, which leads to the sudden drop in ATP levels [32]. However, the mechanism of TisB alone would not be helpful to surviving cells, as the rate of respiration would increase to compensate for the sudden drop in ATP levels. As the cell would use all its internal store of resources to make up for the deficit, it would be unable to regrow when the antibiotic is removed from the environment. We reasoned that there was another mechanism induced by DNA damage that would coincide with the action of TisB to fully shut down the cell until resuscitation and regrowth could occur.

We believe that DNA-damaged induced respiration shutoff is the missing mechanism that allows cells to survive DNA-damaging agents through a damage prevention pathway. Although the phenomenon of DNA damage-induced respiration has been studied since the 1950s, the mechanism of shutoff has remained elusive [42, 43, 49, 112]. The goal of this study was to reaffirm that DNA-damage does result in the shutoff of aerobic respiration. Our second aim was to show that the fluoroquinolone ciprofloxacin induces this shutoff. This study shows that ciprofloxacin does induce respiration cessation, and that this phenotype is dependent on the SOS response. We chose to look into the role of the SOS response using two strains, a strain that is deficient in homologous recombination as well as the SOS response (a recA deletion) and a strain that is only deficient in the induction of the SOS response (lexA3 mutant). We found that after ciprofloxacin treatment, respiration does not stop in either mutant, indicating that it is the induction of the SOS response that is the necessary component for respiration cessation.
As mentioned above, we have previously identified the toxin TisB as an important component of persister cell formation. As TisB affects the PMF we expected that TisB would play no role in cessation of respiration. We show that a TisB deletion has the same phenotype as the wild type strain after ciprofloxacin treatment, indicating a lack of involvement. As expected, when TisB is overexpressed the rate of respiration increases to offset the sudden drop in ATP levels.

We next wanted to know if cessation of respiration would cause cells to become tolerant to other classes of antibiotics. The first antibiotic we tested was the aminoglycoside kanamycin. As aminoglycoside requires the PMF to enter the cell, we reasoned that in cells without an active ETC, the cell would be protected from kanamycin since the gradient would not be formed to drive chemiosmosis. We chose to use low levels of ciprofloxacin to limit the presence of TisB in the membrane, as the overexpression of TisB renders cells tolerant to aminoglycosides [31]. We found that even at low ciprofloxacin concentrations, which will result in fewer DNA breaks, the population is protected from the action of kanamycin. These results indicate that even low levels of DNA damage will protect cells from aminoglycosides, which may have important ramifications in the treatment of infections. It also appears as if the reverse is true. Inhibiting the action of RNA polymerase causes cell to become tolerant to ciprofloxacin. This is most likely due to the reduced activity of gyrase and topoisomerase IV, the targets of ciprofloxacin.

We also wanted to know if ciprofloxacin pretreatment would protect cells from rifampicin. Rifampicin inhibits RNA polymerase [104], although it is unknown what causes rifampicin to be bactericidal. Wild type *E. coli* is not susceptible to rifampicin in
physiological relevant concentrations, so we used a strain of *E. coli* that has increased membrane permeability to allow the drug entry. Thus, this strain has a lower MIC than the wild type. We found that the addition of rifampicin offers protection to cells that have been previously exposed to ciprofloxacin, and inhibits any further killing by either drug.

Another bactericidal antibiotic class in gram negative bacteria is the beta-lactams. However, as beta-lactams are only effective against growing bacteria, we reasoned that cessation of respiration would stop cell growth due to the drop in ATP levels, and the cells would be fully tolerant to any β-lactam drug.

As the mechanism of respiration shutoff is unknown, we decided to begin by classifying the type of mechanism that may be inhibiting respiration. The most likely source of inhibition would be to directly inhibit the ETC by disrupting the path of the electrons. To test this theory, we isolated inverted membrane vesicles. Inverted membrane vesicles were used because all of the proteins will be facing out into the buffer, so in order to measure the consumption of oxygen, an electron donor could simply be added into the buffer, and would not have to transverse across the membrane to activate the ETC. If the mechanism of respiration shutoff directly inhibits the ETC by imbedding into the membrane, then ciprofloxacin treated membrane vesicles would be unable to use oxygen as the terminal electron acceptor. In addition to inhibiting the action of the ETC enzymes, the inhibitor could also prevent the transfer of electrons to ubiquinone or to one of the cytochromes. We found that treated membrane vesicles are still able to reduce oxygen. The results of the membrane vesicle isolation experiments may also be due to the number of branched ETC present in *E. coli* (reviewed in [113,
that are activated under different conditions. Since the membrane vesicles were
isolated from cells grown in rich medium to a higher OD than in previous studies, other
ETCs could have been activated later in the culture. Although this result shows that the
shutoff of respiration is not solely due to an imbedded membrane protein, it does not rule
out the possibility that this is still one component of the shutoff mechanism. Inhibiting
the ETC, as opposed to the tricarboxylic acid cycle, would be a more direct and quicker
way of ensuring full cessation. The mechanism may have more than one component, and
include a membrane associated component that is required for full shutoff. It is also
possibly that the sole inhibitor of the ETC is loosely associated with the membrane, and
upon cell homogenization the protein is no longer associated with the membrane,
allowing the ETC to function normally.

A potential mechanism that would have an impact on the ETC is the loss of
NAD+ to the medium. NAD+ plays a key role in electron transport through the ETC,
NADH dehydrogenase catalyzes the transfer of the hydride ion from NADH. NAD+ is
also required by the two known NAD-dependent DNA ligases, LigA [115-117] and LigB
[118]. As DNA ligases catalyze the formation of phosphodiesterase bonds in the DNA
after replication and during DNA repair processes, this could also lead to stasis of
growth, or perhaps even death if not remedied. It is possible that ciprofloxacin treatment
results in increased permeability of the membrane, however, a previous study showed
that the leakage was unique to NAD+ [51]. Future work could consist of characterizing
the nature of the leakage, and measuring the response of the NAD+ biosynthesis pathway
to this loss would prove interesting. More in depth studies of the membrane structure of
fluoroquinolone-treated cells would also yield clues into the nature of NAD+ leakage, and would also answer important questions about the mechanism of respiration cessation.

3.5 Materials and Methods

Oxygen Consumptions Measurements

Oxygen concentration was measured using the Oxygraph oxygen electrode system (Hansatech Instruments Limited, United Kingdom) attached to a circulating water bath set to 37\(^\circ\)C. The instrument was set up and calibrated per the instructions. Overnight cultures were diluted 1:1000 into fresh MOPS minimal medium with 0.2% glucose supplemented with 0.1% cassamino acids and grown for two hours at 37\(^\circ\)C with aeration. When cell density was approximately 10 x 10\(^6\) colony forming units (CFUs) per milliliter (ml), 1 ml of culture was added to the Oxygraph electrode chamber, and consumption of oxygen was followed over time. The culture was constantly stirred at 60% of the maximum rate. Before the start of measurement, an aliquot was serial diluted and plated on Luria-Bertani (LB) agar to measure CFUs. To measure the effect of ciprofloxacin on oxygen consumption, cells were grown to the same density under the same conditions. Ciprofloxacin was added to a final concentration of 0.1\(\mu\)g/ml or 1.0 \(\mu\)g/ml directly to the Oxygraph chamber immediately after cells were added.

Strain Construction

All bacterial strains used in this study are listed in Table 3-1. \textit{E. coli} K12 MG1655 was used as the wild type in this study. P1 transduction was used to move alleles from donor
strains into the wild type background. Precise deletions were made in MG1655 as described in [90]. The kanamycin resistant cassette was cured using pCP20.

**Survival Assays**

All survival assays were performed in MOPS minimal medium with 0.4% malate. Overnight culture was diluted 1:100 into 3ml of fresh medium and grown for 6 hours at 37°C with aeration (220 rpm). Cultures were treated with 2X MIC (0.02 µg/ml) or with 4X MIC (0.04 µg/ml) of ciprofloxacin for either 1 or 2 hours. After pretreatment, 10X MIC (4 µg/ml) of kanamycin or 100X MIC rifampicin (500 µg/ml) was added. CFUs were measured by washing cells with 1% NaCl to remove antibiotic, serial diluted, and plated on LB agar supplemented with 20mM MgSO4 to neutralize ciprofloxacin carryover. Plates were incubated at 37°C for 36 hours before colonies were counted.

**Table 3-2 Bacterial Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>P1 donor→recipient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>K-12 F− λ−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV1954</td>
<td>lexA300(Def)::spec</td>
<td>AB1157</td>
<td>[93]</td>
</tr>
<tr>
<td></td>
<td>ΔsulA::kan</td>
<td>lexA300(Def)::spec</td>
<td>MV1627</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MV2057</td>
<td>lexA3</td>
<td>DM49→MV2056</td>
<td>[94]b</td>
</tr>
<tr>
<td>TD122</td>
<td>ΔtisAB::FRT</td>
<td></td>
<td>[31, 90]f</td>
</tr>
<tr>
<td>TD127</td>
<td><em>lexA</em>300(Def)::spec</td>
<td>MV1954</td>
<td>[93]</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
<td>---------</td>
<td>-----</td>
</tr>
<tr>
<td></td>
<td>Δ<em>sulA</em>::FRT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT35</td>
<td>Δ<em>recA</em>-srl::Tn10</td>
<td>JJC4027→MG1655</td>
<td>[65]</td>
</tr>
<tr>
<td>AT272</td>
<td><em>imp</em>4213</td>
<td>BE100→MG1655</td>
<td>[119, 120]</td>
</tr>
</tbody>
</table>

*a* Transduced allele is indicated in bold.

*b* Transductants were selected on MOPS/maltose minimal medium, checked for UV-sensitivity and confirmed by sequencing.

*c* kan^R^ cassette was cured by expressing the *flp* recombinase as described in [90].

### NAD+ Assay

An overnight culture was diluted 1:100 in fresh MOPS medium with 0.2% glucose with 0.1% cassamino acids. Cells were grown for 2 hours at 37°C with aeration. 0.1 µg/ml (final concentration) of ciprofloxacin was then added to the cultures and incubated for 2 hours. Cells were spun down and the supernatant was separated and used to measure NAD+ concentration in the medium. NADH/NAD was measured using the Amplite Fluorimetric NAD/NADH ratio assay kit (AAT Bioquest Inc, CA) according to manufacturer’s directions.

### Inverted Membrane Vesicle Isolation

Inverted membrane vesicles were prepared according to the methods described in [121] with minor changes. Overnight cultures of *E. coli* were diluted 1:100 into 1 liter of fresh MOPS medium with 0.2% glucose and 0.1% cassamino acids. Cells were grown for 2 hours at 37°C with shaking. Ciprofloxacin (0.1 µg/ml, final concentration) was added to
one of the cultures and allowed to incubate for 2 hours. Cells were spun down via centrifugation and resuspended in 100 mL of 50 mM potassium phosphate (pH 7.5) with 5 mM magnesium sulfate. Cells were spun down and resuspended in the same buffer with the addition of 1 mM dithiothreitol. Cells were broken by a single passage through a pressure cell homogenizer. Unbroken cells were removed by centrifugation at 48,000g for 10 minutes. The inverted membrane vesicles were collected by centrifugation at 210,000g for 2 hours. Protein concentrations were measured using Bradford reagent (Sigma Aldrich).

**Membrane Vesicle Oxygen Uptake**

For oxygen uptake experiments using membrane vesicle, vesicles were diluted the desired concentration in 50 mM potassium phosphate with 10 mM magnesium sulfate. 5 mM NADH was used as a substrate. NADH was added two minutes after inverted membrane vesicles were added to the chamber to ensure there were not contaminating electron donors. The Oxygraph oxygen electrode system was used for oxygen measurements.
CHAPTER 4 DEVELOPMENT OF A NOVEL SCREEN TO IDENTIFY THE MECHANISM OF RESPIRATION SHUTOFF

4.1 Abstract

Persisters are bacterial cells that are able to tolerate antibiotic treatments without acquiring heritable resistance. Persisters to fluoroquinolones are formed upon addition of the antibiotic and formation is dependent on the SOS response. It has previously been shown that the toxin TisB induces persister formation by forming an anion-specific channel in the inner membrane, dissipating the proton motive force and reducing the concentration of intracellular ATP. However, this mechanism is wasteful as depletion of the proton motive force will activate futile respiration and exhaust the cell’s stored nutrients, leaving the cell unable to resuscitate. In order to counter balance the mechanism of TisB, we reasoned that an additional protein-based mechanism will be induced during DNA damage to stop respiration. Here we describe two genetic screens to elucidate the mechanism of respiration shutoff in cells that have been treated with fluoroquinolones. Using a water soluble tetrazolium salt as an indicator of respiration, we screened both a collection of knockouts and an EMS mutant library to identify mutants that are able to undergo aerobic respiration after being treated with ciprofloxacin.
4.2 Introduction

Persister are drug-tolerant cells that are able to survive antibiotic treatment with acquiring heritable resistance. Persisters contribute to the difficulty of eradicating biofilms [10, 98], and have been implicated in many types of bacterial infections [5, 6, 122, 123]. Understand the mechanism of persister formation is likely to lead to novel approaches for treatment. Progress has been slow in identifying new mechanisms of persister formation due to the redundancy of pathways. Recent work has shown that the SOS plays a major role in persister formation to fluoroquinolone antibiotics [3, 31, 99].

One of the mechanisms identified is the SOS-regulated toxin TisB. TisB functions by forming an anion pore in the inner membrane, dissipating the proton motive force and decreasing intracellular ATP levels [32, 124]. However, this is a wasteful mechanism. As ATP levels drop, the cell will increase respiration to make up for the sudden loss, using up its internal store of energy sources in the process. Thus, when it is time to regrow, the cell will be unable to resuscitate from the persister state. We reasoned that there is another mechanism that coincides with the mechanism of TisB. We hypothesized that this second mechanism is DNA damage induced respiration shutoff. This cessation of aerobic respiration has been shown to be dependent on DNA repair and the SOS response [45, 125] and [chapter 3]. The mechanism of respiration shutoff was never identified, although many possible components have been identified [47, 51, 52, 126]

I developed a screen to identify the mechanism(s) responsible for respiration shutoff using a tetrazolium salt indicator of respiration and an ordered library of *Escherichia coli* deletions. 12 candidates were identified through the screen that may
play a role in the stopping of respiration. The deletion and overexpression phenotypes were further tested to examine their possible role in respiration cessation. Any additional screen was performed using a library of EMS mutagenized strains. This chapter will discuss the initialization of the screen, as well as analyze the candidate genes that were found in both screens.

4.3 Results

Development of WST-8 colorimetric screen

We reasoned that if the mechanism of respiration cessation has been deleted from the wild type, respiration will continue after addition of ciprofloxacin. In order to screen the Keio collection, an ordered library of nonessential knockouts in *E. coli* [89], we needed a simple readout to identify respiring mutants. The first indicator tested was the viability dye alamarBlue (resazurin). Cells that have an active metabolism reduce the blue resazurin to the red-colored resofurin. However, entry into the cells is dependent on the generation of the PMF. Thus when TisB is overexpressed, there is no reduction of resazurin (Figure 7-5).

The next viability dye I tested was WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5(2,4 disulfophenyl)-2H- tetrazolium, monosodium salt]), a water-soluble formazan dye. Upon reduction by dehydrogenases within the cell, WST-8 is reduced to WST-8 formazan, an orange colored product. The reduction of WST-8 can be monitored at 450 nm, allowing us to develop a high throughput screen.

Before screening began, WST-8 was optimized for use as an indicator of aerobic respiration. Cells were grown in MOPS minimal medium with malate as the sole carbon
source to prevent the activity of other dehydrogenases within the cell. A pilot screen was performed with mutants deficient in the SOS response to measure the difference in WST-8 reduction between untreated and ciprofloxacin treated cells (Figure 4-1).

Figure 4-1. WST-8 as an indicator of aerobic respiration. WST-8 is quickly reduced by growing cultures of E. coli, including a strain overexpressing the toxin TisB (tisBOE). When 0.1 µg/ml of ciprofloxacin is added to the culture, respiration stops and WST-8 is not reduced in the wild type and in a strain overexpressing TisB. WST-8 continues to be reduced in SOS deficient strains (ΔrecA and lexA3) that have been treated with ciprofloxacin. Wt, wild type
In untreated cultures, WST-8 is reduced fully in approximately 200 minutes.

To measure reduction of WST-8 in ciprofloxacin treated cultures, growing cultures were pretreated with ciprofloxacin for two hours to ensure full stoppage of respiration. After this incubation period, WST-8 was added and its reduction was followed. The wild type was unable to reduce WST-8, while the SOS deficient strains, lexA3 and a recA deletion strain, were able to continuously reduce WST-8. These results show the reduction of WST-8 is dependent on active respiration in these growth conditions. To further confirm that the reduction of WST-8 is dependent on respiration shutoff, we also looked at the effect TisB overexpression had on reduction. TisB is a toxin that forms an anion pore in the inner membrane, dissipating the proton motive force. Overexpression of TisB had no effect on the reduction of WST-8, further confirming that reduction of WST-8 can be used as an indicator of respiration under these growth conditions.

**Screen Results**

I completed a screen of the Keio deletion collection to identify mutants that are able to respire after the addition of ciprofloxacin. The 12 mutants with the greatest reduction of WST-8 are listed in Table 4-1. There is no common functionality between the 12 candidate genes. In terms of localization, there is one periplasmic protein (FecB), two inner membrane proteins (FxsA and YdjM), and the remainder are either cytoplasmic proteins or their localization remains unknown. Interestingly, there are 4 candidates whose expression is regulated by the LexA repressor (RecX, YafO, YafP and YdjM). As respiration cessation is dependent on an active SOS response, these four candidates were of particularly interest. RecX directly interacts with RecA to inhibit the ATPase and
coprotease functions of RecA [127]. RecX also acts by blocking the extension of RecA filaments on single strand DNA (ssDNA) by capping the 3’ end of the growing filament [128-130]. The deletion of recX in a wild type background does not affect the level of

Table 4-1 Candidates from Keio collection screen.

<table>
<thead>
<tr>
<th>Hit</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspA</td>
<td>Component of aspartate ammonia-lyase (aspartase). Aspartase reversibly catalyzes the amination of fumarate to generate :aspartate</td>
</tr>
<tr>
<td>fecB</td>
<td>Periplasmic binding protein of ferric citrate transporter</td>
</tr>
<tr>
<td>fxsA</td>
<td>Inner membrane protein. Overproduction of FxsA inhibits F exclusion of bacteriophage T7</td>
</tr>
<tr>
<td>kdgR</td>
<td>DNA-binding transcription factor. Regulator of transport and catabolism of 2-keto-3-deoxygluconate</td>
</tr>
<tr>
<td>moaD</td>
<td>Molybdopterin synthase small subunit</td>
</tr>
<tr>
<td>paaI</td>
<td>Thioesterase in phenylacetic acid degradation operon. Functional role is unknown.</td>
</tr>
<tr>
<td>recX</td>
<td>Located downstream of recA. Inhibits RecA recombinase, coproteases and DNA strand exchange activity</td>
</tr>
<tr>
<td>rfaL/waaL</td>
<td>LPS core biosynthesis. No functional role in K12.</td>
</tr>
<tr>
<td>yafO</td>
<td>Toxin of the yafON toxin-antitoxin module. Ribosomal-dependent interferase. Functional role unknown. Downstream of dinB</td>
</tr>
<tr>
<td>yafP</td>
<td>Possible acyltransferase. Located in the same operon as dinB and yafNO under SOS control. Function may be connected to Pol IV</td>
</tr>
<tr>
<td>ydjM</td>
<td>Predicted inner membrane protein. Transcription induced by the SOS response.</td>
</tr>
<tr>
<td>yhaB</td>
<td>Predicted protein. Expression is induced ~50 fold after exposure to biocide polyhexamethylene biguanide</td>
</tr>
</tbody>
</table>
YafNO, along with YafP, is under SOS control and located directly downstream of DinB (Pol IV). YafO is a ribosome-dependent interferase associating with the 50 S subunit in 70 S ribosomes [131]. YafO inhibits protein translation by cleaving mRNA 11-13 bases downstream from the initiation codon [131]. YafP, while in the same operon as YafNO, is not part of the toxin-antitoxin module. While the functional role of YafP remains unknown, it may play a role in the metabolism of genotoxic compounds in natural isolates [132]. YdjM is a putative protein, and its C terminus is located in the periplasm [133].

Analysis of Candidates

To confirm each candidate’s potential role in respiration shutoff, we took single deletions of all candidates and measured their rate of oxygen consumption under normal growth conditions and after the addition of ciprofloxacin. 8 candidates (aspA, fecB, fxsA, kdgR, moaD, paaI, rfaL and yhaB) were found to either have no phenotypic difference from the wild type, or had a delay in phenotype (data only shown for two mutants, Figure 4-2). A recX deletion was found to continue respiration after two hours of ciprofloxacin treatment, the same length of time used in the screen (Figure 4-3). However, after incubation with ciprofloxacin for three hours, oxygen consumption had ceased, indicating that one function of RecX may participate in the shutoff of respiration, but is not required for this phenomenon. It is not clear if this function is directly related to the mechanism shutoff, or if it is through the function of RecF and the effect the deletion will have on RecA.
Figure 4-2. Oxygen consumption of an *aspA* and *fecB* mutant before and after ciprofloxacin treatment. Oxygen consumption was measured in an *aspA* (A) and a *fecB* (B) mutant in exponential phase. Respiration was monitored in an untreated culture and in a culture treated with 10X MIC of ciprofloxacin for two hours.
**Figure 4-3. Role of RecX in respiration shutoff.** When recX is deleted and treated with 10X the MIC of ciprofloxacin for 2 hours, respiration continues (green line). When the recX deletion strain is treated with the same concentration of ciprofloxacin for 3 hours, respiration ceases (purple line). wt, wild type

A deletion of the yafNOP operon was examined next for its role in respiration shutoff. When oxygen consumption was measured after two hours of ciprofloxacin treatment (0.1 µg/ml), respiration continued until the culture has used up all the available oxygen (Figure 4-4, green line). However, after three hours ciprofloxacin treatment, respiration continues at the same rate as the untreated culture for about 1,200 seconds (20 minutes) then begins to slow down. At approximately 3,000 seconds (50 minutes) respiration ceases, about 4 hours after initial ciprofloxacin treatment (purple line).
The next step was to measure the rate of oxygen consumption in a strain overexpressing the protein of interest. Overexpression of YafO is known to cause inhibition of growth [134-136]. When YafO is overexpressed, the rate of respiration slows dramatically (Figure 4-5A). Indeed, even when the plasmid has not been induced, respiration has slowed. This phenotype may be caused by the endoribonuclease function of YafO, rather than a specific mechanism to slow down respiration. However, more experiments are needed to determine the relationship. Previous results showed that overexpression of YafO has no effect on respiration, however these experiments were performed in different medium with a longer generation time (data not shown). When YafP is overexpressed under the same conditions, the rate of oxygen consumption decreases (Figure 4-5B). Overexpression of YafP has a slight effect on growth rate (data not shown), which may be enough to decrease the rate of respiration. From these results it is not clear if the effect YafP has on respiration is a direct result of inhibit respiration, or an artifact of expression of a toxic protein.

YdjM, a SOS-induced inner membrane protein, may also play a role in SOS-dependent respiration shutoff. When an ydjM deletion is treated with ciprofloxacin for two hours, respiration continues until all available oxygen is used up in 1,700 seconds (approximately 30 minutes) (Figure 4-7). However, the rate of oxygen consumption is lower than in the untreated culture (red line). When the ydjM deletion strain is treated for a longer time with the same concentration of ciprofloxacin, the rate of oxygen consumption is similar to that of the treated wild type strain. YdjM may interact with, or recruit, the protein responsible for stopping respiration to the membrane where it is able
to inhibit the electron transport chain. It is also possible that there are two mechanisms for respiration shutoff, and both mechanisms are able to shut off respiration once DNA damage, or SOS induction, reaches a certain threshold. When YdjM is overexpressed, the rate of oxygen consumption slows down (Figure 4-7), but all the oxygen is consumed in the culture over a period of approximately 4000 seconds (66 minutes). It is unclear if longer induction will fully stop respiration, or if a second component is involved. The plasmid expressing YdjM was placed in a strain with the SOS response constitutively expressed (lexA(DEF) mutant), however a similar phenotype was seen (data not shown).

Figure 4-4. Role of YafNOP in respiration shutoff. When the yafNOP operon is deleted, respiration continues normally. When 10X MIC of ciprofloxacin is added 2 hours to the culture, respiration slows down but continues to use all the available oxygen (green line). When the culture is treated with ciprofloxacin for 3 hours (purple line), respiration ceases after an additional 3000 s (approximately 50 minutes). wt, wild type
Figure 4-5. Overexpression of YafP and YafO do not induce complete respiration cessation. When the toxin YafO is overexpressed (A) but the plasmid promoter is not induced (red line) respiration slows. When expression is induced by the addition of an inducer, respiration slows down significantly. When YafP (B) is overexpressed, the rate of respiration slows compared to the empty vector (blue line) and an uninduced plasmid carrying yafP (red line).
**Figure 4-6. Role of YdjM in respiration shutdown.** When \(ydjM\) is deleted and treated with 10X the MIC of ciprofloxacin for two hours (green line) respiration continues. When the wild type (wt) is treated the same length of time, respiration stops (blue line). When \(\Delta ydjM\) is treated with 3 hours of ciprofloxacin (purple) respiration slows down significantly, but does not dully stop. wt, wild type

Both Lex boxes of \(ydjM\) were also deleted to measure the effect that constitutively expressed YdjM may have on respiration. The results resembled the respiration patterns of the wild type (data not shown). It is possible that DNA damage is necessary component of respiration shutdown, or that YdjM is not directly involved.

**EMS mutagenesis library screen**

As the screening of the Keio collection failed to yield any one mutant whose absence was required for cessation of respiration, we decided to screen an EMS mutant library. Each
strain this library contained 10-15 mutations (see Materials and Methods section for detailed explanation). As the EMS mutant library would contain strains with multiple genes with mutations, a strong selection for respiring mutants after DNA damage would allow us to identify a mutant deficient in respiration cessation. We screened

![Graph A](image1)

**Figure 4-7. Rate of respiration in EMS mutant screen candidates.**

Oxygen consumption was measured in the two top hits of the EMS mutant screen for a strain able to respire after ciprofloxacin treatment. After 1 hour of ciprofloxacin treatment, neither strain completely stops respiring.
approximately 2,200 EMS mutants using WST-8 as an indicator of respiration. After the initial screen, 100 mutants remained and were retested using WST-8. After the reduction of WST-8 was measured a second time after ciprofloxacin treatment, two mutants remained. The rate of oxygen consumption before and during ciprofloxacin was measured using the Oxygraph measuring system for both mutants (Figure 4-8). We found that both candidates had a reduced rate of oxygen consumption. The first EMS candidate (Figure 4-8A) had a phenotype that closely resembled the untreated strain. However, after longer ciprofloxacin treatment the phenotype did stop consuming oxygen (data not shown). The second mutant (Figure 4-8B), upon ciprofloxacin treatment, considerably slowed down its rate of respiration.

**Revisiting the EMS screen**

Since the EMS library contained 2,200 mutants, it was possible that a mutant with the desired phenotype had been missed. Recently, the hits from the first screen were revisited to identify mutants with strong WST-8 reduction. One mutant showed a strong phenotype in the first screen, but did not repeat the reduction in the rescreen. This mutant was pulled from the library and its rate of oxygen consumption was measured before and after treatment (Figure 4-9). We found that after one hour of ciprofloxacin treatment, the rate of oxygen consumption closely resembled that of the untreated mutant. This mutant was also able to continue respiring after two hours of ciprofloxacin treatment (Figure 4-9, purple line). We tested the mutant for longer periods of time with ciprofloxacin, and the mutant continued respiring, even after incubation with ciprofloxacin for 4 hours. It is possible that the mutant is resistant to ciprofloxacin and is not affected by the dosage, but
this remains untested. This mutant will be sent for whole genome sequencing to identify mutations involved in respiration shutoff.

**Figure 4-8. EMS mutant is found to respire after ciprofloxacin treatment.** An additional EMS mutant that showed strong WST-8 reduction in the initial screen was tested for its ability to consume oxygen. After 1 hour (red line) and 2 hours (purple line) of ciprofloxacin treatment, the mutant was able to continue consuming oxygen.

### 4.4 Discussion

Persisters are non-growing phenotypic variants of the wild type that are able to survive treatment through different mechanisms [15, 18, 23, 28, 31, 99]. In this study, our goal was to identify another mechanism of persister formation that is dependent on the class of antibiotic. We have previously shown that TisB is able to induce persister formation by its function as an anion channel, dissipating the proton motive force and decreasing intracellular concentrations of ATP to create a persister state [32]. Based on earlier data,
we reasoned that this mechanism is protein-based, which would allow us to screen an *E. coli* deletion library for the mechanism of action. In the past, screening transposon libraries has been successful in isolating proteins involved in specific pathways, such as biofilm formation and sporulation [137]. However, our screen resulted in not a single mutant that had the desired phenotype under all conditions, perhaps due to redundancy of the mechanism. Finding mutants which form fewer persisters has proven to be a challenge due in part to the overlapping mechanisms and the specificity of mechanisms to certain stresses [15, 24, 138]. The same may be true of the mechanisms of respiration shutoff.

It is unclear whether the four genes identified in the screen, *recX*, *ydjM*, *yafO*, *yafP* play a role in respiration shutoff or if the phenotypes associated with them are an artifact of their own function(s). As in the case of RecX, it appears that RecX is most likely not associated directly with the mechanism of respiration shutoff, and its phenotype is most likely an artifact of its interaction with RecA.

With the remaining proteins, the question still remains what their role is in respiration shutoff. As the biological function remains unknown for all three, and the cellular function of YdjM remains unknown. YafO has been shown to be a ribosome-dependent mRNA interferase, and does not have endoribonuclease activity on its own [135]. YafO is associated with the 50 S ribosomal subunit and cleaves mRNAs downstream of the initiation site [135]. The *yafNOP* genes do not appear to play a role in stress induced mutagenesis that requires DinB [139] YafO is also under the control of two different promoters. *yafNOP* has been shown to be in the same operon as *dinB*, which is under SOS control [139, 140]. Although there is an addition Lex box upstream of
yafNOP and after dinB, it does not appear to effect expression of YafNO after MMC treatment when the dinB promoter is deleted [136]. Transcription of yafO is also regulated by an addition reported that is autoregulated by the antitoxin YafN [136]. The biological role of interferases has been a topic of debate, but the prevalent view is that interferases act to control the growth rate of the organism to promote survival (reviewed in [141]). Interferases have also been linked to the formation of persister cells, and overexpression of YafO, as well as other mRNases, have been shown to produce antibiotic tolerant cells [19, 62]. The deletion of yafO does not have an effect on persister levels (unpublished), indicating that it has no effect on survival to FQs, or that it shares the same mechanism with another protein. YafO and YafP are also not required for survival to other DNA damaging agents, including MMC and UVR, nor are they required for protection against phage λ [139]

The action of YafO and the respiration phenotype seen when this toxin is overexpressed brings up an interesting question about SOS-dependent respiration shutoff. The possibility exists that there is not one dedicated mechanism to respiration shutoff. Instead, there are multiple proteins that exert their individual functions, and the end effect is cessation of respiration. Most likely, there are two mechanisms that work to stop respiration. One mechanism prevents the translation of new respiratory proteins, while a second mechanism inhibits the action of the existing ETCs. The role of YafO may be to inhibit the translation to prevent the formation of additional respiratory chains. By preventing the formation of new respiratory chains, the mechanism directly linked to the ETC is able to inhibit the transfer of electrons, thus stopping the transfer of electron to oxygen.
In addition to YafO, YdjM may be the membrane component. Without the inhibition of translation to prevent additional respiratory chains from being activated, YdjM is unable to fully stop respiration. Combining overexpression of YdjM with constitutive chromosome expression of YafO may lead to a complete phenotype without growth inhibition due to artificial overexpression of YafO. Follow up experiments using these genes could include measuring the oxygen consumption rate of double mutants or when both proteins are being constitutive expressed.

Additional clues to the mechanism of respiration shutoff may be found in the EMS mutagenized strain that is able to respire in the presence of ciprofloxacin. Whole genome sequence analysis may identify specific proteins or pathways that play a role in shutting off respiration upon DNA damage.

4.5 Materials and Methods

**WST-8 Screen**

For the pilot screen, overnight cultures were diluted 1:100 into MOPS minimal medium with 0.4% malate and then transferred to a 96-well Costar flat bottom plates and grown for 36 hours at 37°C on a rotator. Cells were then transferred using a replicator into a new 96-well plate with 200µl in each well of fresh MOPS minimal medium with 0.4% malate as the sole carbon source. Cells were allowed to grow for 6 hours at 37°C. Cells were then treated with 0.1µg/ml ciprofloxacin for two hours. 5 µl of WST-8 (Cell Counting Kit 8, Dojindo, Japan) and OD 450 was read to follow reduction of WST-8.

For the screen, the Keio deletion collection was grown for 36 hours in the same MOPS medium for 36 hours at 37°C. The same protocol as written above was followed,
with the exception that plates were incubated with WST-8 overnight and OD 450 was read the next morning to measure the reduction of the compound. The screen was performed in duplicate. Knockouts able to reduce the WST-8 after ciprofloxacin treatment were cherry picked and grown in the same medium in new plates, and underwent confirmation for WST08 reduction after treatment. Deletions that reduced WST-8 in the second round were analyzed further.

**Respiration Assays**

To confirm respiration cessation phenotype, oxygen consumption was measured using the Oxygraph oxygen electrode system (Hansatech Instruments Limited, United Kingdom). Strains were grown 36 hours at 37°C with aeration and then diluted 1:1000 in fresh MOPS medium with 0.4 % malate. Strains were grown for 6 hours before being treated with 0.1 μg/ml ciprofloxacin for 2 hours. After this time, 1 mL of culture was added to the Oxygraph chamber and oxygen consumption was measured over a period of time.

Additional testing was performed in MOPS medium with 0.2% glucose and 0.1% cassamino acids. An overnight culture of cells was diluted 1 to 1000 into 3ml fresh medium and grown for 1.75 hours at 37°C aeration. A 1 ml aliquot was removed for oxygen measurements. Another aliquot was removed for CFU counts.

For overexpression assays, cultures were grown in MOPS medium with glucose and cassamino as outlined above. Expression was induced with 1 mM IPTG for 20 minutes before oxygen levels were measured.
**Strain Construction**

The strains made for this study can be found in Table 4-2. Deletions were made by moving the allele from the Keio collection or relevant strain into MG1655 by P1 transduction.

To construct pZS\(^{*}\)\(djhM\), pZS\(^{*}\)\(yafP\), and pZS\(^{*}\)\(yafO\), the ORF of each gene was cloned into the kpnp/pstI (in boldface type) cloning sites of pZS\(^{*}\)35 [91]. pZS\(^{*}\)\(djhM\) was made using the primers ydjMkpnp (ATTTTAGGTACCAGGAGGCGATATGACGGCAGGTCACCTTCTC) and ydjMpstI (TATTAATCTGCACAGCTATAGTATTTCAACCTGATGTAAATGCCG). pZS\(^{*}\)\(yafP\) was made using the primers yafPkpn (ATTTTAGGTACCAGGAGGCGATGACGGGTTATCAAAACAAAATCTTATTC) and yafPpstI (TATTAATCTGCACAGCTATAGTATTTCAACCTGATGTAAATGCCG). pZS\(^{*}\)\(yafO\) was made using the primers yafOkpn (ATTTTAGGTACCAGGAGGCGATGACGGGTTATCAAAACAAAATCTTATTC) and yafOpstI (TATTAATCTGCACAGCTATCAAAAACAAAATCTTATTC).
Table 4-2 Bacterial Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>P1 donor→recipient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT225</td>
<td>ΔydjM::kan</td>
<td></td>
<td>[89]</td>
</tr>
<tr>
<td>AT236</td>
<td>pZS*ydjM</td>
<td>n/a</td>
<td>[91]</td>
</tr>
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<td>AT203</td>
<td>ΔrecX::kan</td>
<td></td>
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</tr>
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<td>AT242</td>
<td>pZS*YafO</td>
<td>n/a</td>
<td>[91]</td>
</tr>
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<td>AT242</td>
<td>pZS*YafP</td>
<td>n/a</td>
<td>[91]</td>
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<tr>
<td>AT300</td>
<td>ΔyafNOP::kan</td>
<td></td>
<td>[139]</td>
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<td></td>
</tr>
<tr>
<td>ATEMS2</td>
<td>EMS mutant 2</td>
<td>n/a</td>
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</tr>
<tr>
<td>ATEMS3</td>
<td>EMS mutant 3</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>

EMS Mutagenesis Library Construction

EMS mutagenesis protocol was adapted from [97]. Individual colonies were inoculated in fresh Luria-Bertani broth (LBB) and grown overnight at 37°C with shaking. Each overnight culture was diluted 1:500 in 6mL of fresh LBB and grown until an OD600 of 0.2. The cultures were then diluted 1:50 in 6 mL LBB and grown to OD600 of 0.2 three times. After the final growth period, a 10 µl aliquot was taken for serial dilutions and serial plating on Luria-Bertani agar to count colony forming units (CFUs). The cells were collected via centrifugation and washed two times with 0.125M HEPES/KOH buffer (pH 7) and resuspended in 3 mL buffer. 1.5 mL aliquot were made and kept on ice until ethyl methanesulfonate (EMS) was added to tubes. 22.5µl of EMS was added to
each tube, with the exception of the negative control. The cell aliquots were incubated with the EMS for 10 minutes at 37°C. After incubation, the cells were washed 2 times with 0.125M HEPES buffer and resuspended in 1.5 mL LBB. An aliquot was taken for serial diluting and plating to count CFUs. And additional 1.5 mL LBB was added to each culture tubes and cells were incubated overnight at 37°C with shaking. After overnight incubation, CFUs was again measured by serial diluting and plating on LB agar. Dilutions were also plated on LB agar plates containing 100 µg/ml rifampicin (Sigma Aldrich) to measure frequency of mutants in mutagenized culture compared to the untreated culture.

To select for mutants able deficient in respiration cessation, the collection of mutants was grown for 36 hours in MOPS minimal medium with 0.4% malate. The culture was diluted 1:10 into 10 mL fresh medium and grown for 6 hours. Cultures were treated with 0.1 µg/ml ciprofloxacin for 2 hours. Cells were then collected via centrifugation and washed once with 1% NaCl. Cells were resuspended in the same volume of fresh medium and allowed to recover overnight. Selection was repeated twice more. After the third challenge and recovery, cells were sorted into 96-well plates containing 100 µl MOPS medium with 0.4 % malate using a FACS BD Aria II. 1 cell was sorted into each well to form a clonal population. Cultures were grown for 48 hours at 37°C before being diluted into fresh medium for the WST-8 screen, as outline above. Screen was performed in duplicate. The overnight, sorted plates were frozen with 30% glycerol for future work.
Wells with cells able to reduce the WST-8 after ciprofloxacin treatment were cherry picked and placed in new 96 well plates. Potential hits were rescreened using the same protocol to identify false positives.

**EMS Mutant Respiration Assays**

Candidates from the EMS mutant screen were grown overnight and diluted 1 to 1000 into fresh MOPS medium with 0.2% glucose and 0.1% cassamino acids. The cells were grown for two hours at 37°C with shaking, and a 1ml aliquot was removed and placed into the Oxygraph chamber for oxygen consumption measurements. A 10 µl aliquot was also removed for CFU counts, as described previously. Then to the remaining culture 0.1 µg/ml of ciprofloxacin (final concentration) was added, and incubated for an addition 1 hour at 37°C with aeration. After the one hour, another 1ml aliquot was removed for oxygen consumption measurements.
CHAPTER 5 ANALYSIS OF PROTEIN FRACTIONS OF CIPROFLOXACIN TREATED ESCHERICHIA COLI

5.1 Introduction

The mechanism of respiration shutoff has remained unknown since its initial discovery over 50 years ago [41, 43, 142, 143]. Although some possible mechanisms, such as the loss of NAD+ to the medium, have been proposed, the proteins responsible for this shutoff have remained elusive. In the previous chapter, we developed a genetic screen using an indicator of respiration to screen an E. coli knockout library as well as an EMS mutagenesis library to identify proteins which play a role in cessation of respiration due to DNA damage. However, the mutants identified had mixed phenotypes that did not show with certainty that there is one mechanism of respiration shutoff. We decided to examine the mechanism of respiration from a different angle to attempt to identify the mechanism(s) involved.

The field of proteomics has grown substantial since the introduction of 2 dimensional gel electrophoresis (2DE). In the beginning, 2DE gels were difficult to perform and to reproduce, but with the introduction of immobilized pH gradient (IPG) strips and Edman sequencing, 2DE became a useful component of cell biology (reviewed in [144]). In traditional SDS-PAGE, proteins are separated based strictly on size. In 2DE, proteins are first loaded onto IPG strips with a known range of pH. The proteins are then separated by their isoelectric point (pI) during isoelectric focusing (IEF). The IPG strip is then placed in the second dimension, and proteins are separated based on molecular weight. Separating a complex mixture of proteins based on two properties instead of one will allow separation between similarly sized proteins, whereas in SDS-PAGE only one
band would be seen. Further subcellular fractionation of a sample, such as localization fractionation performed in bacteria, can further increase the separation of proteins by decreasing the amount of proteins in the sample.

In the last two decades, 2DE have been used in many fields to differentiate between different populations. Analysis of stress responses using 2DE has been done in yeast, and important components of the stress responses have been identified [145-147]. In *E. coli*, much of the recent work in 2DE has been to determine the localization of proteins. In an impressive study by Lopez-Campistrous *et al.*, 2DE analysis was performed using protein samples that has been fractionated into their subcellular components [148]. In their analysis, they detected 3,200 proteins, annotated over 2,100 of them and then assigned them to the cytoplasm, periplasm, inner or outer membrane. In the same study, the protein profiles of cells experiencing amino acid starvation were also compared to cells grown under normal conditions. Another study compared the proteome of urinary *E. coli* isolates [149] as a possible method for classifying infectious strains. The proteomes of over 40 urinary isolates were compared to each other and to the lab strain MG1655 using 2DE, with the goal of identifying specific pathways and features important in infection of the urinary tract.

In *E. coli*, there have been many studies that performed differential proteomics to study the proteome of the bacterium under stressful conditions, such as glucose-limiting conditions [150, 151] and changing pH [152-154]. Other stress responses analyzed using 2DE include cadmium stress [155], cold shock [156, 157], as well as acid stress and base stress [158]. The proteome of a cell undergoing treatment by fluoroquinolones (FQs), ultra-violet radiation (UVR) or mitomycin C treatment (MMC) have not been studied.
The recent work to identify other genes under control of the LexA repressor have been through the use of microarrays or the detection of the Lex box sequence in the genome [1, 2]. An extensive search of the literature brought up no proteomic studies to study the cell’s response to DNA damage.

We decided that 2DE gel analysis would be helpful in detecting the mechanism of respiration shutoff. We reasoned that by comparing the subcellular fractions of an untreated culture of *E. coli* to a ciprofloxacin-treated one, we could detect differences in the subcellular fractions to use for mass spectrometry (MS) analysis. We were especially interested to look for changes in the inner membrane, as a change in this fraction could potentially disrupt the electron transport chain (ETC). This chapter will describe the preliminary work done to analyze the protein patterns of cells experiencing DNA damage and an induction of the SOS response, and discusses the next steps of this project.

### 5.2 Results

Before performing 2D analysis, it was important to confirm that protein subfractionation would be sufficient for use after the SOS response had been induced. A variety of protocols were performed to identify the protocol that would yield the greatest amount of protein. We wanted to separate the fractions into the periplasmic, cytoplasmic and inner membrane fractions, as these fractions would be most likely to contain information relating to respiration cessation. The results from the fractionation were first analyzed using SDS-
Figure 5-1. Subfractionations of E. coli on SDS-PAGE. The periplasmic fraction (1-3), cytoplasmic fraction (4-6), and the insoluble fraction (7-9) were run on SDS-PAGE to identify any potential differences. The first sample of each fraction is the untreated culture, the second well has been treated with 0.1 µg/ml ciprofloxacin for 1 hour, and the third well has been treated for two hours.

In the periplasmic fraction, the protein isolated from cells that had been treated for two hours with ciprofloxacin shows a marked difference. More specifically, there are three bands that only appear in the sample that was treated with antibiotic or two hours. It is difficult to note any differences in the cytoplasmic or
insoluble fractions, more likely due to the amount of protein in each sample. Mass spec analysis failed to yield cohesive
Figure 5-2. Analysis of periplasmic fraction using 2D electrophoresis. The periplasmic protein fraction was analyzed using 2D gel electrophoresis from an untreated (A) and a ciprofloxacin treated (B) culture.
Figure 5-3. Analysis of cytoplasmic fraction using 2D electrophoresis. The cytoplasmic protein fraction was analyzed from an untreated culture (A) and a ciprofloxacin treated culture (B) using 2D electrophoresis.
Figure 5-4. Analysis of inner membrane proteins using 2d electrophoresis. The inner membrane proteins were separated and analyzed using 2D electrophoresis. An untreated culture (A) was compared to a ciprofloxacin treated culture (B)
results. We next decided to next use 2DE as a way to see a clearer separation between protein bands.

For 2DE analysis, we looked at the cytoplasmic, periplasmic and inner membrane fractions, as the proteins in these three fractions are most likely to be involved in respiration shutoff, as well as the general SOS response. The treated periplasmic fraction (5-2B) contains a greater amount of protein then the untreated periplasmic fraction (Figure 5-2A), even though a similar concentration of protein was loaded onto each IPG strip. The difference may be due to the initial solubilization of the precipitated proteins during the Bradford assays and protein loading.

The results for the cytoplasmic fraction appear in Figure 5-3. Due to protein overloading, the spots are difficult to decipher. The horizontal streaking is due to an overload of protein that was unable to separate during isoelectric focusing (IEF). However, this can easily be remedied by loading a smaller concentration of protein. Alternative gels can be seen in Figure 7-8, where less protein has been added. The final fraction analyzed using 2DE was the inner membrane fraction. Inner membrane fractions are notoriously difficult to analyze using traditional 2DE analysis. Due to their hydrophobic nature, membrane proteins tend to aggregate and demonstrate poor solubility. For preliminary testing, we used the same protocol for this fraction as we did for the cytoplasmic and periplasmic fractions. The results can be seen in Figure 5-4. The untreated fraction shows a clear separation with distinct protein spots. There is some aggregation in the larger protein spots, but overall is shows a clear picture of the inner membrane fraction. The ciprofloxacin treated fraction (Figure 5-4B) shows greater
aggregation and less solubility than the untreated isolated fraction. Possible reasons for these discrepancies will be discussed in the discussion section.

5.3 Discussion and Future Directions

In this chapter, the preliminary data is described to analyze the protein fractions of E. coli cultures that have been treated with the fluoroquinolone ciprofloxacin to identify the mechanism of respiration shutoff. The data show that 2DE can be used to differentiate between a growing cell population and a population treated with ciprofloxacin.

Although these initial gels are useful in determining that 2DE analysis can be used to identify proteins of interest which can then be analyzed using MS, there are a number of experiments and challenges that face this project. The first experiment that needs to be done is to confirm clean fractionation of the samples. This can be performed in one of two ways. The first way would be to use antibodies specific for proteins with known localization for detection in immunoblotting [148, 159]. Common protein targets are GroEL (cytoplasm), TonB (inner membrane), Mbp (periplasmic) and OmpX (outer membrane). Unfortunately, antibodies for these targets are not all commercial available. An additional method would be to perform enzymatic assays to detect the presence of enzymes with known localization. A beta-galactosidase assay can be performed to detect the presence of this enzyme, which is localized to the cytoplasm. An assay to detect the presence of alkaline phosphatase, which is localized to the periplasm. The quality of the fractionations is important when comparing the fractions of two populations to one another. However, it has been shown that complete separation of the subcellular fractions is not possible in gram negative organisms [159, 160]. Nonetheless, it is still
important to show that the same level of fractionation was obtained in all samples, especially when comparing two individual population.

Showing a clean fractionation is especially important in the ciprofloxacin treated membrane. Although it has been show that UVR can induce changes in the cell envelope [161], it is unclear what these changes consist of. When prepping the cells for fractionation, it was clear during subcellular fractionation that the pellet from the ciprofloxacin-treated sample behaved different than the pellet from the untreated culture. After the spin which separates the periplasmic proteins from the rest of the sample, the treated pellet was very loose and resuspended very easily, whereas the untreated pellet was not easily lost or resuspended. The insoluble fraction also differed from the untreated sample, as in displayed none of the usual viscosity. This may be due in part to a change in lipid composition of the membranes during ciprofloxacin treatment. In order to detect differences in the lipids, MS analysis can be used to compare a change in the composition. It is possible this change in lipid composition could have an effect on the physiological processes of the cell.

Another difficulty with the membrane fractions is the hydrophobicity of the proteins. This makes the solubilization of the proteins difficult and results in the formation of aggregates. Using the tradition protocol as described in this chapter may limit the number of membrane proteins that can be detected. There are a number of different approaches that can be taken to maximize the efficiency when using 2DE for separation (reviewed in [162]). Techniques include increasing the use of detergents, such as Triton X-100, in fractionating protocols to increase the separation of membrane proteins, the use of soft IPG strips to reduce aggregation, or increased delipidation.
However, even by employing all of these techniques, 2DE stills fails at separating highly hydrophobic proteins with three or more transmembrane domains [163]. Alternative methods for separation of highly hydrophobic proteins that are gaining in popularity include SDS/SDS-PAGE, 16-BAC/SDS-PAGE and CTAB/SDS-PAGE (reviewed in [162]).

Traditional 2DE stained with Coomassie or silver stain is a more traditional approach for comparing differential populations. However, it allows for differences in final gel images due to protein loading in IEF and in the running of the second dimension gels. An alternative form of 2DE is 2-dimensional differential gel electrophoresis (2D-DIGE) [164]. 2D-DIGE is designed to allow users to run up to three samples on the same gel. The proteins are prepared in a similar fashion to 2DE, however, before IEF each sample is labeled with a different fluorescent dye. The Cy2, Cy3 are Cy5 dye are most commonly used for 2D-DIGE. Once samples have been labeled, they can be mixed and loaded onto IPG strips for use in IEF. Once the gel has been run, each sample will fluoresce a different color, and the samples can be compared from the same gel. By using the same IPG strip and gel for differential comparison, the reproducibility problems between samples will not influence results. It would be preferable for this technique to be used over traditional 2DE, as the samples will be compared on the same gel. This is especially important when looking for specific proteins that are present in only one condition, as it will leave no doubt to whether the protein is present in the second sample.

By using the proteomic tools available to us, identifying the mechanism of respiration shutoff is possible using 2DE or 2D-DIGE analysis. A different procedure that allows for better separation of hydrophobic proteins may be more useful in
uncovering changed in the inner membrane proteome, which may play a key role in the shutoff of respiration. Although incomplete, this technique would allow us not only to identify the mechanism of FQ-induced respiration shutoff, it would also allow us to identify other proteins that whose expression is affected by the addition of a FQ.

5.4 Materials and Methods

Subcellular fractionation
Cultures of *E. coli* MG1655 were fractionated using method 4 described in [159]. Overnight cultures were diluted 1:100 in 1L Luria-Bertani broth and grown for 1.5 hours at 37°C with aeration. 0.1 µg/ml of ciprofloxacin was added to one culture and both cultures were incubated for an additional 2 hours at 37°C with aeration. Cells were then fractionated following method 4. Protein concentrations were measured using Bradford Reagent (Sigma Aldrich).

Gel Electrophoresis
Protein samples were analyzed by SDS-PAGE before 2D gel electrophoresis was performed. Samples were run on 8-12% Bis-Tris gels (Life Technologies, NY).

Protein Precipitation
Fractionated proteins were precipitated using the trichloroacetic acid (TCA)/acetone method. 1 ml cell lysate was added to a 15 ml falcon tube. 8ml of 100% ice cold acetone was added to cell lysate, and the mixture was inverted to mix. 1ml TCA was
then added to each tube, mixed to invert, and placed at -20°C to precipitate for 1 hour. The samples then were then spun down at 20,000x g for 20 minutes at 4°C. The supernatant was discarded and the cells were washed 3 three with 1 ml ice cold acetone at 20,000 x g for 20 minutes at 4°C. After the final wash all acetone was removed and the pellet was allowed to air dry at room temperature to remove any residual acetone. Precipitated proteins were stored at -20°C until used.

2D gel electrophoresis

Proteins were solubilized in 2D sample solution buffer (7M urea, 2M thiourea, 4% (w/v) CHAPS, 40 mM dithiothreital, 0.2% (w/v) ampholytes, 0.001 bromophenol blue) by repeatedly pipetting up and down to break up the protein pellet. The sample was left at room temperature for 30 minutes, with vortexing every 10 minutes to further break up the pellet. The sample was then transferred to a 1.5 ml Eppendorf tube and centrifuged for 10 minutes at room temperature at 14,000 rpm. The supernatant was immediately collected and used immediately for 2D gel analysis. 11 cm nonlinear immobilized pH 3-10 (IPG) strips (Bio-Rad Laboratories, CA) were used for all experiments. 185µl of protein samples were applied to IPG strips and left to rehydrate overnight. Isoelectric focusing (IEF) was run at 20°C using the Protean IEF Cell (Bio-rad laboratories, CA). Strips were stored at -20°C until used for gel electrophoresis. IPG strips were equilibrated for electrophoresis for 10 minutes in equilibration buffer (6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS dissolved in 0.05 M Tris/HCL buffer) with 100 mM of dithiothreitol (DTT). The buffer with DTT was then removed and the strips were placed in equilibration buffer containing 400 mM iodoacetamide. Strips were run using 12%
Bis-Tris gels and run for 1 hour at 180 volts (Criterion Cell, Bio-Rad Laboratories, CA).

Gels were stained with biosafe Coomassie Stain (Bio-Rad Laboratories, CA).
CHAPTER 6 DISCUSSION

Persister cells are a small population of bacterial cells that are able to survive treatment. When a fluoroquinolone antibiotic is used to treat a population of *E. coli*, persister formation is dependent on an active SOS response [28]. Understanding what functions of the SOS response are important in survival to FQs could have the potential to change treatment practices when a patient is suffering from a chronic infection. In the first part of this study, we tested individual deletions of all known SOS genes to identify candidates that play a role in survival to FQs. We identified the repair proteins DinG, UvrD and RuvAB as functions necessary for this survival. DinG is a DNA helicase that is capable of removing D- and R-loops, and its role in the clearing of replication forks has previously been demonstrated [64, 65]. As induction of the SOS response is impaired in a *dinG* mutant, we reasoned that the role of DinG is to clear stalled transcription complexes to allow RecBCD to process the break, and our data shows that through this action DinG allows for the loading of RecA onto ssDNA. UvrD is another DNA helicase that is necessary for persister cell formation. In addition to its helicase function, UvrD prevents the formation of toxic RecF-dependent RecA filaments [67]. UvrD participates in survival by removing these filaments to allow replication restart and repair of the breaks. In a strain deficient in the SOS response, overproduction of UvrD rescues the strain after FQ treatment to levels similar to the wild type, showing that the functions of UvrD are imperative to survival. We found that deletion of *recF* increased survival in our assays, indicating that this is one but role UvrD has in ensuring survival. Both RuvA and RuvB form a complex, and together with RuvC mediate branch migration and the
resolution of Holliday junctions. A *ruvAB* and *recG* double mutant is extremely sensitive to ciprofloxacin, confirming this step of recombination repair is also needed for survival to fluoroquinolones. Our result show that although there may be other mechanisms that shut down the cell to prevent damage, active repair is also required to guarantee survival.

Although repair is necessary for survival, there also exists damage prevention pathways that prevent the cell from accumulating more damage. One such pathway, induced by the TisB toxin [31], dissipates the PMF through the formation of an anion channel [32]. In addition to the action of TisB, we reasoned there is another mechanism that prevents the rate of respiration from increasing. Respiration shutoff has been seen to occur in response to UVR and MMC, and we reasoned that the same will also be true with FQs. We show that the FQ ciprofloxacin does induce cessation of respiration, and this state further prevents killing by another class of antibiotic, the aminoglycosides.

We further looked into the possible mechanism(s) of respiration shutoff. Isolation of inverted membrane vesicles offered no clues as to whether the ETC is directly inhibited, as it is possible multiple components are needed for ETC inhibition. We decided to screen two mutant libraries to attempt to uncover a mechanism. Using an indicator of respiration, four genes of interest were identified using a library of single, precise deletions. Although the single deletion of each candidate lacked the ability to continually respire after ciprofloxacin treatment, the overexpression of the toxin YafO did have an effect on the rate of respiration, presenting the possibility that inhibition of translation plays an important role in the total shutoff. The second screen of a library of EMS mutants yielded one candidate able to respire in the presence of a FQ, and this candidate will be sent for whole genome sequencing to pinpoint any genes or pathways
involved. These results indicate that multiple mechanisms participate to stop aerobic respiration.

Using a different method, a preliminary study using 2DE analysis was used to compare the proteome of a FQ treated culture to an untreated one. Although no protein spots were sent for analysis from the 2DE, this method has the capacity to uncover novel proteins associated with the phenotype of respiration shutoff. This method also has the potential to identify novel genes associated with the SOS response and homologous recombination, as well as assign known proteins to the proper localization.

Overall, this study works to better understand the role the SOS plays in survival to a commonly used classed of antimicrobials. By building on this work, I expect we will uncover more important insights into additional functions of the SOS response that play an important role in survival.
Fig. 7-1. The level of persisters to different antibiotics is dependent on the generation time. When ampicillin (blue bars) or ciprofloxacin (red bars) is used to treat a growing culture of *E. coli* with varying generation times, the amount of killing depends on the generation time. Ampicillin kills faster growing cells for effectively, while ciprofloxacin has a greater effect on slower cultures. The effect of ciprofloxacin may be dependent on the number of chromosomes. The generation time (in minutes) is written under the medium type for clarity.
Figure 7-2 Deletions of many SOS induced genes result in no persister phenotype. Many of the candidate genes tested had no persister phenotype to 10x MIC of ciprofloxacin. BW, wild type

Figure 7-3 SOS induction of a *ruvAB* and *recF* mutant during ciprofloxacin treatment. When *ruvAB* and *recF* are deleted from a strain expressing *lacZ* under the *recA* promoter, expression of the promoter is the same as in the wild type culture, indicating that neither *ruvAB* nor *recF* play a role in induction of the SOS response when cells are treated with ciprofloxacin. 10X the MIC of ciprofloxacin was used. Graphs represent 3 independent experiments, and error bar represent standard error.
Figure 7-4. The deletion of *dinG* has no role in SOS induction in response to *mitomycin C*. When treated with 10X MIC of MMC, the absence of DinG does not affect the induction of the SOS response when compared to the wild type. Experiment was performed as described in Chapter 2. Red, untreated dinG. Orange, Wt treated 4 hrs. Green, *dinG* KO treated 2 hrs. Blue, *dinG* KO treated 4 hrs.
Figure 7-5. Simplistic representation of the mitochondrial electron transport chain.

The aerobic respiratory chain of *E. coli* is similar to that of mitochondria. Adapted from Lehninger *Biochemistry*

Figure 7-6. Oxygen consumption of membrane vesicles during isolation. When oxygen consumption was measured during the isolation procedure, ciprofloxacin treated
vesicles were unable to respire when NADH was added to the buffer (purple line). After cell homogenization, the phenotype disappeared (red line) and resembled an untreated culture (blue line).

**Figure 7-7. Overexpression of TisB prevents reduction of alamarBlue.** When the toxin TisB is overexpressed, resazurin is unable to enter the cell, and no reduction is measured. Since the reduction of resazurin is dependent on the presence of TisB, it could not be used to look for mutants with no respiration shutoff mechanism.
Figure 7-8. WST-8 colorimetric assay. When WST-8 is reduced by a respiring culture of *E. coli* grown in MOPS medium with malate as the carbon source, WST-8* is reduced to a dark orange color. However, when ciprofloxacin is added to the culture (well 7, red box) WST-8 is not reduced and remains a light orange color. When ciprofloxacin is added to a *recA* knockout or a *lexA3* mutant, WST-8 is still reduced (columns 8 and 9, respectfully).
Respiration cessation in *Burkholderia cepacia*. When *B. cepacia* is treated with ciprofloxacin for one hour, respiration slows down but does not completely stop. After two hours of treatment, respiration stops after an additional 30 minutes of incubation at 30°C with aeration. An overnight culture of *B. cepacia* was diluted 1:1000 into fresh LB broth and grown for 5 hours at 30°C with shaking. Ciprofloxacin was added at 10X the MIC. *B. cepacia* has a higher MIC than *E. coli* to ciprofloxacin, as well as a longer generation time, which may explain phenotypic difference in respiration shutoff.
Figure 7-10. 2DE analysis of cytoplasmic fraction. Alternative 2DE gel of the cytoplasmic fraction. The gels compare an untreated sampled (A) with another treated with 10X MIC of ciprofloxacin (B).
REFERENCES


