GENETIC BASIS FOR PERSISTER CELL FORMATION

A Dissertation in
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by
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ABSTRACT

The prevalence of bacterial infections poses a significant threat to public health; however, the advent of antibiotics has largely subdued the lethality of infections treated clinically. Nevertheless, antibiotics are ineffective against both resistant and persistent bacteria, which contribute to chronic and recalcitrant infections. Persister cells comprise a small, multi-drug tolerant sub-population of all bacterial cultures, which is a non-hereditary phenotype that occurs due to a state of metabolic dormancy. The low frequency of persister cell formation makes it difficult to isolate and study persisters, leading to a dearth of information regarding the basis for persister cell formation. In this dissertation, a technique is developed to chemically induce persistence via bacteriostatic compounds that mimic toxins from toxin/antitoxin (TA) systems, which have been implicated in persister cell formation. Through these chemical pre-treatments, it is demonstrated that arrested protein synthesis is the key to persister cell formation and that environmental factors contribute to persistence. Investigation of toxin YafQ of the YafQ/DinJ TA system reveals a regulatory mechanism which increases persistence via reduced levels of the intercellular and interkingdom signal indole. Additionally, phosphodiesterase DosP is found to increase persistence through reduction of the global regulator cyclic adenosine monophosphate and consequentially down-regulation of indole levels, corroborating the effect of indole on persistence through a second regulatory pathway. Owing to the relationship between TA systems and persistence, the physiological significance of the MqsR/MqsA TA system was investigated, revealing an important role for this TA system in tolerance to bile stress through regulation of YgiS, a periplasmic protein. MqsR/MqsA is a well-characterized persister cell formation mechanism, so this result reveals bile as an environmental stress that likely influences persistence. Finally, the anti-cancer drug mitomycin C (MMC) was tested for activity against persister cells because MMC crosslinks DNA through a spontaneous, growth-independent mechanism which should be effective in metabolically dormant persisters. MMC was found to eradicate persister cells with efficacy against both planktonic cultures and highly robust biofilm cultures for a broad range of bacterial species, including commensal *Escherichia coli* K-12 as well as pathogenic species of *E.*
coli, Staphylococcus aureus, and Pseudomonas aeruginosa. Additionally, MMC was effective in an animal model and in a wound model. Therefore, MMC is the first broad-spectrum compound capable of eliminating persister cells, which can be used clinically against recalcitrant infections. In summary, this dissertation reveals arrested protein synthesis as the basis for persistence, characterizes mechanisms of persister cell formation, and discovers a readily applicable clinical treatment to eradicate persisters via repurposing an FDA-approved anti-cancer drug.
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\[ 2^{-(C_t \text{ target, } \Delta mqsRA - C_t \text{ rrsG, } \Delta mqsRA)/2} \cdot 2^{-(C_t \text{ target, wild-type } - C_t \text{ rrsG, wild-type})} \]

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Figure 7.5. MMC eradicates pathogens in suspension and in biofilms. Cell viability of EHEC exponential (turbidity of 2 at 600 nm) (A) and mid-stationary phase (turbidity of 4 at 600 nm) (B) cultures in buffered LB, late-stationary phase cultures (24 h of growth) in M9-glucose (C), and biofilm cultures (24 h of growth) in M9-glucose (D). Cell viability of *S. aureus* exponential (turbidity of 0.8 at 600 nm) (E) and mid-stationary phase (turbidity of 3 at 600 nm) cultures in tryptic soy broth (TSB) (F), and biofilm cultures (24 h of growth) in modified M9-glucose (G). Cell viability of *P. aeruginosa* PA14 exponential phase cultures (turbidity of 2 at 600 nm) in buffered LB (H) and late-stationary phase cultures (24 h of growth) in M9-glucose (I). Cell viability is shown before (black) and after treatment (3 h for planktonic cultures and 24 h for biofilm cultures) with 5 μg/mL...
ciprofloxacin (green) and 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown throughout (n ≥ 2). .................................................. 119

**Figure 7.6.** MMC eradicates pathogens in clinically relevant wound and animal models. Cell viability of EHEC (A), *S. aureus* (B), and *P. aeruginosa* PAO1 (C) mono-cultures and *S. aureus* and *P. aeruginosa* PAO1 co-cultures (D) in an in vitro wound model (24 h of growth). Cell viability is shown before (black) and after 5 h treatment with ciprofloxacin (blue; 5 μg/mL for EHEC and *S. aureus* or 10 μg/mL for PAO1 mono- and co-cultures), ampicillin (green; 100 μg/mL for EHEC and *S. aureus* or 2 mg/mL for PAO1 mono- and co-cultures), and MMC (red; 10 μg/mL for EHEC and *S. aureus* or 15 μg/mL for *P. aeruginosa* PAO1 mono- and co-cultures). * represents eradication beyond the limit of detection. (E) Survival of *C. elegans* after infection with EHEC (days -2 to 0) followed by 6 h exposure to 5 μg/mL ciprofloxacin (green), 100 μg/mL ampicillin (blue), 10 μg/mL MMC (red), or no treatment (white). As a negative control, *C. elegans* was grown on OP50 without antibiotic treatment (black). Means ± s.d. are shown throughout (n ≥ 2). ....... 121
Preface

This dissertation is a compilation of articles published in peer-reviewed journals and a manuscript submitted for publication. Citations are listed below and a more detailed preface is provided at the beginning of each chapter.


- Chapter 7 is adapted from the following submitted manuscript: Kwan, B.W., Chowdhury, N., Wood, T.K. *Combatting bacterial infections by killing persister cells with mitomycin C.*
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Chapter 1. Dissertation Overview

1.1 Motivation

During the last century, the discovery of antibiotics and advent of use in modern medicine has had a significant impact on the treatment of bacterial infections. Nearly all antibiotics were developed based on naturally isolated compounds from organisms in the environment. In recent decades, antibiotic discovery has drastically declined due to exhaustion of the naturally available compounds, so it has become increasingly important to improve upon current antibiotic therapies to advance medicine. There are two main mechanisms for bacterial survival in the face of antibiotics, which are resistance, a hereditary trait conferring elevated antibiotic tolerance in growing cells, and persistence, a non-hereditary phenotype conferring multi-drug tolerance via metabolic inactivity. Bacterial persistence occurs both stochastically (i.e., through fluctuations within a multitude of cellular mechanisms) or through environmental influence to allow a small sub-population of cells to endure lethal antibiotic regimens. These persister cells contribute to chronic and recalcitrant infections, such as cystic fibrosis and tuberculosis. Therefore, this work was undertaken with the intent to:

- Review and determine the cellular basis for multi-drug tolerance of persister cells.
- Characterize the genetic basis for persister cell formation mechanisms.
- Develop a clinically applicable method to eradicate persister cells.

1.2 Overview of Chapters

Chapter 2 encompasses a literature review of persister cells with a focus on studies relevant to the formation of persister cells and their metabolic state. We examine the two main persister cell formation models: toxin/antitoxin (TA) systems and the alarmone guanosine tetraphosphate (ppGpp). Additionally, we compare decades-old research supporting metabolic dormancy as the key to persistence with a few recent opposing studies, and conclude that dormancy remains an appropriate mechanistic model for persistence.
The low frequency of persister cell formation makes it difficult to isolate and study persisters, leading to a dearth of information regarding the basis for persister cell formation. Bacterial persistence is often attributed to a quiescent metabolic state induced by toxins of cellular TA systems\textsuperscript{11, 12}. By mimicking toxins via chemical pre-treatments in Chapter 3, we induced high levels of persistence (10-100\%) from an initial persister population of 0.01\%. Pre-treatment of \textit{Escherichia coli} with the bacteriostatic compounds rifampicin, tetracycline, and carbonyl cyanide \textit{m}-chlorophenyl hydrazine all dramatically increased persistence. Thus, we demonstrate that bacterial persistence results from arrested protein synthesis and that environmental factors contribute to persistence.

Owing to the relationship between TA systems and persistence, in Chapter 4 we investigated the role of toxin YafQ of the YafQ/DinJ \textit{E. coli} TA system on persister cell formation. Under stress, YafQ alters metabolism by cleaving transcripts with in-frame 5’-AAA-G/A-3’ sites, and production of YafQ increased persister cell formation against multiple antibiotics. We demonstrate that YafQ contributes to persistence by reducing cellular indole concentrations via down-regulation of TnaA mRNA and protein levels, and that indole is inversely-related with persister cell formation. Therefore, the intercellular and interkingdom signaling molecule indole influences persistence.

Biofilms are associated with numerous infections and consist of an extracellular polymeric matrix, which can act as a physical barrier to protect bacteria from antimicrobials and/or host defense mechanisms\textsuperscript{13}. Biofilms contain cells with a wide range of metabolic activities, including a high population of persister cells\textsuperscript{14}. Since cyclic diguanylate (c-di-GMP) is an intracellular signal that increases biofilm formation\textsuperscript{15}, in Chapter 5 we sought to determine whether c-di-GMP has a role in bacterial persistence. By examining the effect of 30 genes from \textit{E. coli} involved in c-di-GMP metabolism, we found that DosP (direct oxygen sensing phosphodiesterase) dramatically increases persistence. Using both transcriptomic and proteomic approaches, we determined that DosP increases persistence by reducing indole levels, similarly to YafQ. Despite the role of DosP in breakdown of c-di-GMP, we found that the reduction of indole synthesis was in fact due to breakdown of cyclic adenosine monophosphate (cAMP). Corroborating this result, the reduction of cAMP via CpdA, a cAMP-specific phosphodiesterase,
increased persistence and reduced indole levels similarly to DosP. Therefore, the global regulator cAMP influences persistence via regulation of indole.

Chapter 6 focuses on a study of the MqsR/MqsA type II TA system of E. coli, in which we used phenotype assays to determine that MqsR/MqsA is important for cell growth and tolerance during stress from the bile salt deoxycholate. Using transcriptomics and in vitro assays with purified MqsR, we found that endoribonuclease toxin MqsR degrades YgiS mRNA, which encodes a periplasmic protein that promotes deoxycholate uptake and reduces tolerance to deoxycholate exposure. The importance of reducing YgiS mRNA by MqsR is evidenced by improved growth, reduced cell death, and reduced membrane damage when cells without ygiS are stressed with deoxycholate. We propose that MqsR/MqsA is physiologically important for E. coli to thrive in the gallbladder and upper intestinal tract, where high bile concentrations are prominent. This finding suggests that bile should influence persistence because MqsR/MqsA is a well-characterized persister cell formation mechanism.

In a more clinically applicable study in Chapter 7, we demonstrate that the anti-cancer drug mitomycin C (MMC) eradicates persister cells through a growth-independent mechanism, cross-linking DNA. We find MMC is effective against both planktonic cultures and highly robust biofilm cultures for a broad range of bacterial species, including commensal E. coli K-12 as well as pathogenic species of E. coli, Staphylococcus aureus, and Pseudomonas aeruginosa. Additionally, we demonstrate the efficacy of MMC in an animal model and in a wound model. Therefore, MMC is the first broad-spectrum compound capable of eliminating persister cells, and is a paradigm for the investigation and repurposing of cancer drugs to combat infection. Furthermore, MMC is an FDA-approved compound that is readily situated for clinical use against recalcitrant infections.

Chapter 8 comprises a summary of the major scientific contributions from this work and provides recommendations for the future direction of persister cell research, with the intent of discovering new methods for the eradication of persister cells.
1.3 Summary

This dissertation was motivated by the increasing need to improve clinical treatment of bacterial infections, which was approached through studying multi-drug tolerant persister cells. This study was undertaken to: (i) review and determine the cellular basis for multi-drug tolerance of persister cells, (ii) characterize the genetic basis for persister cell formation mechanisms, and (iii) develop a clinically applicable method to eradicate persister cells. As described herein, these goals were achieved by investigating the persister cell phenotype from several different perspectives using a multitude of scientific techniques, which has furthered the understanding of persister cells and led to a readily applicable treatment to eradicate persisters in clinical infections.

1.4 References

Chapter 2. Review of Current Literature

2.1 Preface

This chapter is adapted from the following publication: Wood, T.K., Knabel, S.J., and Kwan, B.W. *Bacterial persister cell formation and dormancy*. Appl. Environ. Microbiol., 2013. 79:7116-7121. The role of dormancy in persistence was well established in the majority of literature published prior to this review; however, a couple of more recent articles proposed a weakly supported argument that dormancy is not necessary for persistence. Therefore, this review was written with the intent of clarifying that bacterial persistence occurs as a direct result of metabolic dormancy. The following text reviews the clinical ramifications of persistence and the roles of toxin/antitoxin systems and ppGpp in persister cell formation, presents the arguments in favor of and against the role of dormancy in persistence, and provides a general outlook on techniques to eradicate persister cells and where future research should be directed.

2.2 Abstract

Bacterial cells may escape the effects of antibiotics without undergoing genetic change; these cells are known as persisters. Unlike resistant cells which grow in the presence of antibiotics, persister cells do not grow in the presence of antibiotics. These persister cells are a small fraction of exponentially growing cells (due to carryover from the inoculum) but become a significant fraction in the stationary phase and in biofilms (up to 1%). Critically, persister cells may be a major cause of chronic infections. The mechanism of persister cell formation is not well understood, and even the metabolic state of these cells is debated. Here we review studies relevant to the formation of persister cells and their metabolic state and conclude that the best model for persister cells is still dormancy, with the latest mechanistic studies shedding light on how cells reach this dormant state.

2.3 Literature Review

2.3.1 Persister cells and infection

Persister cells, those cells tolerant to antibiotics, usually comprise about 1% in the stationary state and
in biofilms\textsuperscript{1,2}. These persisters cells arise due to a state of dormancy, defined here as a state in which cells are metabolically inactive. This phenotype was first described with \textit{Staphylococcus aureus} in 1942 by Hobby et al.\textsuperscript{3}, who found that 1\% of cells were not killed by penicillin and became persister cells. In 1944, Bigger\textsuperscript{4} found that one in a million \textit{Staphylococcus pyogenes (aureus)} cells was not killed by penicillin and that these surviving cells did not undergo genetic change; hence, these cells are not resistant but instead should be considered phenotypic variants that are tolerant to antibiotics. Bigger also determined that these persister cells are non-growing by showing that penicillin did not effectively kill cells in non-nutritive medium and by showing that they had delays in regrowth in rich medium\textsuperscript{4}; hence, the first lines of evidence that these tolerant cells are dormant came from the original work with antibiotics. Bigger also recognized that penicillin was unable to clear chronic infections due to antibiotic-tolerant cells forming in patients\textsuperscript{4} and coined the term persisters for these antibiotic-tolerant cells\textsuperscript{4}. Also, Chain and Duthie\textsuperscript{5} confirmed in 1945 that penicillin did not completely kill \textit{Staphylococcus} spp. until longer treatments were used and that stationary-phase cells (i.e., non-growing cells) were nearly completely insensitive to penicillin.

Therefore, persister cells comprise a subpopulation of bacteria that become highly tolerant to antibiotics and reach this state without undergoing genetic change\textsuperscript{6}. Also, the number of persister cells depends on the growth stage. Persister cells in biofilms appear to be responsible for the recalcitrance of chronic infections, since antibiotics kill the majority of cells; however, persisters remain viable and repopulate biofilms when the level of antibiotics drops\textsuperscript{6}. Based on decades-old research, persisters are thought to be less sensitive to antibiotics because the cells are not undergoing cellular activities that antibiotics can corrupt, which results in tolerance (i.e., no growth and slow death). In contrast, resistance mechanisms arise from genetic changes that block antibiotic activity, which results in resistance; i.e., cells grow in the presence of antibiotics when they are resistant, whereas persister cells do not grow and are dormant\textsuperscript{1}. Also, this antibiotic tolerance occurs in the biofilms of members of many different genera, including \textit{Escherichia coli} (where they are best studied), \textit{Pseudomonas aeruginosa}, \textit{S. aureus}, \textit{Lactobacillus acidophilus}, and \textit{Gardnerella vaginalis}\textsuperscript{7}.
Note that the rate at which persisters form is a function of inoculum age in that older inocula have more persister cells. Hence, if older inocula are used, there is little difference between wild-type strains and deletion mutants that have elevated persistence. These authors also found, like many others, that the degree of persistence depends on the antibiotic used.

2.3.2 Formation of persister cells via toxin-antitoxin systems

In terms of the genetic basis of persister formation, the main model for the formation of persister cells is that toxin-antitoxin (TA) pairs are primarily responsible, as they induce a state of dormancy that enables cells to escape the effects of antibiotics. TA systems typically consist of a stable toxin (always a protein) that disrupts an essential cellular process (e.g., translation via mRNA degradation) and a labile antitoxin (either RNA or a protein) that prevents toxicity. RNA antitoxins are known as type I if they inhibit toxin translation as antisense RNA or type III if they inhibit toxin activity by binding the toxin protein. Type II antitoxins are proteins that inhibit toxin activity by direct protein-to-protein binding. Type IV protein antitoxins prevent the toxin from binding its target instead of inhibiting the toxin directly, and type V antitoxins are proteins that cleave the toxin mRNA specifically.

TA systems were first linked to persistence in 1983 through ethylmethane sulfate mutagenesis of *E. coli* that led to the identification of high persistence (*hip*) mutants; most notably, one mutant with enhanced persistence due to the *hipA7* gain-of-function mutation was isolated. The *hipBA* locus constitutes a toxin-antitoxin locus, and the HipA toxin inactivates the translation factor EF-Tu by phosphorylating it (other substrates may also exist). The structures of HipA and HipB suggest that the two substitutions of the HipA7 toxin (G22S [substitution of serine for glycine at position 22] and D291A) may cause it to interact poorly with the antitoxin, which would give rise to its enhanced activity that leads to increased persistence. Critically, the two amino acid substitutions of HipA7 render the protein nontoxic, so the mechanism by which HipA7 increases persistence is not known (except for its dependence on guanosine tetraphosphate) and is not via increased toxicity of a TA pair. Hence, work with HipA7, such as that showing persister cells arise stochastically, is not based on HipA7 acting as a toxin.

In 2004, TA systems were linked to persistence by DNA microarrays using a *hipA7* strain and
ampicillin treatment to lyse non-persister cells\textsuperscript{19}. Two percent of the genes with differential expression included those related to the YafQ/DinJ, RelE/RelB, and MazF/MazE TA systems. Overproduction of the toxin RelE led to as much as a 10,000-fold increase in persistence\textsuperscript{19}.

In 2006, TA systems were linked to persistence by DNA microarrays performed on carefully isolated dormant cells\textsuperscript{20}: using a green fluorescent protein (GFP) reporter downstream from a ribosomal promoter, metabolically inactive cells were isolated via fluorescence-activated cell sorting (FACS) based on diminished fluorescence. These dormant cells had 20-fold greater persistence to ofloxacin, so they were shown to be persisters, and DNA microarrays revealed that, compared to transcription levels in actively growing cells, these cells had the largest change in terms of elevated transcription of the toxin gene \textit{mqsR}\textsuperscript{20}. Other TA-related genes with differential transcription included \textit{dinJ}, \textit{yoeB}, and \textit{yefM}.

Only two TA pairs have been directly related to persistence in planktonic cells by their deletion; in contrast, overproducing almost any toxin increases persistence. The first work demonstrating this deletion phenotype was with the MqsR/MqsA TA system: deleting \textit{mqsR}, as well as deleting \textit{mqsRA}, decreased persistence\textsuperscript{21}; these results were corroborated by an independent laboratory\textsuperscript{8}. Later, the type I TA system TisB/IstR-1 was linked to persistence, since deletion of the \textit{tisAB}-\textit{istR} locus reduced persistence\textsuperscript{22}. Subsequently and confirming these two reports, it was shown that deleting multiple TA systems decreases persistence\textsuperscript{23}. Prior to the discovery of \textit{mqsRA} and persistence, the \textit{hipBA} TA locus was reported to be related to persistence via deletion\textsuperscript{19}; unfortunately, this result was retracted\textsuperscript{22}, as the phenotype was due to inadvertent deletion of more than just the TA loci. Also, for biofilm but not for stationary-phase planktonic cells, deletion of the gene that encodes the toxin YafQ decreased persistence to cefazolin and tobramycin 2,400-fold\textsuperscript{24}.

The proposed mechanisms by which TA systems cause persistence are linked to dormancy. For the TisB/IstR-1 system, the TisB toxin decreases the proton motive force and ATP levels, which causes the cells to become dormant\textsuperscript{22}. Cells producing the TisB toxin became persistent to several antibiotics, including ampicillin (a cell wall synthesis inhibitor that kills growing cells), ciprofloxacin (effective in killing non-growing cells), and streptomycin (inhibits protein synthesis). For the MqsR/MqsA\textsuperscript{25-27}, the
increased persistence arises from MqsR toxin cleaving most of the transcripts in the cell (its 5'-GCU cleavage site is found in all but 12 transcripts); hence, MqsR renders the cell dormant by diminishing translation. By selecting for a more-toxic MqsR variant and by utilizing DNA microarrays, it was determined that MqsR also causes persistence by diminishing the ability of the cell to respond to stress. Corroborating the importance of TA systems for persister cell formation, Lon protease has been shown to be necessary for persister cell formation; Lon activity is required to degrade labile antitoxins for type II TA systems where a protein antitoxin inactivates the protein toxin.

Moreover, there appear to be redundant ways to form persister cells. For example, overexpression of toxin TisB is effective for inducing persistence in the exponential phase but is not effective in the stationary phase, suggesting that there are multiple mechanisms available for *E. coli* to enter the persistent state. The authors also proposed that cells try to do at least two things when stressed, (i) activate genes to respond to the stress in the hope of resisting it and (ii) convert part of the population to a dormant state, a bet-hedging strategy that allows a fraction of the population to survive the stress through inactivity. These two different responses are important for pathogens as they face various host-related stresses (oxidants, high temperature, low pH, and membrane-acting agents).

### 2.3.3 ppGpp and persistence

To activate TA systems, the cell must respond to stress, and it appears that this stress response is most likely propagated to TA systems through the alarmone guanosine tetraphosphate (ppGpp). ppGpp is produced via RelA and SpoT (which can also degrade ppGpp) during nutrient limitation (i.e., the stringent response) and other stresses (e.g., acid stress) and serves to change transcription due to direct interactions with RNA polymerase and by its activation of RpoS (σ^S), the stress response sigma factor for the stationary phase, and RpoE (σ^E), the stress response sigma factor for misfolded proteins in the periplasm. ppGpp also directly reduces DNA replication and protein synthesis.

Since TA systems are one of the prominent elements in models of persistence, it is germane that ppGpp was found in 1996 to be required for MazF toxicity; MazF is an endonuclease toxin of the type II MazF/MazE TA system. Hence, this work set the stage for the findings that ppGpp is required for...
persistence. ppGpp was first definitively linked to persistence in 2003 via the HipA toxin\textsuperscript{16}. Using an \textit{E. coli} strain with the gain-of-function mutation hipA7 in which persistence is increased 1,000-fold, it was shown that persistence conferred by the HipA7 allele was both diminished by \textit{relA} knockout and eliminated by \textit{relA spoT} mutation\textsuperscript{16}. Therefore, ppGpp is required for HipA7 to increase persistence. In addition, it was shown that the increase in persistence is a result of cells transitioning to a non-growing state more rapidly\textsuperscript{16}. The link of ppGpp to persistence was rediscovered 8 years later through a study showing that the persistence of \textit{P. aeruginosa} also requires ppGpp\textsuperscript{31}. Also, Amato et al.\textsuperscript{32} provided additional evidence that confirmed the role of ppGpp for persistence in \textit{E. coli}.

\textbf{2.3.4 The argument for dormancy}

The stress response in bacteria is accompanied by a significantly reduced growth rate\textsuperscript{33}. It is thus probable that the increased dormancy in biofilms and the dramatically reduced growth rates of persister cells are the major reasons for the reduced susceptibility of biofilms to antibiotics\textsuperscript{34}; i.e., if antibiotics target translation and if translation is repressed by toxins such as MqsR or RelE\textsuperscript{35}, then some cells can escape the effect of the antibiotic\textsuperscript{36}.

In addition to the original work of Bigger\textsuperscript{4} and that related to TA systems\textsuperscript{20}, Kwan et al.\textsuperscript{37} demonstrated that persister cells are metabolically dormant by showing that cells that lack protein synthesis are tolerant to antibiotics. Recognizing that a major route to persistence is via activation of toxins, the group mimicked a type II endonuclease toxin (e.g., MqsR) by pre-treating cells with rifampicin to curtail transcription and achieved nearly 100\% persister cells from an initial population of 0.01\% (a 10,000-fold increase in persister cells). Hence, cells that are not producing protein are persisters. Corroborating this result, the group also pre-treated the cells with tetracycline, which halts translation, and again converted nearly 100\% of the cells into persister cells\textsuperscript{37}. Similarly, carbonyl cyanide \textit{m-}chlorophenyl hydrazine, which halts ATP synthesis, converted nearly 100\% of the cells into persister cells. Note these three pretreatments led to similar results with two antibiotics, ciprofloxacin (5 \textmu g/mL) and ampicillin (100 \textmu g/mL), and that the pre-treatment only reduced the viable cell population by about one half, so the dramatic increase in persister cells was achieved by converting nearly all of the initial.
exponential culture into persisters. Therefore, these results demonstrated that persister cells lack protein synthesis.

The results of Kwan et al. also demonstrate that stress from extracellular factors like antibiotics (that are encountered by cells in the environment) induces persistence, so cells respond via genetic circuits to increase persistence and persistence can be induced beyond the levels that occur via stochastic generation. Other examples showing persistence increases as a result of extracellular factors include the increase in persistence due to ciprofloxacin, a bactericidal antibiotic shown to induce toxin TisB at subinhibitory concentrations, and the increase due to indole, an interspecies and interkingdom signaling molecule.

Reduced metabolic activity has also been correlated with increased persistence through persister studies performed with metabolic regulators. PhoU is a negative regulator of phosphate metabolism in *E. coli*, and deletion of *phoU* leads to a metabolically hyperactive state with increased expression of numerous genes involved in energy production. While deletion of *phoU* does not affect the initial percentage of persister cells, the *phoU* mutant persisters die more rapidly in the presence of ampicillin, with 100-fold-reduced CFU/mL after 3 h in comparison to their occurrence in the wild type. Similarly, Zhang et al. showed that deletion of *crc*, the catabolite repression protein responsible for regulating metabolism of *P. aeruginosa* cells within biofilms, leads to increased metabolic activity throughout a mature biofilm, causing reduced ciprofloxacin tolerance. Complementation, through vector expression of *crc* in a ∆*crc* host restored normal metabolic activity, thus abating the reduced tolerance. Therefore, low metabolic activity is the key to survival of persister cells. This indicates that the significantly reduced growth rate that accompanies the bacterial stress response and is characteristic of the inner-biofilm subpopulation is the major reason for reduced susceptibility of biofilms to antibiotics.

In 2004, Balaban et al. used an elegant single-cell approach and microfluidics with a *hipA7* strain to investigate whether persister cells form prior to antibiotic treatment. They found that persister cells appeared prior to antibiotic treatment, so there is a fraction of persisters which are formed stochastically. Critically, these persister cells have reduced growth or no growth. The same group extended the single-
cell approach to demonstrate that the duration of the non-growth of persister cells is a function of the activity of the toxin of a TA system.

2.3.5 The argument against dormancy

It has become fashionable in the literature to argue that persister cells are not dormant but, instead, that the persistent state is an active response to stress. However, this line of reasoning actually supports that persister cells are dormant, with the only change being a better understanding of the genetic mechanism by which the cells get to the dormant state; i.e., the cells respond to stress in an active manner (via genetic circuits) only as a means to achieve dormancy, and the current line of research is determining what these circuits are. Hence, the issue of whether persisters are active or passive is really a matter of whether one chooses to analyze the cells in their response to stress or analyze the cells once a dormant state is achieved. Although the majority of cells respond actively to stress, it is only the dormant cells which demonstrate persistence.

In 2011, Nguyen et al. confirmed the much-earlier work of Korch et al. on the necessity of ppGpp for persistence with HipA7 by demonstrating a modest decrease of 68-fold in persistence upon deleting relA and spoT in P. aeruginosa. Nguyen et al. also reported similar results as Korch et al. in regard to decreased persistence using similar E. coli relA and spoT mutants. Nguyen et al. argued that the necessity of ppGpp implied an active response, whereas an alternative interpretation of their results is that ppGpp is required to activate a cell response that leads to arrested growth. In P. aeruginosa, Nguyen et al. found that the reduction of 4-hydroxy-2-alkylquinoline and the production of catalase and superoxide dismutase were important for the ppGpp effect. What was not considered was the effect of the simulated stringent response on TA systems in P. aeruginosa and their effect on dormancy.

Orman and Brynildsen utilized FACS in an attempt to differentiate non-dormant cells that were actively dividing and metabolically active from those dormant cells that were not and to relate this “dormancy status” to persistence. To accomplish this, they constructed a chromosomally integrated T5 promoter (T5p)-mCherry (red fluorescent) mutant under the control of a strong isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter in order to detect whether or not cells were actively
dividing/reproducing. Also, to differentiate cells that were metabolically active from those that were not, they added RedoxSensor green prior to FACS. They concluded from their flow cytometry data that non-growing (red) cells are more likely to bepersisters than growing (non-red) cells but that persisters can be found in the normally-dividing subpopulation. They interpreted this to mean that persistence is far more complex than dormancy and that their data point to additional characteristics needed to define the persister phenotype. However, to carry out their assays, they inoculated stationary-phase cells of *E. coli* incubated in LB medium at 37°C for 24 hours into LB medium for 2.5 hours prior to the FACS and persister cells assays, with the assumption that this resulted in all cells being in the exponential phase. However, Jõers et al. demonstrated that stationary-phase cells of *E. coli* grown in LB medium at 37°C, when used as the inoculum, do not rapidly resuscitate and can lead to 5% of the culture having persister cells after 2.5 hours at 37°C. Therefore, instead of analyzing only exponential-phase cells, Orman and Brynildsen very likely carried over high numbers of dormant persister cells into their FACS and persister cell assays. While both Keren et al. and Orman and Brynildsen demonstrated that the ability of a normally replicating cell to form a persister is lost after continuous exponential-phase propagation, Orman and Brynildsen did not use these types of cells in the above-described FACS assays. Therefore, pre-existing persister cells would be expected in their assays at time zero. Also, out of 500,000 cells that were subjected to FACS, their overall conclusion that dormancy is not necessary for persistence was based on their finding that out of approximately 100 persister cells, only approximately 20 of them sorted as metabolically active or actively growing; i.e., the vast majority (80 of them) were metabolically inactive. Given the inherent inaccuracy of FACS (they showed that the error with their FACS method was 0.2% or 1,000 cells) and the fact that no explanation of how the boundaries between red and non-red and green and non-green in the FACS were established, it seems very possible that if the green fluorescence boundary had been increased and/or the red fluorescence boundary decreased, 100% of the persister cells would have been characterized as non-dividing and non-metabolically active and thus classified as dormant. Hence, their claim that dormancy is not necessary or sufficient for persistence is suspect.

The study of Hofsteenge et al. found that environmental isolates of non-pathogenic *E. coli* have
different tolerances to different antibiotics. Based on this observation, they concluded that there may be distinct physiological states of dormancy. However, this conclusion cannot be directly drawn from these results without demonstrating that the different antibiotic-tolerant populations do in fact exhibit differing states of dormancy. Without this important analysis, it seems more likely that different tolerance levels to antibiotics could result from varying levels of induced persistence, since antibiotics are known to affect persistence.\textsuperscript{22,37}

It has been argued that the study of Wakamoto et al.\textsuperscript{51} with \textit{Mycobacterium smegmatis} implies that persister cells are not dormant. This study showed that cells surviving lethal treatment with the prodrug isoniazid were metabolically active. However, isoniazid requires activation by the catalase KatG, so the cells tolerant to isoniazid were simply cells with low levels of KatG activity. In effect, these tolerant cells were never exposed to a lethal antibiotic treatment because the isoniazid remained inactive. Hence, the metabolic activity observed for cells surviving isoniazid is not indicative of metabolic activity in persister cells and this report is a special case of a prodrug requiring activation. Therefore, there is little evidence indicating that persister cells are not dormant but a wealth of evidence indicating persister cells are dormant.

2.3.6 Preventing persistence and waking persisters

There has been some success in killing persister cells by adding glycolysis intermediates (e.g., pyruvate) which serve to generate a proton motive force that makes the cells more susceptible to aminoglycosides;\textsuperscript{52} note that these authors did not show that these compounds revert persister cells. These results suggest that persister cells are primed for more-active metabolism, since providing glycolysis intermediates stimulates metabolic activity. Also, the fact that TA systems are closely related to persistence\textsuperscript{2,9} and many if not all slow metabolism due to free toxins (e.g., endoribonucleases) argues that if toxin activity could be controlled, persistence may be controlled.

Since persister cells are likely dormant, it makes sense to try to wake them to make them susceptible to antibiotic treatments. By screening 6,800 chemicals in a random chemical library, 3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate was identified as a compound that
wakes persister cells at 25 µM\(^53\). However, the mechanism was not determined. Also, Pan et al.\(^54\) found a new use for brominated furanones that have been studied for inhibiting quorum sensing by finding that (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5\(H\))-one wakes \(P.\ aeruginosa\) planktonic and biofilm persister cells. They also determined that the compound is effective with mucoid \(P.\ aeruginosa\)^\(^55\). In addition to the limited success using chemical compounds to wake persisters, there has also been some success using Trp-/Arg-containing antimicrobial peptides to kill persisters\(^56\). Antimicrobial peptides do not function like traditional antibiotics, which disrupt cellular processes, but rather act by directly disrupting cell structure, with activity against bacteria, viruses, and fungi\(^57\). In summary, little is known about how persister cells awaken from a dormant state to become susceptible to antibiotics, and few methods have been devised to kill persisters.

2.4 Conclusions

Given the strong link between ppGpp, TA systems, and persistence, it would be informative to investigate whether changes in ppGpp lead to direct changes in the transcription of TA systems and thereby activate toxins (when toxins are studied with physiologically relevant copy numbers). Also, it is important to demonstrate how external stress results in changes in ppGpp concentrations. Clearly, overproduction of most toxins increases persistence, but how physiological levels of toxins induce persistence is not yet clear and needs to be addressed. Since TA systems control other TA systems to form a cascade related to persistence\(^14, 58\) and both toxins\(^26, 59\) and antitoxins\(^60, 61\) are global regulators, TA systems form an intricate part of how the cell responds to stress. Clearly what is needed is a way to keep toxins inactive and antitoxins active, but given the large number TA systems in many strains, this is a formidable challenge. With an improved understanding of how persister cells form, we may be in a better position to wake them and make them more susceptible to antibiotics.

2.5 Acknowledgements

This work was supported by the NIH (grant R01 GM089999) and the Grace Woodward Foundation. T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University.
2.6 References


Chapter 3. Lack of Metabolic Activity in Dormant Cells Causes Multidrug Tolerance

3.1 Preface

This chapter is adapted from the following publication: Kwan, B.W., Valenta, J.A., Benedik, M.J., and Wood, T.K. *Arrested protein synthesis increases persister-like cell formation.* Antimicrob. Agents Chemother., 2013. 57:1468-1473. In this study, several chemical compounds were used as pre-treatments to arrest protein synthesis, which results in bacterial persistence. These results demonstrate the direct correlation between metabolic dormancy and the persister cell phenotype.

3.2 Abstract

Biofilms are associated with a wide variety of bacterial infections and pose a serious problem in clinical medicine due to their inherent resilience to antibiotic treatment. Within biofilms, persister cells comprise a small, bacterial subpopulation that exhibits multidrug tolerance to antibiotics without undergoing genetic change. The low frequency of persister cell formation makes it difficult to isolate and study persisters, and bacterial persistence is often attributed to a quiescent metabolic state induced by toxins that are regulated through toxin-antitoxin systems. Here we mimic toxins via chemical pre-treatments to induce high levels of persistence (10-100%) from an initial population of 0.01%. Pre-treatment of *Escherichia coli* with (i) rifampicin, which halts transcription, (ii) tetracycline, which halts translation, and (iii) carbonyl cyanide m-chlorophenyl hydrazine, which halts ATP synthesis, all increased persistence dramatically. Using these compounds, we demonstrate that bacterial persistence results from halted protein synthesis and from environmental cues.

3.3 Introduction

Within every bacterial population there is a small sub-population that survives when challenged with a lethal antibiotic treatment. This sub-population of cells, known as persisters, exhibits a nonhereditary, multidrug tolerance. Regrowth of these persisters, by inoculation into fresh media, produces a population both genetically identical to the original culture and equally susceptible to antibiotic treatment. Persisters are found in all phases of cell growth, with a frequency of 0.0001 to 0.001% of the
population in exponential-phase cultures and as high as 1% in bacterial biofilms and stationary-phase cultures\textsuperscript{3,4}. Biofilms consist of an extracellular polymeric matrix, which in some cases acts as a physical barrier to protect bacteria from antimicrobials and/or host defense mechanisms, and also contains cells with a wide range of metabolic activities\textsuperscript{5}. Biofilms are associated with numerous infections\textsuperscript{6}, including recalcitrant infections of cystic fibrosis, and gingivitis or infections on indwelling devices\textsuperscript{3,7}. Persister cells are the key to biofilm resilience because survival of just a few persisters allows the biofilm to repopulate\textsuperscript{8}. Persisters also cause recurrence of tuberculosis infections\textsuperscript{7}, which are particularly difficult to treat in the lungs. Therefore, persister cell research is necessary in order to devise more effective medical treatments to combat infectious diseases.

Treatment of bacterial infections is complicated by both bacterial persistence and resistance, two distinct phenomena occurring through unrelated mechanisms\textsuperscript{3}. Resistance is caused by genetic variations, which result in the alteration of antibiotic targets to reduce antibiotic binding and efficacy\textsuperscript{3}. As a result, resistant bacteria are able to survive and even grow in the presence of antibiotics. Persistence is believed to arise from stochastic, physiological differences\textsuperscript{9} that induce a state of dormancy, or low metabolic activity\textsuperscript{3}. This is supported by the naturally higher frequency of persister cells in the stationary phase as opposed to the exponential phase. It is thought that persisters neither grow nor die in the presence of antibiotics because antibiotics are unable to corrupt cellular processes in a cell with globally reduced metabolism\textsuperscript{3}.

Toxin-antitoxin (TA) systems are prevalent in the bacterial genome (at least 37 in \textit{Escherichia coli})\textsuperscript{10}, and are perceived as a redundant, genetic basis for the formation of persisters from normal cells\textsuperscript{11,12}. TA systems typically consist of two genes encoding a stable toxin, which disrupts an essential cellular process, and an unstable antitoxin, which mediates the effect of the toxin\textsuperscript{7}. MqsR/MqsA is one such well-characterized TA system\textsuperscript{13-19} that is important to persistence\textsuperscript{20-22}. MqsR, the toxin, is an RNase\textsuperscript{18} which cleaves nearly all mRNA\textsuperscript{23}, and overproduction of MqsR was shown to significantly increase persistence\textsuperscript{20-22}; deletion of \textit{mqsR} resulted in decreased persistence, which was the first time the absence of a toxin was shown to affect persistence\textsuperscript{21}. TisB/IstR-1 is another TA system in which the toxin TisB
decreases ATP levels and induction of *tisB* transcription through the SOS response increases persistence. 

Persister cells are typically described as dormant, nondividing cells with globally reduced metabolism; however, it is not clear what exactly a persister cell is or what the extent of dormancy is. A significant step in understanding the persistence phenotype was the demonstration that cells exhibiting low levels of translation are more likely to be persisters. An unstable green fluorescent protein variant was inserted into the chromosome under the control of the ribosomal *rrnB*P1 promoter, which is highly expressed under conditions of rapid growth. Cells from an exponential-phase culture were individually sorted based on the level of fluorescence to isolate the “dimmer” cells with low levels of translation. These dimmer cells were 20-fold enriched in persisters, suggesting a correlation between persisters and reduced protein synthesis.

Induction of the SOS response by a low-concentration fluoroquinolone pre-treatment, increased the persister population that tolerated lethal antibiotic exposure. This suggests that induced persistence is a side effect of antibiotic treatment and can be triggered as a response to the environment. Based on the significant increase in persistence observed with overproduction of the toxin MqsR and with ciprofloxacin as an environmental trigger for the SOS response, we hypothesized that perhaps compounds which reduce protein synthesis could be used to mimic the effects of toxins, like MqsR and TisB, to produce persister cells. Hence, in this study, we disrupted protein synthesis via rifampicin and tetracycline and disrupted energy production via carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP).

Corroborating our hypothesis, use of these compounds to inhibit transcription, inhibit translation, and inhibit ATP synthesis, induced persistence in a large percentage of the population (10-100%), in comparison to a maximum of ~1% observed in stationary-phase cultures. Based on our results, we conclude that the level of bacterial persistence is proportional to the level of halted protein synthesis and, additionally, have designed a novel method for inducing a high level of persistence within any bacterial population by utilizing a chemical pre-treatment. The ability to work with a culture consisting of almost entirely persister cells is a useful tool for future persister research.
3.4 Results and Discussion

For this study, ciprofloxacin and ampicillin were the bactericidal antibiotics chosen for treatments to isolate persister cells. Ciprofloxacin, a fluoroquinolone, binds to DNA and affects the activity of DNA gyrase, inhibiting DNA replication. Ciprofloxacin is effective at killing cells in all phases of growth, making it an appropriate antibiotic to isolate persister cells regardless of whether pre-treatment affects replication. Ampicillin, a β-lactam, binds to transpeptidase and carboxypeptidase, inhibiting cell wall synthesis. The disruption of these enzymes leads to lethal defects of the cell wall and also cell lysis during the process of cell replication. It should be noted that ampicillin has reduced efficacy in treating non-growing or slow-growing cells. Ampicillin was used in this study, in addition to ciprofloxacin, to isolate persister cells and test for multidrug tolerance of pre-treated cultures, indicative of bacterial persistence.

3.4.1 Rifampicin pre-treatment increases persistence

Rifampicin is a bacteriostatic antibiotic which inhibits mRNA synthesis by binding and inactivating RNA polymerase, thereby preventing transcription. We reasoned that rifampicin pre-treatment could be used to increase persistence by inhibiting transcription, consistent with overproduction of MqsR, which cleaves nearly all mRNA. Hence, we tested whether a pre-treatment of E. coli with rifampicin would increase persistence. To pre-treat the cells, we exposed cells to rifampicin (100 µg/mL) during the mid-exponential phase for 30 min. Rifampicin pre-treatment conditions were optimized for maximum survivability, and altering the concentration and duration led to varied survivability. Our results showed that cultures pre-treated with rifampicin displayed significantly increased persistence (~1,000-10,000-fold increase over untreated cultures), with 59% of the pre-treated population surviving ciprofloxacin treatment and 69% surviving ampicillin treatment (Fig. 3.1).
Figure 3.1. Persistence induced with rifampicin, tetracycline, and CCCP pre-treatments. Cell survival (%) after treatment with (a) ciprofloxacin (5 µg/mL) and (b) ampicillin (100 µg/mL) for 3 h. Exponential phase cultures of *E. coli* K-12 BW25113 wild-type were pre-treated with rifampicin (100 µg/mL for 30 min), tetracycline (50 µg/mL for 30 min), and CCCP (50 µg/mL for 3 h) prior to antibiotic exposure. Data from four independent cultures are shown along with one standard deviation.

To verify our results, we observed that pre-treatment only caused a modest amount of cell death, with 57% of the initial population surviving rifampicin exposure, confirming that persistence is not artificially increased by eradication of non-persisters via pre-treatment. We also tested whether the formation of spontaneous resistant mutants affected our results by checking for growth of the persister cultures plated on LB agar plates containing ampicillin or ciprofloxacin, respective of prior treatment. Our results showed that spontaneous resistant mutants contribute to less than 0.0001% of the induced persister population, with no observable growth up to the limit of detection. An important consideration, regarding the choice of antibiotics, is that pre-treatment may lead to a state of diminished growth, similar to stationary phase, which would reduce the efficacy of ampicillin. However, increased survival to ciprofloxacin after rifampicin treatment confirms the persistence phenotype because ciprofloxacin eradicates cells with reduced growth. Therefore, we achieved a consistent increase in persistence to
antibiotics of two separate classes.

### 3.4.2 Rifampicin-induced persisters show a characteristic lag in revival and slow loss of viability

To provide further evidence that the rifampicin pre-treatment induced persistence, we performed a revival assay of cultures pre-treated with rifampicin since persister cells exhibit a lag before resumption of normal growth\(^{25}\). As expected for persister cells, we found that rifampicin-pre-treated cultures displayed a lag of ~4 h (Fig. 3.2). The delay before growth following exposure to rifampicin has previously been described as a “post-antibiotic effect”, caused by the lack of mRNA\(^ {36} \). The lag before resumption of normal growth, reduced levels of mRNA, and increased survival to antibiotics are all characteristics that are associated with both persister cells and rifampicin-treated cells. These correlations, suggest that the “post-antibiotic effect” of rifampicin is physiologically the same as that in persister cells, so we conclude that rifampicin pre-treatment induces a persister-like state.

![Figure 3.2](image_url)

**Figure 3.2.** Revival of rifampicin-, tetracycline-, and CCCP-induced persisters. Resumption of growth (monitored with turbidity) for persister subsequent to pre-treatments. Exponential phase cultures of *E. coli* K-12 BW25113 wild-type were pre-treated with rifampicin (100 µg/mL for 30 min), tetracycline (50 µg/mL for 30 min), and CCCP (50 µg/mL for 3 h) prior to growth in fresh LB media. Representative data from two independent cultures are shown along with one standard deviation.

Since antibiotic treatment of bacterial cultures produces bi-phasic cell death, with persister cells surviving extended periods of time, exhibiting slow, steady cell death\(^ {37} \), we tested the rifampicin-pre-treated cells to see if they demonstrated this slow loss of viability that is characteristic of persister cells.
Hence, we exposed pre-treated cultures to ciprofloxacin for a prolonged period, quantifying the number of viable cells at several time points. Our results reflect slow, steady cell death for up to 8 h of antibiotic treatment (Fig. 3.3), which is characteristic of persister cell cultures. Therefore, our rifampicin-induced persisters are “true” persisters.

![Figure 3.3. Prolonged antibiotic exposure of rifampicin-induced persisters. Cell survival (%) during prolonged treatment with ciprofloxacin (5 µg/mL). Exponential phase cultures of *E. coli* K-12 BW25113 wild-type were pre-treated with rifampicin (100 µg/mL for 30 min) prior to antibiotic exposure. Data from four independent cultures are shown along with one standard deviation.](image)

**3.4.3 Rifampicin induces persistence by arresting transcription**

Our hypothesis is that rifampicin increases persistence by halting protein synthesis via inhibition of transcription. This suggests that rifampicin-resistant mutants would no longer show a rifampicin-induced increase in persistence. Three independent spontaneous rifampicin-resistant mutants behaved consistently with this prediction. These strains showed no effect of rifampicin pre-treatment, producing less than a 4-fold difference in antibiotic persistence with and without pre-treatment, in comparison to a 13,000-fold increase in persistence for the pre-treated wild-type culture. Rifampicin-resistant mutants yielded between 0.0001 to 0.002% persistence, identical to the magnitude of survival for the wild-type parent without pre-treatment.

Another important consideration was to check whether the induced persistence is actually the result of arrested transcription, rather than a cellular response from the inhibition of RNA polymerase.
Merodiploid strains (two independent strains) containing both wild-type and rifampicin-resistant (rpoB+/rpoB3) alleles were compared to the wild type (rpoB+/rpoB+) for persister formation upon rifampicin pre-treatment. If rifampicin induces persistence through inhibition of transcription, then carrying a rifampicin-resistant allele will allow continued transcription and should reduce the effect of pre-treatment. Without pre-treatment, both the rpoB+/rpoB3 and rpoB+/rpoB+ merodiploids showed the same magnitude of antibiotic persistence (0.2 to 2%). However, rifampicin pre-treatment increased persistence ~100-fold for the rpoB+/rpoB+ strains, with a reduced effect of ~10-fold for the rpoB+/rpoB3 strains. Therefore, the presence of a rifampicin-resistant allele in the merodiploids reduced persistence ~10-fold, allowing us to conclude that rifampicin pre-treatment induces persistence by inhibition of transcription and not via a cellular signal from the wild-type polymerase.

3.4.4 Tetracycline induces persistence similarly to rifampicin

Based on our findings that rifampicin induces persistence through inhibited transcription, we decided to test whether inhibiting translation would also induce persistence. We found that pre-treatment with tetracycline, a translation inhibitor, significantly increased persistence. Cultures were pre-treated with tetracycline in the same manner as with rifampicin (conditions optimized for maximum survivability), resulting in 47% survival to ciprofloxacin (5 µg/mL) and 21% survival to ampicillin (100 µg/mL) (Fig. 3.1). A majority of cells (60%) survived the tetracycline exposure, confirming that persistence was not artificially increased, and a reduction in protein synthesis increases persistence.

We also performed a revival assay and found that tetracycline-pre-treated cultures displayed a lag in revival of ~2 h (Fig. 3.2). The “post-antibiotic effects” of tetracycline, a combination of the lag before growth, reduced protein levels (from inhibition of protein synthesis), and increased survival to antibiotics, are consistent with rifampicin pre-treatment, so we conclude that tetracycline pre-treatment also produces persister cells. The corroboration of results from rifampicin and tetracycline pre-treatments suggests that inhibiting protein synthesis is the key to making persister cells. Preliminary tests with streptomycin (100 µg/mL) and trimethoprim (10 µg/mL) had a similar effect, both displaying ~1% survival to ampicillin (100 µg/mL), but pre-treatments with these compounds were not optimized.
3.4.5 Carbonyl cyanide m-chlorophenyl hydrazine (CCCP) induces persistence

After our success at inducing persistence through mechanisms acting similar to toxin MqsR, we hypothesized that mimicking the toxin TisB would also induce persistence. Since the toxin TisB decreases cellular ATP\(^2\), we tested pre-treatment with the metabolic poison CCCP, an uncoupling agent that inhibits ATP synthesis\(^3\). Using a pre-treatment of CCCP to reduce cellular ATP (optimized for maximum survivability), we observed 76% persistence to ciprofloxacin and 64% persistence to ampicillin, similar to the results with rifampicin and tetracycline (Fig. 3.1). As with the other pre-treatments, 47% of cells survived CCCP exposure, confirming that persistence was not artificially increased and a reduction in metabolism increases persistence.

3.4.6 CCCP-induced persisters show characteristic lag in revival

As with the rifampicin and tetracycline pre-treatments, we performed a revival assay following CCCP pre-treatment to characterize the induced survivability as persistence. Our results showed a lag in revival of ~0.5-1 h (Fig. 3.2) following CCCP pre-treatment, which is shorter than that induced by antibiotics. We reason that the recovery time is reduced because CCCP diminishes metabolic activity without directly inhibiting mRNA or protein synthesis. Therefore, we conclude that CCCP pre-treatment does in fact induce persistence.

3.5 Conclusions

Persister cells have frequently been characterized as dormant cells in a state of low metabolism\(^12, 20, 25\), although few experiments have been performed to verify this. Several TA systems have been suggested as the basis of persister cell formation\(^12, 22\). In this study we have shown that bacterial persistence can be induced with pre-treatments that affect transcription and translation. We first attempted to mimic the effects of the toxin MqsR\(^2\) and found that temporary exposure of cells to rifampicin, to inhibit transcription, significantly increased subsequent survival to treatment with the bactericidal antibiotics ciprofloxacin and ampicillin (Fig. 3.1), two different classes of bactericidal antibiotics. This shows that the rifampicin-induced survivor cells exhibit multidrug tolerance, which is indicative of bacterial persistence\(^2\). The order of magnitude of these persister cells was consistently between 10 to 100%, a
considerably higher level than the ~0.01% persistence we found in exponentially growing cells (Fig. 3.1) and the ~1% persistence observed in stationary-phase cultures\textsuperscript{3,4}. This high increase in percentage of persister cells can have several applications, considering the low frequency of naturally forming persisters.

Similarly to our rifampicin pre-treatment, we found that tetracycline, which binds the ribosome to inhibit translation, also produced a high level of persistence (Fig. 3.1). Since both rifampicin and tetracycline effectively prevent translation, the corroboration of these results shows that lack of protein synthesis is a key to bacterial persistence. Our conclusion is consistent with previous research that indicates a correlation between persister cells and reduced protein synthesis\textsuperscript{20}. It was shown that cells exhibiting low levels of translation were 20-fold more persistent than a normal culture\textsuperscript{20}. With our pre-treatments, we have induced persistence in nearly 100% of the cell population by blocking protein synthesis (~1,000- to 10,000-fold increased persistence). Our results suggest that persisters are cells exhibiting essentially a complete lack of protein synthesis.

Pre-treating cells with rifampicin or tetracycline to induce persistence was effective at imitating the effect of overexpressing the toxin MqsR. However, it is important to note that rifampicin and tetracycline block all protein synthesis, while MqsR allows continued translation of 14 mRNA lacking GCU cleavage sites\textsuperscript{23} and transcripts whose GCU sites are blocked by mRNA secondary structure or protein binding. Therefore, it seems that naturally formed persisters arrest protein synthesis, except for the continued synthesis of a few select proteins. These specific proteins may have an important role in maintaining the viability of the persisters, or may be coincidental, so further study is warranted.

After success with mimicking the effect of MqsR, we also tried to mimic another toxin linked to persister cell formation, TisB\textsuperscript{24}. We did this by testing whether the uncoupling agent CCCP had a similar effect on persistence when used as a pre-treatment. We found that this metabolic poison induced persistence along the same order of magnitude as that observed for pre-treatments with rifampicin and tetracycline (Fig. 3.1). Based on our supposition that bacterial persistence is the general lack of protein synthesis, we postulate that the inhibition of ATP synthesis from CCCP\textsuperscript{30} forces the cells to halt
metabolic activity and become persister cells. We conclude that any TA system capable of inhibiting protein synthesis, either directly (e.g. inhibition of transcription), or indirectly (e.g. inhibition of ATP synthesis), provides a means for persister cell formation.

Combination therapy with multiple antibiotics is often used to treat infections as an added measure to prevent development of bacterial resistance and to achieve antibiotic synergism\(^3\). Rifampicin\(^4\) and tetracycline\(^5\) have often been used in combination therapy against infections from a wide range of microorganisms. The results of this study suggest that the use of these two compounds may induce persistence and lead to a reduced or failed treatment from additional antibiotics.

Our newfound ability to produce a culture consisting almost entirely of persister cells allows utilization of techniques designed to study population-wide characteristics. Previously, the low frequency of persister cells has made it difficult to isolate enough cells for transcriptome and proteome experiments. Using chemical pre-treatments to produce high-persister cultures will be useful in studying additional aspects of persistence; for example, we can better study gene expression of persister cells to determine the extent of dormancy required for persistence. Abundant persisters also allow us to more easily study how persister cells revive and determine methods for altering revival. Gaining a better understanding of the extent of dormancy required for persistence and uncovering the dynamics of revival will allow research to progress in designing methods to eliminate persisters.

As with all biological studies, laboratory conditions need to be evaluated with regard to naturally occurring conditions. In this study, chemical pre-treatment may introduce unwanted physiological responses that are not relevant to the natural persister phenotype; the pre-treatment compounds mimic the activity of toxins, so the high levels of persistence arise as a direct consequence of the chemical pre-treatments. Caution is also advised for studying revival of these chemically induced persisters because it is not known whether resumption of growth is distinct from that of natural persistence (i.e., regulated by TA systems). Therefore, this induced state of dormancy should be regarded as a persister-like state and distinguished from dormancy of naturally forming persister cells. Other methods may also be used to generate high frequencies of persister cells (e.g., overexpression of a toxin from an inducible plasmid); for
example, persistence has been induced in exponential cell cultures through overexpression of \( mqsR^{21} \), \( tisB^{24} \), and \( relE^{42} \) at the same order of magnitude achieved in this study. However, the method developed in this study can be applied to wild-type cultures in non-supplemented media and provides another viable method to study persisters.

Persister cell formation was first shown to be a stochastic process\(^{25} \), thought to result from fluctuations in gene expression and protein levels within individual cells of an isogenic population\(^{43} \). Recent studies have suggested that persistence can also arise as a result of environmental factors, including the presence of ciprofloxacin\(^{24} \), a bactericidal antibiotic, or indole\(^{44} \), an intercellular signaling molecule. Many antibiotic compounds are naturally occurring in the environment, are produced by various microorganisms, and are used to provide a competitive advantage against neighboring species\(^{45} \). The significant increase in persistence we observe, after pre-treatment with rifampicin, tetracycline, and CCCP, clearly shows several ways in which bacterial persistence is induced via environmental pressure, rather than solely via a stochastic event. Hence, it is apparent that while persistence can result from stochastic fluctuations, it is also directly affected by the presence of numerous extracellular molecules. The response of persister cell formation, when faced with the presence of these natural inhibitory compounds, likely serves as an evolutionarily developed mechanism for bacteria to subsist despite chemical pressure from competing organisms.

3.6 Materials and Methods

3.6.1 Bacterial strains and growth conditions

Bacterial strains used are listed in **Table 3.1**. All experiments were conducted at 37°C in lysogeny broth (LB) medium\(^{46} \), with shaking at 250 rpm (liquid cultures).

3.6.2 Minimum inhibitory concentration (MIC) assay

The MIC of *E. coli* K-12 BW25113 to rifampicin, tetracycline, ampicillin, and ciprofloxacin was determined by incubating freshly inoculated cultures in LB broth for 16 h with varying concentrations of each antibiotic and observing inhibition of growth based on lack of turbidity. Experiments were performed with at least three independent cultures.
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<td>MB411 F’110-1 metB&lt;sup&gt;R&lt;/sup&gt; rpoB3 (Rif&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>MB4817</td>
<td>MB411 F’110-1 metB&lt;sup&gt;R&lt;/sup&gt; rpoB3 (Rif&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Rif<sup>R</sup> is rifampicin resistance.

### 3.6.3 Persister cell viability assay

The number of persister cells was determined based on an assessment of cell viability after antibiotic treatment by serially diluting cultures in 0.85% NaCl solution, plating 10 µL drops on LB agar, and counting colonies<sup>48</sup>. Both ciprofloxacin<sup>31</sup> (5 µg/mL) and ampicillin<sup>32</sup> (100 µg/mL) treatments were used for 3 h except for the prolonged treatment with ciprofloxacin where 8 h was used. Concentrations were chosen to be at least 10x the MIC (ciprofloxacin, 0.01 to 0.05 µg/mL; ampicillin, 5 to 10 µg/mL) to minimize survival of potential spontaneous resistant mutants, and treatments lasted 3 h to ensure eradication of non-persisters. Cultures of **E. coli** K-12 BW25113 were diluted 1:1,000 in fresh LB broth from a 16-h overnight inoculum and grown to mid-exponential phase (turbidity of 0.8 at 600 nm) before either adding antibiotics (ciprofloxacin or ampicillin) and quantifying the number of persisters or pre-treating with compounds that reduce protein synthesis and then adding antibiotics (ciprofloxacin or ampicillin) and quantifying the number of persisters. Pre-treated cultures were exposed to rifampicin (100 µg/mL for 30 min), tetracycline (50 µg/mL for 30 min), or CCCP (50 µg/mL for 3 h). Cultures were centrifuged and resuspended in fresh LB broth to remove the pre-treatment compounds prior to quantifying persister cells after exposure to ciprofloxacin or ampicillin. The pre-treatment concentrations and durations were optimized to produce the highest survivability for this study. Concentrations of rifampicin (100 µg/mL) and tetracycline (50 µg/mL) were both significantly higher than the MICs, 10 to 20 µg/mL and 0.5 to 1 µg/mL, respectively. Experiments were performed with at least four independent cultures.
3.6.4 Persister revival assay

The time required for the revival of induced persister cells was determined based on growth curves for cultures after pre-treatment. Cultures of *E. coli* K-12 BW25113 were diluted 1:1,000 in fresh LB broth from a 16-h overnight inoculum and grown to mid-exponential phase (turbidity of 0.8 at 600 nm) before being pre-treated with rifampicin (100 µg/mL for 30 min), tetracycline (50 µg/mL for 30 min), or CCCP (50 µg/mL for 3 h). Following pre-treatment, cultures were centrifuged and resuspended in fresh LB broth to remove the pre-treatment compounds and then diluted to a turbidity of 0.1 at 600 nm. Experiments were performed with at least four independent cultures.

3.6.5 Merodiploid construction

The merodiploid strains were constructed by conjugation between a multiply auxotrophic donor carrying F’110 or F’110-1 and selecting for Met’ prototrophy with MB411. Rifampicin resistance was scored for confirmation. Persistence was tested as above, except with 25 µg/ml rifampicin pre-treatment, a concentration allowing normal growth of the resistant merodiploid, with at least three independent cultures.

3.7 Acknowledgements

This work was supported by the NIH (grant R01 GM089999). T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University.

3.8 References

Chapter 4. Toxin YafQ of the YafQ/DinJ Toxin/Antitoxin System Increases Persistence through Reduced Indole Signaling

4.1 Preface

This chapter is adapted from the following publication: *Hu, Y.*, *Kwan, B.W.*, Osbourne, D.O., Benedik, M.J., and Wood, T.K. *Toxin YafQ increases persister cell formation by reducing indole signalling*. Environ. Microbiol., 2014. doi: 10.1111/1462-2920.12567. (*These authors contributed equally.*) Toxin/antitoxin (TA) systems are bi-stable systems, which provide an ideal mechanism for stochastic formation of persister cells. In this study, toxin YafQ of the YafQ/DinJ TA system is shown to increase persistence by reducing levels of indole. These results demonstrate that persister cell formation can be modulated through the environment (i.e., through the inter-cellular signal indole).

4.2 Abstract

Persister cells survive antibiotic and other environmental stresses by slowing metabolism. Since toxins of toxin/antitoxin (TA) systems have been postulated to be responsible for persister cell formation, we investigated the influence of toxin YafQ of the YafQ/DinJ *Escherichia coli* TA system on persister cell formation. Under stress, YafQ alters metabolism by cleaving transcripts with in-frame 5’-AAA-G/A-3’ sites. Production of YafQ increased persister cell formation with multiple antibiotics, and by investigating changes in protein expression, we found that YafQ reduced tryptophanase levels (TnaA mRNA has 16 putative YafQ cleavage sites). Consistently, TnaA mRNA levels were also reduced by YafQ. Tryptophanase is activated in the stationary phase by the stationary-phase sigma factor RpoS, which was also reduced dramatically upon production of YafQ. Tryptophanase converts tryptophan into indole, and as expected, indole levels were reduced by the production of YafQ. Corroborating the effect of YafQ on persistence, addition of indole reduced persistence. Furthermore, persistence increased upon deleting *tnaA*, and persistence decreased upon adding tryptophan to the medium to increase indole levels. Also, YafQ production had a much smaller effect on persistence in a strain unable to produce indole. Therefore, YafQ increases persistence by reducing indole, and TA systems are related to cell signaling.
4.3 Introduction

Persisters are predominantly dormant cells\(^1\) that are highly tolerant to antibiotics without undergoing a genetic change\(^3\). Though persister cell numbers are low (they are absent in exponentially growing cultures\(^3\)) and reach a maximum of about 1% in the stationary phase and in biofilms\(^4\)\(^-\)\(^5\), these antibiotic-tolerant bacterial subpopulations have been implicated as the culprits for recurrent infections\(^6\). Unlike resistant cells which grow in the presence of antibiotics due to genetic changes, persisters survive antibiotic treatments since these cells are not undergoing the metabolic activities that antibiotics inhibit\(^6\). Therefore, understanding the mechanism of persister cell formation is important to derive strategies for controlling bacterial infections. However, the molecular mechanisms involved in the formation and waking of persister cells are not understood well.

Bacterial toxin/antitoxin (TA) systems appear to constitute the primary mechanism of persister cell formation, as they may be used to induce a state of dormancy\(^5\)\(^-\)\(^7\). There are well-established links between the frequencies of persister cells in *Escherichia coli* populations and the chromosomal TA gene modules HipA/HipB and RelE/RelB\(^8\)\(^-\)\(^9\). For example, the first gene linked to persisters\(^10\), HipA, is a toxin that was named for its high persistence (hip) mutants\(^9\), and its ectopic expression causes multidrug tolerance\(^8\). Additional evidence that TA systems are related to persister cell formation was found by deleting the gene that encodes toxin MqsR of the MqsR/MqsA TA system and showing a reduction in persistence\(^11\). Subsequently, inactivation of 10 TA systems that utilize endoribonuclease toxins in *E. coli* was also shown to reduce persistence\(^12\).

Similarly, deletion of the gene encoding the YafQ toxin of the YafQ/DinJ TA pair reduced the persistence of biofilm cells but not that of planktonic cells\(^13\). Moreover, transcriptional profiling of persisters isolated from planktonic cell cultures indicates that *dinJ-yafQ* is induced\(^8\); hence, the YafQ/DinJ TA system, like other TA systems, is linked to persister cell formation. Toxin YafQ is a specific endoribonuclease that blocks translation elongation through mRNA cleavage at in-frame 5’-AAA-G/A-3’ sequences via its association with the 50S ribosomal subunit\(^14\).

Although some persister cells arise stochastically\(^15\), stress appears to activate persister cell formation
since cells that are less fit to deal with stress have increased persistence\textsuperscript{16}. Furthermore, persistence increases as a result of stress from ciprofloxacin\textsuperscript{17}, rifampicin\textsuperscript{17}, tetracycline\textsuperscript{2}, and carbonyl cyanide \textit{m}-chlorophenyl hydrazone\textsuperscript{2}. Therefore, cell stress, and thus the general stress response master regulator RpoS\textsuperscript{18}, are important for persister cell formation.

One of the compounds regulated by RpoS is the cell signal indole\textsuperscript{19}. Indole is produced by a large number of Gram-positive and Gram-negative bacterial species, including \textit{E. coli}\textsuperscript{20}. Indole acts as a signal in \textit{E. coli} by activating genes such as \textit{gabT} and \textit{astD}\textsuperscript{21}; this signal is primarily active at low temperatures whereas autoinducer-2 is the primary signal in the gastrointestinal tract\textsuperscript{22}. Indole also acts as an intercellular signal by reducing the pathogenicity of cells that do not synthesize it\textsuperscript{23} and by influencing the biofilm of other cells\textsuperscript{24}, and acts as an interkingdom signal by tightening epithelial cell junctions\textsuperscript{25}. Moreover, indole was shown initially to increase antibiotic resistance by activating efflux pumps\textsuperscript{26, 27}, and later to increase the antibiotic resistance of neighboring cells\textsuperscript{28}. Therefore, indole is intimately related to RpoS and antibiotic resistance.

Like indole, TA systems have also been related to RpoS. Toxin MqsR enriches RpoS transcripts through differential mRNA decay\textsuperscript{29}, and antitoxin MqsA represses \textit{rpoS} transcription and influences the response to oxidative stress by binding to a palindrome in the \textit{rpoS} promoter\textsuperscript{30}. Hence, during non-stress conditions, antitoxin MqsA serves to limit RpoS, while during stress, toxin MqsR serves to increase RpoS. Similarly, antitoxin DinJ influences the general stress response by indirectly regulating the translation of RpoS transcripts via direct repression of \textit{cspE}\textsuperscript{31}; cold-shock protein CspE enhances translation of RpoS mRNA. Therefore, TA systems are intimately related to RpoS and the stress response.

Given that the YafQ/DinJ TA system actively participates in the general stress response regulated by RpoS\textsuperscript{31} and that persister cell formation is triggered by stress\textsuperscript{2, 17}, we focused here on how YafQ influences persister cell formation. We found that YafQ significantly reduces both RpoS and TnaA which results in reduced levels of indole. We also show that the reduction in indole leads to increased persistence. Additionally, the effect of YafQ on persistence is reduced in a strain unable to synthesize indole. Hence, toxin YafQ increases persister cell formation by reducing levels of the extracellular signal


indole.

4.4 Results and Discussion

4.4.1 YafQ increases persister cell formation

Since the production of toxins such as MqsR\textsuperscript{11} and RelE\textsuperscript{8} increase persister cell formation, we investigated the possible impact of YafQ, a homolog of RelE, on persistence to see if this toxin also increases persister cell formation. We used a \(\Delta yafQ\) host so that YafQ could be studied in a host that does not produce background levels of YafQ, and we used ampicillin (100 µg/mL) and ciprofloxacin (5 µg/mL) as representative antibiotics of two different classes, β-lactams and fluoroquinolones, respectively. As expected, YafQ produced from pCA24N-\(yafQ\) increased persister cell formation to both ampicillin (980-fold) and ciprofloxacin (43-fold) (Fig 4.1A), indicating that YafQ has an important role in persister cell formation.

4.4.2 YafQ reduces TnaA

Toxins have been shown to control metabolism by differential mRNA decay\textsuperscript{32,33} and YafQ regulates gene expression at the post-transcriptional level through its endoribonuclease activity\textsuperscript{14}, so we hypothesized that production of YafQ should alter global protein expression. Hence, we investigated the impact of producing toxin YafQ via a proteome analysis so that changes in mRNA cleavage could be studied in terms of how they impact final protein levels. We identified 23 proteins with levels altered by producing YafQ (Table 4.1 and representative two dimensional electrophoresis (2DE) gel image in Fig 4.2). All of the excised proteins spots resulted in a positive identification with all Mascot Molecular Weight Search (MOWSE) protein scores\textsuperscript{34} over 100 (Table 4.1), indicating significant matches\textsuperscript{35}. Of these, some ribosomal subunit proteins (RplQ and RpsF) and outer membrane proteins (OmpA and OmpX) were increased by producing YafQ, while several stress-related proteins (CspC, CspE, DnaK, HupA, HdeB, AhpC, and SodA) were reduced by producing YafQ. Critically, the levels of tryptophanase (TnaA), which produces indole during the stationary phase\textsuperscript{36,37}, and MdtE, an indole derivative exporter\textsuperscript{38}, were reduced when YafQ was produced.
Figure 4.1. YafQ increases persister cell formation and reduces TnaA. (A) Persister cell formation for BW25113 ΔyafQ/pCA24N-yafQ and BW25113 ΔyafQ/pCA24N after inducing YafQ production via 1 mM IPTG for 2 h and after treating with 100 µg/mL of ampicillin or 5 µg/mL of ciprofloxacin for 3 h. Persister data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance as determined using a Student’s t-test (P < 0.05). (B) The upper panel (Western blot) shows TnaA levels as detected by a His-tag antibody for BW25113 ΔtnaA/pCA24N-tnaA/pBS(Kan)-yafQ (YafQ+, lanes 2 and 4) and BW25113 ΔtnaA/pCA24N-tnaA/pBS(Kan) (YafQ-, lanes 3 and 5). The corresponding whole cell lysates were visualized by SDS-PAGE (lower panel). Both yafQ and tnaA were induced by 1 mM IPTG for 2 h. Two independent cultures were used for each strain and both replicates are shown. “M” indicates the protein ladder with protein standards covering a molecular weight range from 11 to 170 kDa. Lane 6 is the negative control (NC) where TnaA is absent (BW25113 ΔtnaA/pCA24N). The red arrow indicates the protein band for TnaA.

To verify the proteomic analysis, we investigated TnaA levels during YafQ production using a Western blot. Consistent with the proteomic results, TnaA was decreased by YafQ (Fig. 4.1B). This reduction in TnaA was significant enough to be visible in the SDS-PAGE (Fig. 4.1B). These results are reasonable since there are 16 putative in-frame YafQ-cleavage cites (5’-AAA-G/A-3’) in the coding region of tnaA (Fig. 4.3), which suggests that YafQ production leads to TnaA mRNA decay. These results also suggest that the reduction in TnaA by YafQ is direct and independent of RpoS since expression of tnaA was via a non-native promoter for the Western experiment.
Table 4.1. Summary of proteins with levels changed by producing YafQ. Changes in protein levels for BW25113 ΔyafQ/pCA24N-yafQ vs. BW25113 ΔyafQ/pCA24N after growth to a turbidity of 1.0 and production of YafQ for 1 h via 1 mM IPTG.

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**Figure 4.2.** 2-DE gels with and without YafQ production. Representative 2DE gel images of soluble proteins from (A) BW25113 ΔyafQ/pCA24N-yafQ and (B) BW25113 ΔyafQ/pCA24N after growth to a turbidity of 1.0 and production of YafQ for 1 h via 1 mM IPTG. The first dimension of separation was performed using 7 cm pH 3–10 IPG strips, followed by SDS-PAGE with 12% polyacrylamide gels in the second dimension, and Coomassie G-250 staining. The identified proteins with more than two fold up and down-regulation are labeled with red and black circles, respectively. The number near the circle refers to the protein ID in Table 4.1 (e.g., spot 22 is TnaA).

To determine directly if YafQ reduces the tnaA transcript levels, we quantified tnaA mRNA using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) after producing YafQ (i.e., for BW25113 ΔyafQ/pCA24N-yafQ vs. BW25113 ΔyafQ/pCA24N) and found that YafQ reduced tnaA transcripts significantly (10.6 ± 0.2-fold). Therefore, YafQ cleaves tnaA mRNA, which results in a reduction of TnaA.
Figure 4.3. The tnaA encoding region. Sequences 500 bp upstream of the translation start of tnaA are in blue font. The putative, in-frame YafQ-cleavage sites (5′-AAA-A/G-3′) are highlighted in green. The primer pair used in qRT-PCR to investigate tnaA mRNA levels is highlighted in yellow. The boxes indicate the -35 and -10 promoter regions. The Shine-Dalgarno sequence is in red.

4.4.3 YafQ reduces RpoS

Since antitoxin MqsA of the MqsR/MqsA TA system and antitoxin DinJ of the YafQ/DinJ system have been found to influence the response of the cell to oxidative stress via the stationary-phase sigma factor RpoS, we investigated the possible role of toxin YafQ on rpoS transcription and RpoS protein levels to see if this toxin influences RpoS. Using qRT-PCR to investigate whether YafQ affects rpoS, we
found that rpoS transcript levels were not changed upon YafQ production. In contrast, production of YafQ dramatically reduced RpoS levels (Fig. 4.4A). The reduction in RpoS levels is expected since both CspC and CspE were found via the proteomics analysis to be reduced upon YafQ production (Table 4.1) and both positively regulate RpoS levels. Therefore, YafQ reduces RpoS post-transcriptionally, likely by reducing CspC and CspE.

Figure 4.4. YafQ reduces RpoS and indole. (A) The upper panel (Western blot) shows RpoS levels as detected by an anti-RpoS antibody for BW25113 ΔyafQ/pCA24N-yafQ (YafQ+, lanes 2 and 4), and BW25113 ΔyafQ/pCA24N (YafQ-, lanes 3 and 5). The corresponding whole cell lysates were visualized by SDS-PAGE (lower panel). yafQ was induced from pCA24N-yafQ via 1 mM IPTG for 2 h. Two independent cultures were used for each strain and both replicates are shown. “M” indicates the protein ladder with protein standards covering a molecular weight range from 11 to 170 kDa. Lane 6 is the negative control (NC) where RpoS is absent (BW25113 ΔrpoS). (B) Indole production of BW25113 ΔyafQ/pCA24N-yafQ and BW25113 ΔyafQ/pCA24N strain after 30 and 60 min induction of yafQ by 1 mM IPTG. Error bars indicate standard error of mean (n = 2).

4.4.4 YafQ reduces indole

Given that RpoS stimulates indole production and YafQ reduces both RpoS and TnaA, we investigated whether YafQ reduces indole levels. As expected, YafQ reduced indole production (Fig. 4.4B). In comparison to YafQ, a smaller reduction in indole levels was found upon producing another endonuclease, toxin MqsR, for 60 min (-1.9 ± 0.3 fold for MqsR vs. -2.9 ± 0.1 fold for YafQ) despite a higher presence of cleavage sites within TnaA mRNA (18 MqsR cleavage sites). This confirms that YafQ is important for regulating indole, and that degradation of TnaA mRNA is not simply due to random
occurrence of cleavage sites. Therefore, YafQ reduces indole due to its reduction of TnaA and RpoS. Note that indole regulation of persistence is independent of YafQ toxicity as addition of 1 mM indole did not alter the reduction in growth seen upon producing YafQ.

4.4.5 Indole decreases persister cell formation

Indole acts as an intercellular and interkingdom signal, influencing multiple aspects of bacterial physiology and has proven to be an important factor in the transition to the stationary phase. Since YafQ reduced both TnaA and its product indole, we investigated whether indole affects persister cell formation. We found that persister cell formation against multiple antibiotics is repressed upon addition of 0.5 to 2 mM indole in both the exponential (Fig. 4.5A) and the stationary phase (Fig. 4.5B and C). Note that intracellular levels of indole as high as 60 mM have been reported as well as extracellular levels of 0.6 mM; hence, the concentrations of indole tested are physiologically relevant.

Figure 4.5. Indole reduces persister cell formation. (A) Persister cell formation of BW25113 ΔtnaA grown to a turbidity of 0.6 (exponential stage) in LB medium, contacted with indole at 0, 0.5, 1, and 2 mM for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin for 2 h. (B and C) Persister cell formation of BW25113 ΔtnaA grown to a turbidity of 2.0 (stationary phase) in LB medium, contacted with indole at 0, 0.5, 1, and 2 mM for 2 h, adjusted to a turbidity of 1.0, and exposed to (B) 100 µg/mL ampicillin or (C) 5 µg/mL ciprofloxacin for 2 h. For both panels, the data are the average of two independent cultures, and one standard deviation is shown. The asterisk indicates statistical significance as determined using a Student’s t-test (*: P < 0.05, **: P < 0.055).

We reasoned that since indole reduces persistence, then persistence should increase in a tnaA knockout strain that lacks indole production, compared with the wild-type strain. As expected, we found that persistence increases 6.4 ± 0.2 fold upon deleting tnaA (BW25113 ΔtnaA vs. BW25113). Furthermore, production of indole via TnaA should decrease persistence and a 2.4 ± 0.3 fold decrease in persistence was found when TnaA was produced (i.e., for BW25113 ΔtnaA/pCA24N-tnaA vs. BW25113
We also reasoned that since the addition of tryptophan to the medium increases indole levels\(^4\), persistence should decrease for the wild-type strain upon the addition of tryptophan. As expected, addition of 3.5 mM tryptophan to LB medium resulted in both a 3.3-fold increase in extracellular indole (0.69 ± 0.001 mM to 2.1 ± 0.04 mM) and a 19 ± 3.6 fold decrease in persistence for BW25113 (Fig. 4.6A). These four sets of experiments demonstrate clearly that indole reduces persister cell formation.

\[ \Delta tnaA/pCA24N \]

Figure 4.6. YafQ-derived persistence decreases in the presence of indole from tryptophan and is dependent on indole. (A) Persister cell formation of BW25113 wild-type grown to stationary phase in LB medium with 0 (“LB”) and 3.5 mM additional L-tryptophan (“LB + Trp”) to increase indole concentrations, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin for 2 h. The asterisk indicates statistical significance as determined using a Student’s t-test (P < 0.05). (B) Persister cell formation for production of YafQ in the BW25113 wild-type strain and for production of YafQ in the BW25113 \( \Delta tnaA \) host. Cultures were grown to early stationary phase in LB medium with 3.5 mM additional L-tryptophan, exposed to 1 mM IPTG for 3 h to induce YafQ production, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin for 2 h. Persister data are the average of two independent cultures, and one standard deviation is shown. “YafQ-” indicates strains with the empty plasmid pCA24N and “YafQ+” indicates strains with pCA24N-yafQ.

\[ 4.4.6 \text{ YafQ persistence is dependent on indole regulation} \]

Through independent experiments, we have shown that YafQ increases persistence, YafQ reduces indole, and indole negatively regulates persistence. However, we sought to determine the significance of indole regulation with respect to YafQ mediated persistence. To test this, persistence from production of YafQ was determined using the wild-type and \( \Delta tnaA \) (i.e., unable to synthesize indole) hosts under conditions in which indole is normally produced (i.e., stationary phase). As expected, we found that the
effect of YafQ production on persistence was decreased by 21 ± 5 fold in the ΔtnaA host compared with the wild-type strain (Fig. 4.6B), demonstrating that the increased persistence from producing YafQ is dependent on the downregulation of indole synthesis. This experiment also confirms the earlier result that deletion of tnaA increases persistence by showing an increase of 12 ± 3 fold (i.e., for BW25113 ΔtnaA/pCA24N vs. BW25113/pCA24N) (Fig. 4.6B). Note that this dependence on indole was specific for toxin YafQ since the reduction in persistence by toxin MqsR was not affected by deleting tnaA (5 ± 1% persistence for BW25113/pCA24N-mqsR vs. 3.9 ± 0.2% persistence for BW25113 ΔtnaA/pCA24N-mqsR). Furthermore, simultaneous deletion of both rpoS and tnaA, in comparison to sole deletion of tnaA, did not appreciably change persistence when YafQ was produced, so the effect of RpoS on persistence is via TnaA (3.8 ± 0.7 fold increased persistence from YafQ production for BW25113 ΔrpoS ΔtnaA/pCA24N-yafQ vs. BW25113 ΔrpoS ΔtnaA/pCA24N compared with a 3.7 ± 0.1 fold increased persistence from YafQ production for BW25113 ΔtnaA/pCA24N-yafQ vs. BW25113 ΔtnaA/pCA24N).

4.5 Conclusions

In this study, we show clearly that production of toxin YafQ increases persister cell formation (Fig. 4.1A) and the mechanism is through a dramatic reduction of TnaA (Fig. 4.1B and qRT-PCR results) that leads to reduced indole levels (Fig. 4.4B). Furthermore, YafQ also reduces RpoS (Fig. 4.4A); hence, the reduction in RpoS and TnaA protein levels by YafQ appear to work in concert to lower indole levels. However, the primary means by which YafQ increases persistence is via a reduction in indole, since deletion of both rpoS and tnaA did not affect persistence appreciably in comparison to deletion of tnaA. Furthermore, the regulation of tnaA by RpoS is complex43, so other factors may be involved. For example, RpoS is important at the start of the stationary phase, but its levels decrease in this growth phase, so it is important for genes as a transient regulator44.

Together with the results that exogenous indole addition reduces persistence (Fig. 4.5), that persistence increases in a ΔtnaA strain (Fig. 4.6B), that TnaA reduces persistence, and that tryptophan addition reduces persistence (Fig. 4.6A), we also demonstrate that persistence and indole levels are
inversely proportional. Additionally, persistence from YafQ production is reduced in a host that is unable to synthesize indole ($\Delta tnaA$) (Fig. 4.6B), confirming the importance of indole regulation to the YafQ persister mechanism. A schematic of our current understanding of how toxin YafQ influences persister cell formation is shown in Fig. 4.7.

**Figure 4.7.** Schematic of how YafQ increases persistence by reducing indole. Under antibiotic stress, free toxin, YafQ, is released due to the degradation of antitoxin DinJ by protease Lon, and YafQ cleaves $tnaA$ mRNA at its 5'-AAA-A/G-3' sites. RpoS levels are also reduced indirectly by YafQ (through a reduction in CspC and CspE), leading to further repression of $tnaA$. The decreased TnaA levels lead to a reduction in indole production, which increases persister formation. The lightning bolt indicates stress, $\rightarrow$ indicates induction, and $\perp$ indicates repression.

Our results differ from a previous report indicating that indole increases persistence$^{45}$. However, different strains and antibiotics were used which may address the apparent discrepancy in the results. Furthermore, our result that the signal indole reduces persistence follows from previous findings that indole increases drug resistance through RpoS-dependent induction of drug efflux$^{26,27}$ (i.e., cells with less
indole are less resistant to antibiotics), and cells that are less resistant to stress are more likely to become persisters. Moreover, the inverse relationship between indole and persistence is also reasonable since indole reduces biofilm formation in *E. coli* and is reduced in biofilms. Since persister cells are more prevalent in biofilms, cells should reduce indole levels in order to increase persistence in biofilms. Hence, our results provide insights into an important physiological role for TA systems: as cells are stressed in biofilms, toxins are activated and indole cell signaling is diminished to facilitate entry of cells into the persister state. Although speculative, our results further suggest that *E. coli* in biofilms may progressively use several TA systems to first activate RpoS (e.g., via MqsR/MqsA) and then deactivate RpoS (e.g., via YafQ/DinJ).

In summary, our results demonstrate how some toxins increase persistence, which is important because production of nearly all toxins increases persistence. In addition, our results demonstrate the importance of indole in biofilm cell physiology as a signal for controlling when cells become persistent in biofilms. Hence, our results cement the role of TA systems in biofilms and link TA systems to cell signaling through indole. Clearly, since not all cells produce indole, there are indubitably additional mechanisms that contribute to persister cell formation.

4.6 Materials and Methods

4.6.1 Bacterial strains, P1 transduction, plasmids, and growth conditions

The bacterial strains and plasmids are listed in Table 4.2. Lysogeny broth (LB) at 37°C was used for all the experiments. We used the Keio collection for isogenic mutants, and pBS(Kan) and pCA24N for expressing genes in *E. coli*. P1 transduction was used to create the double deletion strain, BW25113 ΔrpoS ΔtnaA (Table 4.2). Prior to P1 transduction, the kanamycin resistance cassette was removed from BW25113 ΔrpoS by using plasmid pCP20. After P1 transduction to add ΔtnaA, both gene deletions were verified by DNA sequencing using primers SrpoS-F/-R and StnaA-F/-R (primers are shown in Table 4.3). For construction of pBS(Kan)-yafQ, yafQ was PCR-amplified from *E. coli* MG1655 chromosomal DNA as a template, using primers yafQ-KpnI-F and yafQ-SacI-R (Table 4.3). The PCR product was cloned into the multiple cloning site of pBS(Kan) after double digestion with restriction enzymes.
enzymes KpnI and SacI. The yafQ gene in pBS(Kan) is under the control of a lac promoter. The pBS(Kan)-yafQ plasmid was confirmed by DNA sequencing with primer pBS(Kan)-seq (Table 4.3). Cell growth was assayed using the turbidity at 600 nm. Kanamycin (50 µg/mL) and chloramphenicol (30 µg/mL) were used to maintain the pBS(Kan)-based and pCA24N-based plasmids, and ampicillin (100 µg/mL) was used to maintain pCP20.

**Table 4.2. E. coli bacterial strains and plasmids used in Chapter 4.**

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<tr>
<td>MG1655</td>
<td>F- : : ilvG rfb-50 rph-1</td>
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<td>BW25113</td>
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<td>BW25113 ΔrpoS tnaA Ω KmR</td>
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<tr>
<td>DH5α</td>
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**Plasmids**

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<td>This study</td>
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<tr>
<td>pCP20</td>
<td>ApR, CmR, FLP, λ, cI857, λ, pR Rep +</td>
<td>57</td>
</tr>
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</table>

CmR, KmR, and ApR are chloramphenicol, kanamycin, and ampicillin resistance, respectively.
<table>
<thead>
<tr>
<th>Name</th>
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</tr>
<tr>
<td>r: ACTTAAACAACCGCTGCCG</td>
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</table>

**4.6.2 Persister assay**

Persister levels were determined by counting the number of colonies that grew on solid media after washing and serially diluting the cells after exposure to the antibiotic. To determine the number of persister cells from producing YafQ, overnight cultures were diluted to a turbidity of 0.05 and grown in LB medium with chloramphenicol (30 µg/mL) to a turbidity of 1.0, then 1 mM IPTG was used to induce *yafQ*. After 2 h, cells were washed, adjusted to a turbidity of 1.0 in LB, and were exposed to 100 µg/mL ampicillin or 5 µg/mL ciprofloxacin with 1 mM IPTG for 3 h. Cells were washed and diluted by $10^2$ to $10^7$ via 10-fold serial dilution steps in 0.85% NaCl solution and applied as 10 µl drops on LB agar to determine cell viability. For BW25113 Δ*tnaA*, cells were grown to a turbidity of ~0.6 or ~2.0 at 600 nm, and indole was added at 0, 0.5, 1, and 2 mM (from 500 mM stock; dimethylformamide was used as negative control). After 2 h, cells were washed, adjusted to a turbidity of 1.0 in LB, and were exposed to 100 µg/mL ampicillin or 5 µg/mL ciprofloxacin for 2 h. For YafQ persistence in Δ*tnaA* or Δ*rpoS* Δ*tnaA* hosts, cells were grown in LB supplemented with additional L-tryptophan (3.5 mM) to a turbidity of ~2.0
at 600 nm, and 1 mM IPTG was used to induce yafQ expression. After 3 h, cells were washed, adjusted to a turbidity of 1.0 in LB, and exposed to 100 µg/mL ampicillin for 2 h.

4.6.3 Protein sample preparation and two-dimensional gel electrophoresis (2DE)

For proteomic analysis, BW25113 ΔyafQ/pCA24N-yafQ and BW25113 ΔyafQ/pCA24N were grown to a turbidity of 1.0, then 1 mM IPTG was added to express yafQ. After induction for 1 h, cells were washed with and resuspended in TE buffer with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). After centrifugation at 17,000 × g for 10 min to remove debris, the supernatants were used to determine the protein concentration via the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA). Salt was removed from the protein samples via a Bio-Rad ReadyPrep 2-D cleanup Kit. 2DE was performed using a Protean IEF cell and mini electrophoresis system (Bio-Rad). To ensure 2DE reproducibility and to prevent variations occurring due to the technique, all 2DE gels were performed under the same electrophoresis conditions, and each sample was run on duplicate 2D gels. Protein (200 µg) was mixed with rehydration buffer (Bio-Rad) and loaded onto an immobilized pH gradient (IPG) strip (7 cm pH 3-10, Bio-Rad). After an overnight rehydration, the IPG strip was focused by applying current for 15 min at 250 V followed by 2 h with voltage ramping linearly to 4,000 V to reach 20,000 Vh and then the samples were frozen at -80ºC. Prior to the second-dimension SDS-PAGE, the focused strip was equilibrated for 15 min with equilibration buffer (6 M urea, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 2% SDS, and 0.002% bromophenol blue) containing 2% dithiothreitol and for another 15 min with same solution containing 2.5% iodoacetamide. For the second-dimension separation, the IPG strips were positioned on 12% polyacrylamide gels, and the proteins were separated at 100 V for 2 h at room temperature. Gels were washed in ultrapure water and fixed in 20% methanol and 10% acetic acid solution for 30 min. ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used to quantify the change in the density of the spots on the 2D gel. Duplicate gels of each sample were analyzed.

4.6.4 Mass spectrometry (MS)

2D gels were visualized with Coomassie G-250 to identify differentially expressed proteins. The
protein spots of interest were manually excised from gels. The excised gel plugs were approximately 2 mm in diameter and 1.5 mm in thickness. Trypsin digestion was performed following standard protocols consisting of a series of washing and dehydrating steps using 25 mM ammonium bicarbonate and acetonitrile, respectively. The gel spots then were digested with trypsin at 37°C for 18 h. Trypsin-digested samples were desalted, mixed with the matrix-assisted laser desorption/ionization (MALDI) matrix, and spotted onto a MALDI target plate. Samples were cleaned with 70% acetonitrile and 0.1% formic acid using C18 Ziptips (Millipore, Billerica, MA) prior to spotting.

All MALDI-MS and MS-MS experiments were performed using a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA). The MS data for the MALDI plates were acquired using the reflectron detector in positive mode (700-4500 Da, 1900 Da focus mass) using 800 laser shots (40 shots per subspectrum) with internal calibration. The collision gas was air at the medium pressure setting, with 1 kV of collision energy applied across the collision cell. All MS data were searched against the NCBInr database using the GPS Explorer V2.1 (Applied Biosystems) software. A Mascot MOWSE protein score of greater than 100 obtained with the Mascot search engine (Matrix Science, U.K.) was considered as a significant match. Two spots were analyzed to confirm the generated MS data.

4.6.5 Indole assay

Extracellular indole was measured with *E. coli* BW25113 ΔyafQ/pCA24N-yafQ and BW25113 ΔyafQ/pCA24N as described previously. The strains were cultured overnight in LB with kanamycin (50 µg/mL) and chloramphenicol (30 µg/mL). Overnight cultures were then inoculated into LB medium containing chloramphenicol (30 µg/mL) with an initial turbidity of 0.05. When cells grew to a turbidity of 1.0, 1 mM IPTG was added to induce yafQ expression. Extracellular indole concentrations were measured after 30 min or 60 min induction of yafQ. For the effect of tryptophan addition, BW25113 was cultured in LB with and without tryptophan (720 µg/mL) for 6 h.

To measure extracellular indole, 1 mL of cell-free culture fluid was mixed for 2 min with 0.4 mL of Kovac’s reagent (10 g of *p*-dimethylaminobenzaldehyde, 50 mL of HCl, and 150 mL of amyl alcohol), 100 µl of the reaction mixture was diluted in 900 µl of HCl-amyl alcohol solution (50 mL of HCl and 150
mL of amyl alcohol), and the absorbance at 540 nm was measured. Concentrations were calculated based on a calibration curve.

4.6.6 qRT-PCR

After isolating RNA using RNALater™ (Life Technologies, Carlsbad, CA) and an ethanol-dry ice quick cooling method during cell harvest, 50 ng of total RNA was used for qRT-PCR using the Power SYBR® Green RNA-to-Ct™ I-Step Kit and the StepOne™ Real-Time PCR System (Applied Biosystems). Primers were designed using Primer3Input Software (v0.4.0) and are listed in Table 4.3. The housekeeping gene *rrsG* was used to normalize the gene expression data. The annealing temperature was 60°C for all the genes in this study. To investigate the *rpoS* and *tnaA* mRNA changes by producing YafQ, overnight cultures of BW25113 Δ*yafQ*/pCA24N-yafQ and BW25113 Δ*yafQ*/pCA24N were inoculated into LB medium containing chloramphenicol (30 µg/mL) with an initial turbidity of 0.1 and grown to a turbidity of 2.0, then 1 mM IPTG was added for 2 h to induce yafQ until a turbidity ~3.0.

4.6.7 Western blot analysis

To investigate the influence of YafQ on RpoS protein levels, Western blots and SDS-PAGE were performed as described previously30. To investigate how YafQ affects TnaA levels, BW25113 Δ*tnaA*/pCA24N-tnaA/pBS(Kan)-yafQ and BW25113 Δ*tnaA*/pCA24N-tnaA/pBS(Kan) were grown until a turbidity ~1.0, and 1 mM IPTG was added to induce both *tnaA* and *yafQ*. After inducing for 2 h, cells were washed and resuspended in TE buffer with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). Samples were sonicated twice using a 60 Sonic Dismembrator (Fisher Scientific) at level 4 for 15 s. Soluble protein samples in supernatants were obtained by centrifuging the cell pellets at 17,000 × g for 4 min. Total protein was quantified using a Pierce BCA Protein Assay kit (Fisher Scientific), and 2 µg was loaded into each well of a 12% SDS-PAGE gel. The protein was transferred to a PVDF membrane, which was blocked with 4% BSA in TBST (10 mM Tris pH 7.5, 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature. The Western blots were probed with a 1:2000 dilution of primary antibodies against a His tag (Cell Signaling Technology, Beverly, MA), and then with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibodies
For SDS-PAGE, 12 µg of each protein sample was loaded, and the gel was stained with Coomassie blue G-250. To investigate how YafQ affects RpoS levels, BW25113 ΔyafQ/pCA24N-yafQ and BW25113 ΔyafQ/pCA24N were grown to a turbidity of 2.0, then 1 mM IPTG was added to induce yafQ for 2 h. Cells were processed as described previously and a 1:2,000 dilution of anti-RpoS monoclonal primary antibody (Neoclone, Madison, WI) was used for the Western blot.

4.7 Acknowledgements

This work was supported by the ARO (W911NF-14-1-0279). We are grateful for the Keio and ASKA strains provided by the National Institute of Genetics of Japan and for the help of Michael J. McAnulty with P1 transduction.

4.8 References


42. Li, G. and K.D. Young, Indole production by the tryptophanase TnaA in Escherichia coli is determined by the amount of exogenous tryptophan. Microbiology, 2013. 159(Pt. 2): p. 402-410.


Chapter 5. Phosphodiesterase DosP Increases Persistence through Reduced cAMP and Subsequent Indole Signaling

5.1 Preface

This chapter is adapted from the following publication: *Kwan, B.K., *Osbourne, D.O., Hu, Y., Benedik, M.J., and Wood, T.K. Phosphodiesterase DosP increases persistence by reducing cAMP which reduces the signal indole. Biotechnol. Bioeng., 2014. doi: 10.1002/bit.25456. (*These authors contributed equally.) In this study, the phosphodiesterase DosP, which cleaves both cyclic diguanylate and cyclic adenosine monophosphate (cAMP), was found to decrease indole via down-regulation of cAMP, subsequently leading to increased persistence. This result corroborates the relationship between indole and persistence from Chapter 4, and implicates a role for the general regulatory molecule cAMP in persister cell formation.

5.2 Abstract

Persisters are bacteria that are highly tolerant to antibiotics due to their dormant state and are of clinical significance owing to their role in infections. Given that the population of persisters increases in biofilms and that cyclic diguanylate (c-di-GMP) is an intracellular signal that increases biofilm formation, we sought to determine whether c-di-GMP has a role in bacterial persistence. By examining the effect of 30 genes from *Escherichia coli*, including diguanylate cyclases that synthesize c-di-GMP and phosphodiesterases that breakdown c-di-GMP, we determined that DosP (direct oxygen sensing phosphodiesterase) increases persistence by over a thousand fold. Using both transcriptomic and proteomic approaches, we determined that DosP increases persistence by decreasing tryptophanase activity and thus indole. Corroborating this effect, addition of indole reduced persistence. Despite the role of DosP as a c-di-GMP phosphodiesterase, the decrease in tryptophanase activity was found to be a result of cyclic adenosine monophosphate (cAMP) phosphodiesterase activity. Corroborating this result, the reduction of cAMP via CpdA, a cAMP-specific phosphodiesterase, increased persistence and reduced
indole levels similarly to DosP. Therefore, phosphodiesterase DosP increases persistence by reducing the interkingdom signal indole via reduction of the global regulator cAMP.

5.3 Introduction

It has long been established that lethal antibiotic treatments are unable to kill a small fraction of persistent bacteria\(^1\). This insensitivity to antibiotic treatment is not due to any inherent or developed resistance as cultures grown from these persister cells show the same sensitivity to the antibiotic as the parent culture\(^2, 3\). The persister phenotype has been exhibited in all bacteria tested\(^4\), but the mechanisms underlying persistence have yet to be fully elucidated. However, it is clear that persisters are metabolically dormant\(^5, 6\), and that toxins of toxin/antitoxin pairs increase persistence by inhibiting metabolic activity\(^7, 8\).

Biofilm formation provides protection to bacteria against environmental stress, and greater numbers of persister cells are found in biofilms and stationary-phase cultures in comparison to exponential-phase cultures\(^4\). Though the majority of biofilm cells are sensitive to antibiotics, persisters account for the resilience of biofilms, as the high proportion of persister cells in a biofilm allows survival of the population\(^3\).

Environmental signaling plays a role in persistence as demonstrated by \textit{Pseudomonas aeruginosa}, in which the quorum sensing-linked molecules \(N\)-(3-oxo-dodecanoyl)-\(L\)-homoserine lactone and pyocyanin increase persistence in exponential-phase cultures\(^9\). Since quorum sensing and biofilm formation invoke high levels of persistence, we reasoned that cyclic diguanylate (c-di-GMP), which increases biofilm formation\(^10\), may play a role in modulating persistence. Two molecules of GTP are converted to c-di-GMP by diguanylate cyclases (DGCs), which contain GGDEF domains, and c-di-GMP is degraded into linear di-GMP (pGpG) by phosphodiesterases (PDEs), which contain EAL or HD-GYP domains. The importance of c-di-GMP in bacterial physiology and its tight regulation is evident by the presence of numerous DGCs and PDEs in a given strain; for example, in \textit{E. coli} K-12 there are 12 proteins with a GGDEF domain, 10 proteins with an EAL domain, and 7 proteins with both EAL and GGDEF domains in a single polypeptide\(^11, 12\). Additionally, our lab recently discovered a biofilm dispersal protein (BdcA)
that binds c-di-GMP but does not act as a phosphodiesterase\textsuperscript{13}.

DosP, named as the direct oxygen sensing phosphodiesterase\textsuperscript{14}, is a 90 kDa protein with an NH\textsubscript{2}-terminal heme sensor-PAS-PAS-containing domain and a COOH-terminal GGDEF-EAL catalytic phosphodiesterase domain in which the GGDEF domain is inactive\textsuperscript{15}. DosP cleaves both c-di-GMP\textsuperscript{16} and cAMP\textsuperscript{17}, although c-di-GMP is proposed as the physiological substrate due to higher activity towards c-di-GMP than cAMP\textsuperscript{16}. Nevertheless, DosP is important for maintaining cAMP levels\textsuperscript{18}, which are tightly regulated at low intracellular concentrations\textsuperscript{19}. DosP is active as a tetramer and is strongly inhibited by CO, NO, and etazolate, a selective cAMP PDE inhibitor\textsuperscript{17}. The heme binding domain of DosP is 60% homologous to the PAS oxygen sensing domain of FixL, an oxygen responsive biological sensor in rhizobia\textsuperscript{20}. The catalytic activity of DosP is therefore oxygen dependent\textsuperscript{21} and is enhanced 17 fold when saturated with O\textsubscript{2}\textsuperscript{14}. DosP is also activated during entry into stationary phase and is positively regulated by RpoS, the stationary phase sigma factor\textsuperscript{12}.

Environmental signaling is also conveyed by the cell signal indole. In \textit{E. coli}, indole regulates several cellular processes including conferring multidrug resistance\textsuperscript{22}, increasing plasmid stability\textsuperscript{23}, decreasing motility\textsuperscript{24, 25}, and decreasing biofilm formation\textsuperscript{26-29}. Indole is also an interspecies signal for bacterial biofilm formation and virulence\textsuperscript{26, 28, 30, 31} and an interkingdom signal between bacteria and epithelial cells in the gastrointestinal tract\textsuperscript{32}. In \textit{E. coli}, indole is produced by tryptophanase (TnaA), which degrades tryptophan into indole, pyruvate, and ammonia\textsuperscript{33}. Indole is primarily imported by Mtr\textsuperscript{34} and exported by the AcrEF-TolC multidrug efflux system\textsuperscript{35}, although it is possible for small amounts\textsuperscript{36} of indole to cross the \textit{E. coli} membrane independently of both transporters\textsuperscript{37, 38}.

One positive regulator of indole is cyclic adenosine monophosphate (cAMP), a regulatory molecule used by species across multiple kingdoms. In bacteria, cAMP controls protein synthesis primarily through catabolite mediated repression of transcription\textsuperscript{39}. In \textit{E. coli}, cellular cAMP levels correspond inversely to the carbon source, resulting in low concentrations in the presence of a readily metabolizable carbon source (e.g., glucose)\textsuperscript{39}. cAMP binds to CRP, the catabolite receptor protein in \textit{E. coli}, to form the cAMP-CRP transcriptional regulator\textsuperscript{39}, which controls a large regulon and acts as a positive regulator for
the *tna* operon, responsible for indole synthesis.40

In this work, we find that the phosphodiesterase DosP increases persistence via decreased activity of tryptophanase which results in reduced levels of the signal indole. This regulation of tryptophanase is mediated by cleavage of the cellular signal cAMP by DosP. The involvement of regulatory signals cAMP and indole lends credence to the involvement of numerous pathways in persister cell formation.

5.4 Results and Discussion

5.4.1 DosP increases persistence

To determine if proteins associated with c-di-GMP play a role in persistence, we quantified the number of persister cells surviving ampicillin treatment for 29 isogenic deletion mutants lacking genes encoding diguanylate cyclases and phosphodiesterases. We identified 12 proteins potentially related to persistence (DosC, YdeH, YeaP, YedQ, DosP, Gmr, CsrD, YhjK, BluF (YcgF), YdiV, YjcC, and YliE) based on changes in persistence of 3-fold or greater. We further tested these proteins by producing the 12 proteins via IPTG-inducible plasmids from the ASKA Collection and determining persistence upon production of the DGCs and PDEs. We found that producing DosP caused the most significant difference, increasing persistence to both ampicillin (4200 ± 400 fold) and ciprofloxacin (62 ± 3 fold) (Fig. 5.1A) without affecting overall cell growth (specific growth rate of BW25113/pCA24N-dosP of 0.66 ± 0.09 h⁻¹ vs. 0.74 ± 0.06 h⁻¹ for BW25113/pCA24N). The consistent trend across multiple antibiotic classes demonstrates the multidrug tolerance that is the trait of persister cells; hence, DosP increases persistence dramatically.

5.4.2 DosP reduces biofilm formation

DosP is an active phosphodiesterase which degrades c-di-GMP to linear pGpG.14,16 We reasoned that if DosP is active *in vivo* as a phosphodiesterase, production of DosP should reduce biofilm formation since c-di-GMP increases biofilm formation in many bacteria.42 Corroborating this hypothesis, the production of DosP reduced mature biofilm formation by -25 ± 12 fold with low levels of DosP (from the uninduced leaky P₅₅₋₅₊₅_promoter) and eradicated biofilm formation with high levels of DosP (1 mM IPTG induction) (Fig. 5.1B). This confirms that DosP is an active phosphodiesterase that reduces c-di-GMP in
Figure 5.1. DosP is an active phosphodiesterase in vivo that reduces indole. (A) Persister cell formation for BW25113/pCA24N-dosP and BW25113/pCA24N grown to a turbidity of 1.0 in LB medium, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin or 5 µg/mL ciprofloxacin for 3 h. (B) Biofilm formation for BW25113/pCA24N-dosP and BW25113/pCA24N after 24 h of static growth at 37°C with and without 1 mM IPTG induction in LB medium. (C) Extracellular and intracellular indole concentrations for BW25113/pCA24N-dosP and BW25113/pCA24N after 2 h of DosP production via 1 mM IPTG. All data are averaged from two independent cultures and one standard deviation is shown.

5.4.3 BdcA also increases persistence

Since DosP reduces c-di-GMP and increases persistence, we tested persistence from production of BdcA, which sequesters c-di-GMP and causes biofilm dispersal. We found that producing BdcA from pCA24N-bdcA to reduce c-di-GMP increased persistence by 430 ± 80 fold relative to the empty plasmid. However, production of other c-di-GMP-related proteins did not affect persistence as expected based on
DosP and BdcA; i.e., other PDEs did not necessarily increase persistence and DGCs did not necessarily reduce persistence. For example, GGDEF protein YeaP unexpectedly increased persistence by 9 ± 4 fold. Furthermore, unlike phosphodiesterase DosP, EAL protein YahA reduced persistence by -261 ± 30 fold. Therefore, these results suggested that c-di-GMP levels may not be the mechanism by which DosP increases persistence.

5.4.4 DosP inhibits tryptophanase

To investigate further the relationship between DosP and persistence, we performed a microarray study to determine the effect of DosP production on the transcriptome. We found differential expression (≥4-fold) of 72 transcripts (Table 5.1), including repression of genes involved in tryptophan synthesis (the precursor for indole\(^3\)), indole synthesis, and indole import: \(trpE\) (-8.0 fold), \(trpL\) (-7.5 fold), \(trpD\) (-6.5 fold), \(tnaA\) (-7.5 fold), \(tnaL\) (-6.1 fold), and \(mtr\) (-4.9 fold).

Table 5.1. Microarray data for DosP production. Summary of the largest changes in gene expression as a result of producing DosP (i.e., BW25113/pCA24N-dosP vs. BW25113/pCA24N). Differentially-expressed genes regulated by reduced cAMP-CRP are in bold.

<table>
<thead>
<tr>
<th>Gene</th>
<th>b number</th>
<th>Fold Change</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acid Biosynthesis, Catabolism, and Transport</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(trpE)</td>
<td>b1264</td>
<td>-8.0</td>
<td>Anthranilate synthase, (trp) operon</td>
</tr>
<tr>
<td>(trpL)</td>
<td>b1265</td>
<td>-7.5</td>
<td>(trp) operon leader peptide</td>
</tr>
<tr>
<td>(trpD)</td>
<td>b1263</td>
<td>-6.5</td>
<td>Anthranilate phosphoribosyl transferase, (trp) operon</td>
</tr>
<tr>
<td>(tnaA)</td>
<td>b3708</td>
<td>-7.5</td>
<td>Tryptophanase, makes indole, (tna) operon</td>
</tr>
<tr>
<td>(tnaL)</td>
<td>b3707</td>
<td>-6.1</td>
<td>Tryptophanase leader peptide, (tna) operon</td>
</tr>
<tr>
<td>(mtr)</td>
<td>b3161</td>
<td>-4.9</td>
<td>Tryptophanase and indole permease (import)</td>
</tr>
<tr>
<td>(astC)</td>
<td>b1748</td>
<td>-4.9</td>
<td>Arginine catabolic pathway</td>
</tr>
<tr>
<td>(dppA)</td>
<td>b3544</td>
<td>-4.9</td>
<td>Binding component of dipeptide ABC transporter (import)</td>
</tr>
<tr>
<td>(dppD)</td>
<td>b3541</td>
<td>-4.6</td>
<td>ATP binding component of dipeptide ABC transporter (import)</td>
</tr>
<tr>
<td>(glnH)</td>
<td>b0811</td>
<td>-4.6</td>
<td>Component of glutamine ABC transporter (import)</td>
</tr>
<tr>
<td>(sdaC)</td>
<td>b2796</td>
<td>-4.6</td>
<td>Serine proton-symporter (import)</td>
</tr>
<tr>
<td>(gcvH)</td>
<td>b2904</td>
<td>-4.9</td>
<td>H-protein of glycine cleavage system</td>
</tr>
<tr>
<td>(gcvT)</td>
<td>b2905</td>
<td>-4.9</td>
<td>T-protein of glycine cleavage system</td>
</tr>
<tr>
<td>(gcvP)</td>
<td>b2903</td>
<td>-4.0</td>
<td>P-protein of glycine cleavage system</td>
</tr>
<tr>
<td>(cstA)</td>
<td>b0598</td>
<td>-4.0</td>
<td>Peptide transporter (import), induced by CsrA during carbon starvation</td>
</tr>
<tr>
<td>(aspA)</td>
<td>b4139</td>
<td>-4.0</td>
<td>Aspartate-ammonia lyase</td>
</tr>
<tr>
<td><strong>Carbohydrate Catabolism and Transport</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(gatC)</td>
<td>b2092</td>
<td>-7.5</td>
<td>Galactitol-specific enzyme IIC of phosphotransferase system</td>
</tr>
<tr>
<td>(gatB)</td>
<td>b2093</td>
<td>-7.5</td>
<td>Galactitol-specific enzyme IIB of phosphotransferase system</td>
</tr>
<tr>
<td>(gatD)</td>
<td>b2091</td>
<td>-6.5</td>
<td>Galactitol-1-phosphate dehydrogenase</td>
</tr>
<tr>
<td>(gatZ)</td>
<td>b2095</td>
<td>-6.5</td>
<td>GalYZ subunit</td>
</tr>
<tr>
<td>(gatA)</td>
<td>b2094</td>
<td>-5.7</td>
<td>Galactitol-specific enzyme IIA of phosphotransferase system</td>
</tr>
<tr>
<td>(gatY)</td>
<td>b2096</td>
<td>-4.6</td>
<td>GalYZ subunit</td>
</tr>
<tr>
<td>(acs)</td>
<td>b4069</td>
<td>-8.0</td>
<td>Acetyl-CoA synthetase</td>
</tr>
<tr>
<td>(yjeH)</td>
<td>b4068</td>
<td>-6.1</td>
<td>Cotranscribed with (acs)</td>
</tr>
</tbody>
</table>
Additionally, we performed a proteomic study with DosP using the same conditions and found that...
one of the most significantly reduced proteins was tryptophanase (TnaA, -33.1 fold, Table 5.2), the enzyme responsible for indole synthesis. Hence, both the microarray and proteomic studies indicated that DosP either directly or indirectly reduces cellular tryptophanase.

**Table 5.2.** Proteomics data for DosP production. Summary of the largest changes in protein levels as a result of producing DosP (i.e., BW25113/pCA24N-dosP vs. BW25113/pCA24N).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession</th>
<th>Fold Change</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TnaA</td>
<td>Q5UES8</td>
<td>-33.1</td>
<td>Tryptophanase</td>
</tr>
<tr>
<td><strong>Replication</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YiiU</td>
<td>C3SIZ2</td>
<td>-57.2</td>
<td>Cell division, coordinate FtsZ for division</td>
</tr>
<tr>
<td><strong>Phosphate Allocating Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upp</td>
<td>E2QPW1</td>
<td>-14.3</td>
<td>Uracil phosphoribosyltransferase</td>
</tr>
<tr>
<td>Ndk</td>
<td>E2QPY4</td>
<td>-4.6</td>
<td>Nucleoside diphosphate kinase, involved in stringent response</td>
</tr>
<tr>
<td><strong>Stress Response Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SspA</td>
<td>C3SRY7</td>
<td>-20.2</td>
<td>Induced by stringent starvation, inhibits accumulation of H-NS</td>
</tr>
<tr>
<td>Dps</td>
<td>E2QII0</td>
<td>-9.9</td>
<td>Starvation stress response</td>
</tr>
<tr>
<td>KatG</td>
<td>E2QIX4</td>
<td>-7.7</td>
<td>Catalase, oxidative stress response</td>
</tr>
<tr>
<td>UspE</td>
<td>C3TBD7</td>
<td>-5.3</td>
<td>Resistance to UV irradiation, stress inducible</td>
</tr>
<tr>
<td><strong>Global Regulatory Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hns</td>
<td>C3TCN2</td>
<td>-5.4</td>
<td>Global transcriptional regulator</td>
</tr>
<tr>
<td>IhfA</td>
<td>Q14F23</td>
<td>-3.2</td>
<td>Global transcriptional regulator</td>
</tr>
<tr>
<td><strong>Chaperone/Translation/Housekeeping Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YhpC</td>
<td>C3SSN2</td>
<td>-14.9</td>
<td>Assists in maturation of 30S ribosomal subunit</td>
</tr>
<tr>
<td>FusA</td>
<td>C3SQS7</td>
<td>-14.1</td>
<td>Elongation factor G, facilitates ribosomal translocation</td>
</tr>
<tr>
<td>ClpB</td>
<td>E2QQ51</td>
<td>-5.9</td>
<td>ATP dependent protease</td>
</tr>
<tr>
<td>GroL</td>
<td>Q548M1</td>
<td>-4.3</td>
<td>Hsp60 chaperone for protein folding</td>
</tr>
<tr>
<td>RhlB</td>
<td>E2QHV7</td>
<td>-3.5</td>
<td>RNA helicase of degradosome</td>
</tr>
<tr>
<td>TufB</td>
<td>E2QFI4</td>
<td>-3.4</td>
<td>Elongation factor Tu, coordinates charged tRNA</td>
</tr>
<tr>
<td>HisO</td>
<td>E2QFQ1</td>
<td>3.6</td>
<td>Hsp33, expressed under heat shock and activated by oxidative stress</td>
</tr>
<tr>
<td>Tig</td>
<td>E2QGI0</td>
<td>2.3</td>
<td>Chaperone involved in folding nascent cytosolic proteins</td>
</tr>
<tr>
<td><strong>Metabolic Proteins</strong></td>
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<td></td>
</tr>
<tr>
<td>AceE</td>
<td>E2QF44</td>
<td>-53.6</td>
<td>Component of pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>AceA</td>
<td>E2QJ37</td>
<td>-37.8</td>
<td>Isocitrate lyase, glyoxylate cycle</td>
</tr>
<tr>
<td>AceB</td>
<td>E2QJ36</td>
<td>-13.4</td>
<td>Malate synthase, glyoxylate cycle</td>
</tr>
<tr>
<td>FabB</td>
<td>E2QI14</td>
<td>-9.8</td>
<td>Fatty acid biosynthesis</td>
</tr>
<tr>
<td>GapA</td>
<td>C3T6W2</td>
<td>-8.5</td>
<td>G3P dehydrogenase, required for glycolysis</td>
</tr>
<tr>
<td>SucD</td>
<td>C3TIK7</td>
<td>-5.4</td>
<td>Succinyl-CoA synthase</td>
</tr>
<tr>
<td>Pta</td>
<td>E2QPF8</td>
<td>-4.9</td>
<td>Acetate metabolism</td>
</tr>
<tr>
<td>AtpH</td>
<td>C3SL92</td>
<td>-3.3</td>
<td>Component of ATP synthase</td>
</tr>
<tr>
<td>DapD</td>
<td>E2QF93</td>
<td>-2.6</td>
<td>Lysine biosynthetic pathway</td>
</tr>
<tr>
<td><strong>Ribosomal Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RpsA</td>
<td>C3TGB2</td>
<td>-7.9</td>
<td>S1 component of 30S ribosomal subunit</td>
</tr>
<tr>
<td>RpsF</td>
<td>C3SFQ7</td>
<td>-5.4</td>
<td>S6 component of 30S ribosomal subunit</td>
</tr>
<tr>
<td>RplD</td>
<td>C3SQU7</td>
<td>-4.8</td>
<td>L4 component of 50S ribosomal subunit</td>
</tr>
<tr>
<td>RpsG</td>
<td>C3SQS2</td>
<td>-3.5</td>
<td>S7 component of 30S ribosomal subunit</td>
</tr>
<tr>
<td>RplA</td>
<td>C3SIC2</td>
<td>2.6</td>
<td>L1 component of 50S ribosomal subunit</td>
</tr>
<tr>
<td>RplF</td>
<td>C3SR17</td>
<td>2.0</td>
<td>L6 component of 50S ribosomal subunit</td>
</tr>
<tr>
<td><strong>Miscellaneous and Uncharacterized Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TorC</td>
<td>E2QJN9</td>
<td>-8.5</td>
<td>Inner membrane cytochrome</td>
</tr>
<tr>
<td>ElaB</td>
<td>C3T2J7</td>
<td>-6.3</td>
<td>Unknown function</td>
</tr>
<tr>
<td>YbfN</td>
<td>E2QI69</td>
<td>-3.5</td>
<td>Unknown function</td>
</tr>
</tbody>
</table>
In addition to regulation of tryptophanase-related genes, the transcriptomic and proteomic results both suggest down-regulation of genes encoding several major components related to the tricarboxylic acid cycle and glyoxylate cycle: aceE, acs, yjcH, actP, sdhC, sdhD, sdhA, sucB, sucC, sucD, aceA, and aceB. The results also indicate down-regulation of genes encoding several stress-related proteins involved in starvation (dps, sspA, and ndk), oxidative (katE and katG), acid (hdeA and hdeB), and UV (uspE) stress responses. Therefore, DosP causes a general reduction in cellular metabolism and stress responses, which may contribute to persistence through dormancy.

5.4.5 DosP and BdcA decrease indole

Based on the inhibition of tryptophanase by DosP, we measured indole concentrations and found that producing DosP reduced extracellular indole by -3.9 ± 1.4 fold and intracellular indole by -2.9 ± 0.8 fold (Fig. 5.1C). Corroborating this result, inactivating DosP increased extracellular indole by 2.0 ± 0.3 fold and intracellular indole by 2.0 ± 0.02 fold. We also tested whether the decreased tryptophanase activity observed with DosP was unique by producing other c-di-GMP-related proteins and found that there was no significant difference in extracellular indole concentrations for DGCs AdrA (1.3 ± 0.2 fold) and DosC (1.1 ± 0.2 fold) or for PDEs Gmr (-1.3 ± 0.04 fold) and YjcC (1.4 ± 0.2 fold). The DGC AdrA substantially increases c-di-GMP levels13, while DosC serves as the DGC component of the DosP-DosC c-di-GMP module and also increases c-di-GMP15, 44. Conversely, both PDEs Gmr (YciR)11 and YjcC reduce c-di-GMP via cleavage. Unlike the DGCs and PDEs, we found that c-di-GMP binding protein BdcA reduced extracellular indole (-2.0 ± 0.1 fold), although less significantly than DosP. The trends in both indole and persistence are not consistent among the DGCs and PDEs, again demonstrating that c-di-GMP is not correlated with persistence.

5.4.6 DosP increases persistence via reduced indole

Since DosP reduced indole, we sought to test whether the signal indole was mediating persistence. We recently found that addition of indole to a ΔtnaA strain decreased persister formation with multiple antibiotics in a dose-dependent manner by up to -52 ± 1 fold with 2 mM indole (Fig. 5.2A and B)45. The indole concentrations tested were consistent with previous studies regarding the role of indole signaling in
biofilm formation\textsuperscript{26}, multidrug export\textsuperscript{22}, and stress responses\textsuperscript{46} (concentrations of 0.5-2.0 mM). Furthermore, transient intracellular concentrations are reported to reach as high as 60 mM\textsuperscript{47}. Additionally supporting the importance of indole signaling in persistence, absence of the indole importer Mtr increased persistence 28 ± 11 fold (Fig. 5.2C). Corroborating the role of indole in DosP-mediated persistence, when TnaA and DosP were produced concurrently from two IPTG inducible plasmids (i.e., pBS(Kan)-dosP and pCA24N-tnaA) in the same host, DosP no longer induced persistence (Fig. 5.2D). Together, these three sets of experiments show conclusively that indole inversely regulates persistence and that DosP increases persistence via regulation of TnaA.

5.4.7 cAMP is cleaved by DosP and modulates persistence

Since there was no consistent relationship between c-di-GMP and persistence and since DosP was originally characterized as a cAMP phosphodiesterase\textsuperscript{17}, we investigated the possibility that DosP may modulate tryptophanase activity and persistence through cAMP cleavage. If this is the case, it would suggest that BdcA has the same effects through an uncharacterized binding affinity for cAMP. Based on the microarray conducted for production of DosP (Table 5.1), 37 of the 72 significantly affected transcripts showed differential expression consistent with a reduction in the cAMP-CRP transcriptional regulator. Critically, cAMP-CRP is a positive regulator for transcription of the \textit{tna} operon\textsuperscript{40}, responsible for indole synthesis.

Initially, we tested the impact of cAMP on persistence through CpdA, a cAMP-specific phosphodiesterase\textsuperscript{48}. We found that production of CpdA (i.e., reduced cAMP) increased persistence 235 ± 15 fold (Fig. 5.3A). Furthermore, absence of the single adenylate cyclase (Δ\textit{cyaA}) producing cAMP in \textit{E. coli}\textsuperscript{14} increased persistence 19 ± 4 fold (Fig. 5.3B). Similar to results with DosP and BdcA, production of CpdA reduced extracellular indole (-9 ± 1.1 fold) and intracellular indole (-8 ± 0.2 fold) (Fig. 5.3C), suggesting analogous cAMP reduction. Also, a cAMP enzyme immunoassay was performed to verify that DosP reduced intracellular cAMP (-2 ± 0.1 fold) whereas the positive control CpdA essentially eliminated all cAMP (-323 ± 81 fold) (Fig. 5.3D). Hence, cAMP inversely controls persistence through direct regulation of indole. CpdA eliminated nearly all cAMP yet did not increase persistence as significantly as
DosP, suggesting that cAMP regulation may not be the only mechanism through which DosP increases persistence.

**Figure 5.2.** DosP induces persistence via reduced indole. Persister cell formation for the following strains and conditions: (A and B) BW25113 ΔtnaA grown to a turbidity of 2.0 in LB medium, exposed to 0, 0.5, 1, and 2 mM indole for 2 h, adjusted to a turbidity of 1.0, and exposed to (A) 100 µg/mL ampicillin or (B) 5 µg/mL ciprofloxacin for 2 h, (C) BW25113 Δmtr and wild-type grown to a turbidity of 1.0 in LB medium and exposed to 100 µg/mL ampicillin for 3 h, and (D) BW25113/pCA24N/pBS(Kan), BW25113/pCA24N/pBS(Kan)-dosP, and BW25113/pCA24N-tnaA/pBS(Kan)-dosP grown to a turbidity of 1.0 in LB medium supplemented with 2 mM L-tryptophan, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin for 3 h. All data are averaged from at least two independent cultures and one standard deviation is shown.
Figure 5.3. cAMP is cleaved by DosP and regulates indole and persistence. Persister cell formation for the following strains and conditions: (A) BW25113/pCA24N-cpdA and BW25113/pCA24N grown to a turbidity of 1.0 in LB medium, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin for 3 h and (B) BW25113 ΔcyaA and wild-type grown to a turbidity of 1.0 in LB medium and exposed to 100 µg/mL ampicillin for 3 h. (C) Extracellular and intracellular indole concentrations for BW25113/pCA24N-cpdA and BW25113/pCA24N after 2 h of CpdA production via 1 mM IPTG. (D) Cellular cAMP concentrations for BW25113/pCA24N-dosP, BW25113/pCA24N-cpdA, and BW25113/pCA24N after 2 h of induction via 1 mM IPTG. (E) Persister cell formation for BW25113/pCA24N-dosP and BW25113/pCA24N grown to a turbidity of 1.0 in LB medium with and without 1% (w/v) glucose, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 µg/mL ampicillin for 3 h. All data are averaged from at least two independent cultures and one standard deviation is shown.
Since glucose inhibits intracellular cAMP accumulation, we also tested the effect of glucose supplementation on persistence from DosP production to determine the extent to which DosP persistence is dependent on reducing cAMP. We found that glucose reduced DosP-mediated persistence by \(-25 \pm 6\) fold (Fig. 5.3E), a result which clearly demonstrates the cAMP dependence of DosP persistence while also suggesting the existence of additional DosP persistence mechanisms. In addition to cAMP cleavage, DosP may further contribute to persistence through the general down-regulation of metabolism noted in the transcriptomic and proteomic studies (Tables 5.1 and 5.2). Nonetheless, the distinctly reduced effect of DosP on persistence with low intracellular cAMP (i.e., with glucose) and the consistent results for both CpdA and DosP suggest that DosP increases persistence by cleaving cAMP, to reduce \(tna\) operon transcription and indole synthesis.

5.5 Conclusions

Our results show that cAMP inversely regulates persistence by inducing the \(tna\) operon which increases indole. The phosphodiesterase DosP has activity against both c-di-GMP and cAMP, and here DosP degradation of cAMP leads to reduced indole concentrations and increased persistence. DosP activity is directly dependent on oxygen concentrations, which suggests that DosP mediates persister cell formation with changing oxygen availability. Our proposed regulatory pathway (Fig. 5.4) implicates both the internal regulator cAMP and the external signal indole as mediators of persistence.

It was previously proposed that DosP may signal increased oxygen concentrations present when cells exhibit slow metabolism. Since producing DosP leads to higher persistence, then perhaps DosP contributes to the increased persistence observed in slow growing cultures. Additionally, DosP activity may be regulated in cells located within different layers of biofilms, which contain a gradient of oxygen levels. Oxygen content is higher in the top layer of biofilms due to air exposure, so DosP may act as a mechanism to maintain a persister sub-population among cells in the outer layer of biofilms.
Figure 5.4. Schematic for persistence induced by DosP production. DosP is a phosphodiesterase that is activated by oxygen and cleaves both c-di-GMP and cAMP. cAMP and CRP form a transcriptional regulation complex which induces expression of the tna operon. TnaA synthesizes indole from L-tryptophan, and indole reduces persistence. Curved arrows indicate enzymatic reactions, → indicates induction, and ⊥ indicates inhibition.

CAMP concentrations fluctuate based on carbon metabolism and exogenous addition of cAMP has been purported to increase persistence. Since CAMP uptake occurs via facilitated diffusion (i.e., energy-independent transport), exogenous CAMP is likely internalized. Therefore, CAMP was proposed to increase persistence by stimulating RelA expression to produce the stringent response alarmone ppGpp, which was previously implicated in multiple persister formation pathways. In contrast, our results show that decreasing cellular CAMP via phosphodiesterases increases persistence via diminished indole. Therefore, the specific role of CAMP in persistence may be complex, likely due to the vast size of the CAMP-CRP regulon (188 operons). Versatile involvement of CAMP in persistence provides bacterial populations with mechanisms for maintaining persister subpopulations across a number of different...
growth conditions. Therefore, cAMP adds to the redundancy of persister mechanisms, similarly characterized for toxin/antitoxin systems\(^6\), and ensures the presence of a persistent population to prevent sterilization of a bacterial population.

Our results indicating that indole reduces persistence are in contrast to those reported previously\(^36\) but are consistent with our recent results involving endoribonuclease toxin YafQ of the \(E. \ coli\) YafQ/DinJ toxin/antitoxin system\(^45\); we found that YafQ increases persistence by decreasing indole concentrations, resulting from a reduction in \(t\)naA\(^45\). Since \(t\)naA is repressed in \(E. \ coli\) biofilm cultures\(^54\), our findings suggest that the total cell population contributes to high persister levels in biofilms by reducing intercellular signaling through indole. This is also reasonable since indole reduces biofilm formation\(^26\)-\(^29\).

**5.6 Materials and Methods**

**5.6.1 Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this study are described in Table 5.3. Cultures were grown in lysogeny broth (LB)\(^55\) at 37°C with shaking unless indicated otherwise. Kanamycin (50 μg/mL) and chloramphenicol (30 μg/mL) were utilized to maintain the pCA24N- and pBS(Kan)-based plasmids. The pCA24N-\(dosP\) plasmid was sequenced to confirm the presence of \(dosP^+\).

**5.6.2 Persister cell formation assay**

Persistence was determined by comparing cell viability before and after lethal antibiotic treatment. Overnight cultures were inoculated into LB medium with appropriate antibiotics to maintain plasmids and grown to a turbidity of 1.0 at 600 nm. For strains containing plasmids, gene expression was induced with IPTG (1 mM) for 2 h and cultures were adjusted to a turbidity of 1.0 at 600 nm in fresh LB medium. Cells were then exposed to 100 μg/mL ampicillin or 5 μg/mL ciprofloxacin for 2 to 4 h. The antibiotic concentrations used were at least 10-fold greater that the minimum inhibitory concentration for the wild-type host strain\(^5\) to ensure that any altered resistance of the strains used was not a contributing factor in the persister results. To measure cell viability, samples were taken before and after antibiotic treatment, washed and serially diluted in 0.85% (w/v) NaCl solution, plated on LB agar, and grown overnight at 37°C to determine CFU/mL\(^56\). Experiments were performed with at least two independent cultures.
Table 5.3. Bacterial strains and plasmids used in Chapter 5.

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Cm<sup>R</sup> and Km<sup>R</sup> are chloramphenicol and kanamycin resistance, respectively.

### 5.6.3 DNA microarrays

Overnight cultures of BW25113/pCA24N-dosP and BW25113/pCA24N were inoculated into LB
medium with appropriate antibiotics to maintain plasmids, grown to a turbidity of 1.0 at 600 nm, and
induced with IPTG (1 mM) for 90 min to produce DosP. Cell pellets were isolated and flash-frozen in
ethanol/dry ice. RNALater buffer (Applied Biosystems, Foster City, CA, USA) was added to stabilize
RNA during preparation. Total RNA was isolated from cells as described previously\textsuperscript{54} using a bead beater
(Biospec, Bartlesville, OK, USA). cDNA synthesis, fragmentation, and hybridization to \textit{E. coli} GeneChip
Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA; P/N 900551) were performed as described
previously\textsuperscript{60}. Genes were identified as differentially expressed if the expression signal ratio was higher
than the standard deviation (1.72) and the P-value for comparing two chips was less than 0.05\textsuperscript{61}. The
whole-transcriptome dataset is available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/)
through accession number GSE47427.

5.6.4 Proteomics

Overnight cultures of BW25113/pCA24N-dosP and BW25113/pCA24N were inoculated into LB
medium with appropriate antibiotics to maintain plasmids, grown to a turbidity of 1.0 at 600 nm, and
induced with IPTG (1 mM) for 2 h to produce DosP. Cell pellets were isolated and flash frozen in
ethanol/dry ice. Total soluble protein was extracted using B-PER (Bacterial Protein Extraction Reagents;
Thermo Fisher Scientific, Waltham, MA, USA). In summary, 1 g of cell pellet was lysed by suspending
in 4 mL of B-PER containing 100 µg/mL lysozyme (Thermo Fisher Scientific), 5 Kunitz units/mL DNase
I (Qiagen, Hilden, Germany), and 12.5% (v/v) EDTA-free protease inhibitor cocktail (Sigma-Aldrich, St.
Louis, MO, USA). The lysate was centrifuged and the supernatant, containing the soluble fraction, was
collected. Samples were normalized using IR spectroscopy and 100 µg of protein was labeled with a
Tandem Mass Tag Kit (Thermo Fisher Scientific). Proteins were digested with 2 µg of sequencing grade
trypsin (Promega, Madison, WI, USA) overnight at 37ºC. The digestion mixtures were filtered with 50
kDa MWCO centrifugal filters (EMD Millipore, Billerica MA, USA). Samples were dried using a
Speedvac. The digestion mixtures were reconstituted in 100 µL of 4% (v/v) aqueous acetonitrile with
0.1% (v/v) formic acid. The mixture (2 µL) was loaded onto an Acclaim PepMap100 trapping column
(100 µm x 2 cm, C18, 5µm, 100 Å, Thermo Fisher Scientific) at a flow rate of 20 µL/min using 4% (v/v)
aqueous acetonitrile as a mobile phase. Peptides were separated on an Acclaim PepMap RSLC column (75 μm x 15 cm, C18, 2 μm, 100 Å, Thermo Fisher Scientific) with a 90 minute 4%-50% (v/v) linear gradient of acetonitrile in water containing 0.1% (v/v) formic acid. A Dionex Ultimate 3000 nano-LC system (Thermo Fisher Scientific) was used to deliver the gradient solution at 300 nL/min. Data was acquired over a 40 to 2000 m/z range with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) using the following data-dependent parameters: full FT MS scan at a resolution of 60,000 followed by 10x ion trap MS2 scans on the most intense precursor ions with CID activation. Charge states of +2 or higher were used to select precursors for MS2; monoisotopic precursor selection was enabled, and the isolation window was 2 m/z.

Proteome Discoverer 1.3 (Thermo Fisher Scientific) was used to process the mass spectra using the following search parameters: precursor tolerance 10 ppm, fragment tolerance 0.8 Da (ion trap), dynamic modifications including oxidation (+15.995 Da, M) and deamidation (+0.984 Da, N, Q), and a static modification with carbamidomethyl (+57.021 Da, C). The absolute XCorr threshold and the peptide without protein threshold were set to zero in the peptide scoring option. Proteins were identified from the Uniprot E. coli K-12 database (March, 2013). The generated msf file was loaded into Scaffold 4.0 (Proteome Software, Inc., Portland, OR, USA) and searched with X!Tandem (Global Proteome Machine) against the E. coli K-12 database.

### 5.6.5 Indole assays

Overnight cultures were inoculated into LB medium with appropriate antibiotics to maintain plasmids, and grown to a turbidity of 1.0 at 600 nm. Strains containing plasmids were induced with IPTG (1 mM) for 2 h. Cells were pelleted and the supernatant was collected (i.e., extracellular sample). Cells were resuspended in fresh LB medium and sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific, Hampton, NH, USA). Samples were pelleted to remove debris and the supernatant was collected (i.e., intracellular sample). Extracellular and intracellular indole concentrations were measured spectrophotometrically based on absorbance at 540 nm using Kovac's reagent and HCl-amyI alcohol as described previously27. Experiments were performed with at least two independent cultures.
5.6.6 Biofilm formation

Overnight cultures were diluted to a turbidity of 0.05 at 600 nm in LB medium with or without 1 mM IPTG and with appropriate antibiotics to maintain plasmids. Cultures were grown for 24 h at 37°C in 96-well plates (300 µL/well). Biofilm formation was assayed using crystal violet staining as described previously. Cell growth (turbidity at 620 nm) was used to normalize the total biofilm formation (absorbance at 540 nm). Data points were averaged from at least 12 replicate wells using at least two independent cultures.

5.6.7 Intracellular cAMP levels

Overnight cultures were inoculated into LB medium with appropriate antibiotics to maintain plasmids, grown to a turbidity of 1.0 at 600 nm, and induced with IPTG (1 mM) for 2 h. Cells were pelleted, resuspended in fresh LB medium, sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific), and centrifuged to remove debris. cAMP was acetylated and quantified using a competitive enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

5.7 Acknowledgements

This work was supported by the ARO (W911NF-14-1-0279). T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University. We are grateful for the Keio and ASKA strains provided by the Genome Analysis Project in Japan. We acknowledge the assistance from Tatiana N. Laremore (Penn State Proteomics and Mass Spectrometry Facility, University Park, PA) in performing the proteomic study.

5.8 References


Chapter 6. The MqsR/MqsA Toxin/Antitoxin System Protects *Escherichia coli* during Bile Acid Stress

6.1 Preface

This chapter is adapted from the following publication: Kwan, B.K., Lord, D.M., Peti, W., Page, R., Benedik, M.J., and Wood, T.K. *The MqsR/MqsA toxin/antitoxin system protects Escherichia coli during bile acid stress*. Environ. Microbiol., 2014. doi: 10.1111/1462-2920.12749. Persister cell formation is a well-characterized role for toxin/antitoxin (TA) systems (e.g., MqsR/MqsA), so this study focused on determining physiological conditions under which the MqsR/MqsA system is important. These results demonstrated a role for MqsR/MqsA in bile acid tolerance of *E. coli*, which may have implications for persister cell levels in the gastrointestinal tract.

6.2 Abstract

Toxin-antitoxin (TA) systems are ubiquitous within bacterial genomes, and the mechanisms of many TA systems are well characterized. As such, several roles for TA systems have been proposed, such as phage inhibition, gene regulation, and persister cell formation. However, the significance of these roles is nebulous due to the subtle influence from individual TA systems. For example, a single TA system has only a minor contribution to persister cell formation. Hence, there is a lack of defining physiological roles for individual TA systems. In this study, phenotype assays were used to determine that the MqsR/MqsA type II TA system of *Escherichia coli* is important for cell growth and tolerance during stress from the bile salt deoxycholate. Using transcriptomics and purified MqsR, we determined that endoribonuclease toxin MqsR degrades YgiS mRNA, which encodes a periplasmic protein that promotes deoxycholate uptake and reduces tolerance to deoxycholate exposure. The importance of reducing YgiS mRNA by MqsR is evidenced by improved growth, reduced cell death, and reduced membrane damage when cells without *ygiS* are stressed with deoxycholate. Therefore, we propose that MqsR/MqsA is physiologically important for *E. coli* to thrive in the gallbladder and upper intestinal tract, where high bile concentrations are prominent.
6.3 Introduction

Most bacterial chromosomes contain numerous genetic elements encoding multi-component toxin/antitoxin (TA) systems\(^1\)-\(^3\). TA systems typically consist of a stable protein toxin paired with a labile RNA or protein antitoxin\(^4\). These toxins disrupt essential cellular processes (e.g., translation via specific mRNA degradation), and the toxic activity is prevented when a sufficient amount of antitoxin is present\(^4\). Depending on the interaction between the toxin and antitoxin elements, TA systems are classified as type I through type V, which represent known mechanisms. Type I TA systems consist of an RNA antitoxin that inhibits translation of the toxin via antisense RNA binding\(^4\). In type II and type III TA systems, activity of the protein toxin is inhibited by direct binding from a protein antitoxin or RNA antitoxin, respectively\(^4\). Protein antitoxins in type IV systems prevent toxicity by protecting the target from the toxin\(^5\), and in type V systems, protein antitoxins prevent toxicity by specifically cleaving mRNA of the toxin\(^6\).

TA systems were first discovered as plasmid addiction modules in which the antitoxin and a toxin are encoded on a plasmid, eliminating plasmid-free cells\(^7\). Roles for TA systems have since been proposed as selfish alleles, gene regulation, growth control, persister cell formation, programmed cell arrest, programmed cell death, and anti-phage measures\(^8\). The role of TA systems in the formation of multidrug tolerant persister cells has been of particular interest in recent years due to relevance within bacterial infection models. TA systems appear to be a highly redundant mechanism for persister formation, with only a few TA systems affecting persistence upon deletion from the chromosome\(^9\)-\(^11\) and a requirement of several simultaneous TA system deletions to more significantly impact persistence\(^12\). Many TA systems are autoregulatory\(^4\), and due to the regulatory nature of TA elements, some antitoxins exhibit a gene-specific regulatory effect via promoter binding of loci distinct from the TA loci\(^13\)-\(^15\) and some toxins exhibit a general regulatory effect via post-transcriptional differential mRNA decay\(^16\)-\(^19\). Hence, TA systems regulate global transcriptional networks (e.g., the general stress response\(^14\), \(^20\)) and are also controlled within transcriptional regulons triggered by specific environmental cues (e.g., the SOS response\(^11\), \(^21\), \(^22\) and the stringent response\(^23\)-\(^28\)).
In *Escherichia coli* there are at least 39 toxin/antitoxin systems\(^6\)\(^{29,30}\) including MqsR/MqsA, a type II TA system in which toxin MqsR is an mRNA endonuclease and antitoxin MqsA binds MqsR to prevent toxicity\(^{31}\). MqsR was first characterized as a quorum sensing-related regulator of biofilm formation\(^{16,32}\). MqsR specifically cleaves mRNA at primarily 5'-GCU sites\(^{28}\), and through this specificity, MqsR was found to regulate another TA system, the GhoT/GhoS type V TA system\(^6\)\(^{19}\) which shows that there is a hierarchy in TA systems. MqsA contains a helix-turn-helix DNA binding domain which allows MqsA to autoregulate expression of the *mqsRA* operon\(^{31,33}\). Through this DNA binding domain, MqsA also regulates the general stress response through repression of the stationary phase sigma factor RpoS\(^{14}\) and regulates biofilm formation through repression of biofilm regulator CsgD\(^{15}\), thus MqsA is a regulator of other cell regulators. Therefore, it is clear that the MqsR/MqsA system has regulatory roles to increase the stress response (which includes reducing metabolism through mRNA decay and activating toxin GhoT and RpoS) and to increase biofilm formation during oxidative stress. However, since all but 14 of the *E. coli* transcripts have 5'-GCU sites\(^6\), other regulatory roles of the MqsR/MqsA TA system are also possible, including regulating the cell’s inhabitation of the GI tract.

In humans, bile is secreted after ingestion of food and contains bile acids, which are amphipathic molecules that aid in solubilizing lipids to digest fats\(^{34}\). Bile acids are synthesized in the liver from cholesterol and the majority of bile is stored in the gallbladder\(^{35}\). During digestion, the gallbladder secretes bile into the duodenum (i.e., immediately after the stomach in the digestive tract)\(^{35}\) where the pH typically ranges from 5.3 to 6.3\(^{36}\), and the majority of bile is recycled to the liver in the ileum (i.e., end of the small intestine)\(^{37}\). Bile contains both primary bile acids (e.g., cholic acid and chenodeoxycholic acid) and secondary bile acids derived from primary bile acids by intestinal bacteria (e.g., lithocholic acid and deoxycholic acid)\(^{35}\). In order to increase water solubility, bile acids are often conjugated with glycine or taurine\(^{37}\). Human bile typically contains ~40 mM (~2%) bile salts at a pH of 7.5 to 8.0, and bile is 5-10-fold concentrated in the gallbladder\(^{35}\), reaching concentrations as high as 300 mM (~15%)\(^{34}\). In contrast, bile acids are diluted to sub-millimolar concentrations within the lower intestine\(^{38}\).

In bacteria, bile acids act as a detergent to cause membrane damage, and susceptibility of cells to bile
acids is dependent on the protein and fatty acid composition of the membrane. Antimicrobial activity of bile serves to limit bacterial cell growth in the small intestine, which regulates the bacterial terrain. Gram-negative bacteria are significantly more resistant to bile acid stress than Gram-positive bacteria, and *E. coli*, a very resistant species, is often isolated from the gallbladder (i.e., highest bile acid concentration) in humans. In *E. coli*, lipopolysaccharide structure (*rfa* locus) and efflux pumps (*AcrAB, EmrAB, and MdtABCD*) are important for bile acid resistance, and porins (*OmpF and OmpC*) affect bile acid susceptibility. Additionally, promoters for some oxidative stress genes (*micF and osmY*) and a DNA damage protection gene (*dinD*) are induced upon exposure of *E. coli* to bile acids, indicating that the oxidative stress response may be triggered by bile acid stress. MicF is a small RNA that represses *ompF* at high osmolarity, which restricts uptake of large solutes (e.g., bile salts) across the outer membrane due to the smaller pore size of OmpC compared to OmpF (1.08 nm versus 1.16 nm). After crossing the outer membrane, unconjugated bile acids are largely protonated due to the acidic pH of the periplasm, thus allowing permeation across the inner membrane.

In this study we discovered that the MqsR/MqsA TA system is important for managing the stress related to bile acids. Based on transcriptomics, we determined that MqsR reduces YgiS (an uncharacterized, predicted periplasmic binding protein) through mRNA cleavage in the presence of bile, and that YgiS reduces growth with bile by promoting its uptake. Therefore, we have determined another physiological role for the MqsR/MqsA TA system. We posit that *E. coli* uses MqsR/MqsA in order to improve growth and survival with high bile acid concentrations that are relevant to cells living in the gut and/or gallbladder, an ecological role for TA systems that has not been previously described.

### 6.4 Results and Discussion

#### 6.4.1 MqsR/MqsA decreases metabolism during deoxycholate stress

The PortEco phenotypic database provides scores for growth phenotypes from screening a library of single gene mutants in *E. coli* against a panel of 324 different chemical treatments. Hence, we searched the PortEco database for significant positive (increased growth) or negative (reduced growth) scores involving mutants for *mqsR* and *mqsA* in order to determine chemical stresses which may be influenced...
by the MqsR/MqsA TA system. The database indicated that a strain lacking mqsR had reduced growth with fusidic acid (score: -3.11 with 50 µg/mL and -2.32 with 20 µg/mL) and radicicol (score: -2.25 with 10 µM and -2.15 with 5 µM)\textsuperscript{44}. Fusidic acid is a fusidane class antibiotic that inhibits bacterial protein synthesis via inhibition of elongation factor G, with bacteriostatic activity at low concentrations and bactericidal activity at high concentrations\textsuperscript{45}. Radicicol is a resorcylic acid lactone mycotoxin that exhibits antibiotic activity and is a potent inhibitor of the HSP90 family of proteins\textsuperscript{46}, including HtpG in E. coli\textsuperscript{47}. Additionally, a strain with a hypomorphic mutation in mqsA (i.e., reduced activity) was indicated to have increased growth with ampicillin (score: 3.07 with 1 µg/mL) and reduced growth with deoxycholate (score: -2.50 with 2%)\textsuperscript{44}. Ampicillin is a β-lactam antibiotic that inhibits peptidoglycan synthesis, preventing cell wall repair and causing cell lysis during division\textsuperscript{48}. Deoxycholate (also known as deoxycholic acid) is a bile acid that is formed as a metabolic byproduct of intestinal bacteria\textsuperscript{35}.

To determine whether MqsR and/or MqsA do in fact affect cell growth during stress with these four compounds, we performed a metabolic activity assay which utilizes tetrazolium dye. Tetrazolium dye is reduced to the purple compound formazan due to NADH produced during cellular respiration\textsuperscript{49}. A BW25113 strain lacking both mqsR and mqsA and without any antibiotic selection marker\textsuperscript{13} (hereafter referred to as ΔmqsRA) was grown alongside the wild type in media containing fusidic acid (0, 10, 50, and 250 µg/mL) (Fig. 6.1A), radicicol (0, 2, 10, and 50 µM) (Fig. 6.1B), ampicillin (0, 0.2, 1.0, and 5.0 µg/mL) (Fig. 6.1C), or deoxycholate (0, 0.4, 2.0, and 4.0%) (Fig. 6.1D). Cultures were monitored spectrophotometrically at 590 nm, gauging overall metabolism based on both cell division (i.e., cell density) and cellular respiration (i.e., reduced tetrazolium dye). Treatments with fusidic acid, radicicol, and ampicillin did not show any significant difference in metabolism; however, the ΔmqsRA mutant displayed higher metabolic activity than the wild type when grown with both 2% and 4% deoxycholate. We performed the assay using additional concentrations of deoxycholate (0, 3.0, 4.5, and 5.0%) to verify our initial result and check for concentration dependence, and we obtained consistent results with the ΔmqsRA mutant displaying the most significant increase in metabolic activity during stress with 4.5% deoxycholate (Fig. 6.1E). Additionally, upon performing the assay with the detergent Triton-X100 (0,
0.5, 2.0, and 5.0%), we observed no differences in metabolism (Fig. 6.1F), demonstrating that the phenotype was not due to the detergent activity of deoxycholate. These results indicate that in wild-type E. coli, MqsR/MqsA reduces metabolic activity during bile acid stress from deoxycholate.

6.4.2 MqsR/MqsA improves growth during deoxycholate stress

Since the metabolic activity assay is a combined measurement of cell division and cellular respiration, we sought to distinguish the two metabolic characteristics by monitoring growth based solely on cell density (i.e., no tetrazolium dye). Deoxycholate gelation is a known issue in bacteriological media, and as such we developed a growth medium (referred to here as HEPES-glucose) in which deoxycholate did not undergo gelation (to concentrations above 20%). Using this medium we performed a growth curve comparing the ΔmqsRA mutant with the wild type in the presence of 4.5% deoxycholate and found that the ΔmqsRA mutant consistently grew worse than the wild-type (Fig. 6.2A), suggesting that MqsR/MqsA improves growth during deoxycholate stress. Additionally, there was only a very small difference in growth for the ΔmqsRA mutant grown in the HEPES-glucose medium without any stress (Fig. 6.2B) or with either ampicillin (1 µg/mL) or fusidic acid (50 µg/mL) (Fig. 6.2C). These results confirmed that the reduced growth of the ΔmqsRA mutant was specific to deoxycholate stress. The previously observed increased metabolic activity (i.e., cell division and respiration) of the ΔmqsRA mutant with deoxycholate (Fig. 6.1D and E) despite reduced growth (i.e., cell division) (Fig. 6.2A) indicates that cells without MqsR/MqsA exhibit significantly elevated cellular respiration during deoxycholate stress. Therefore, we hypothesized that MqsR/MqsA may reduce the susceptibility to deoxycholate by reducing cellular metabolism, which results in improved overall population growth.
Figure 6.1. MqsR/MqsA reduces metabolic activity specifically during deoxycholate stress. Comparison of metabolic activity and growth measured in the presence of tetrazolium dye by optical density at 590 nm for BW25113 ΔmqsRA (○) and wild-type (●) when exposed to (A) fusidic acid (0, 10, 50, and 250 µg/mL), (B) radicicol (0, 2, 10, and 50 µg/mL), (C) ampicillin (0, 0.2, 1.0, and 5.0 µg/mL), (D and E) deoxycholate (0, 0.4, 2.0, 3.0, 4.0, 4.5, and 5.0%), or (F) Triton-X100 (0, 0.5, 2.0, and 5.0%). Data are averaged from two independent cultures and one standard deviation is shown.
Figure 6.2. MqsR/MqsA improves growth and YgiS reduces growth specifically during deoxycholate stress. Comparison of growth in HEPES-glucose medium measured by turbidity at 600 nm for BW25113 ΔmqsRA (○), ΔygiS (▼), ΔoppA (△), and wild-type (●) when exposed to (A) 4.5% deoxycholate, (B) no added stress, (C) 1 µg/mL ampicillin (left panel) or 50 µg/mL fusidic acid (right panel). Data are averaged from three independent cultures and one standard deviation is shown.

6.4.3 MqsR decreases YgiS mRNA

MqsR regulates the GhoT/GhoS TA system via directed endonuclease activity against mRNA of antitoxin GhoS\(^{19}\) and MqsA is a global regulator, controlling the general stress response via RpoS\(^{14}\) and biofilm physiology via CsgD\(^{15}\) by binding specific DNA palindromes. Additionally, regulation of several genes via RpoS affects deoxycholate tolerance in Salmonella enterica, demonstrating a multiplicity of bile stress-related regulatory targets\(^{51}\). Therefore, the MqsR/MqsA system likely affects deoxycholate tolerance through regulatory activity of either the toxin MqsR or antitoxin MqsA. To test this, we
performed a DNA microarray study comparing gene expression in the $\Delta mqsRA$ mutant compared to the wild type during 4.5% deoxycholate stress. We found that in the $\Delta mqsRA$ mutant, $ygiS$ was the most significantly induced gene (14-fold), while few other genes had significantly altered expression (Table 6.1). The microarray data were confirmed through quantitative real-time reverse-transcription PCR (qRT-PCR), showing increased expression of $ygiS$ (3.6 ± 0.4 fold) and relatively little difference in expression of two control genes ($katE$: 1.4 ± 0.7 fold and $osmY$: 2.0 ± 1.6 fold) (Table 6.2). $YgiS$ is an uncharacterized protein, and $ygiS$ is located directly downstream of the $mqsRA$ operon. BLAST analysis shows that $YgiS$ is most closely related to OppA, an oligopeptide ABC transporter periplasmic binding protein, although the identity is only 44%. Critically, the $YgiS$ transcript contains 43 5’-GCU sites. Migration of mRNAs$^{52}$ and proteins$^{53}$ away from the chromosomal gene location is limited; therefore, the close proximity to the $mqsRA$ operon (i.e., high local concentration of MqsR) and the high amount of MqsR-specific endonuclease cleavage sites means that $ygiS$ mRNA is a prime substrate for MqsR. Therefore, it is likely that MqsR reduces $YgiS$ by degrading $YgiS$ mRNA.

Table 6.1. Microarray data for stress with 4.5% deoxycholate. Summary of the largest fold changes in gene expression for BW25113 $\Delta mqsRA$ vs. BW25113 wild-type stressed with 4.5% deoxycholate.

<table>
<thead>
<tr>
<th>Gene</th>
<th>b number</th>
<th>Fold change</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$ygiS$</td>
<td>b3020</td>
<td>13.9</td>
<td>Uncharacterized protein, putative ABC transporter, directly downstream of $mqsRA$</td>
</tr>
<tr>
<td>$ybeT$</td>
<td>b0647</td>
<td>13.9</td>
<td>Unknown function, conserved outer membrane protein</td>
</tr>
<tr>
<td>$sfmD$</td>
<td>b0532</td>
<td>6.5</td>
<td>Putative outer membrane transport chaperone</td>
</tr>
<tr>
<td>$yebT$</td>
<td>b1834</td>
<td>2.1</td>
<td>Putative outer membrane protein</td>
</tr>
<tr>
<td>$lysP$</td>
<td>b2156</td>
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<td>Lysine uptake permease</td>
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<td>$lysC$</td>
<td>b4024</td>
<td>2.6</td>
<td>Aspartokinase III, lysine biosynthesis</td>
</tr>
<tr>
<td>$yeeU$</td>
<td>b2004</td>
<td>2.1</td>
<td>$cbeA$, antitoxin of CbtA/CbeA system</td>
</tr>
<tr>
<td>$dapB$</td>
<td>b0031</td>
<td>2.0</td>
<td>Reductase involved in lysine biosynthesis</td>
</tr>
<tr>
<td>$citG$</td>
<td>b0613</td>
<td>2.0</td>
<td>Synthesizes prosthetic group to activate citrate lyase</td>
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<td>$flgD$</td>
<td>b1075</td>
<td>2.0</td>
<td>Flagellar hook protein biosynthesis</td>
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<td>$yfK$</td>
<td>b4183</td>
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<td>Unknown protein</td>
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<td>Transcriptional activator of $rhaBAD$ and $rhaT$</td>
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<td>1.9</td>
<td>Colanic acid export channel lipoprotein</td>
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<td>$speF$</td>
<td>b0693</td>
<td>1.9</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>$katE$</td>
<td>b1732</td>
<td>1.4</td>
<td>Hydroperoxidase II, oxidative stress response</td>
</tr>
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<td>$osmY$</td>
<td>b4376</td>
<td>1.4</td>
<td>Hyperosmotically inducible periplasmic protein</td>
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Repressed genes

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<tr>
<td>yodB</td>
<td>b1974</td>
</tr>
<tr>
<td>yfdP</td>
<td>b2359</td>
</tr>
<tr>
<td>nkr</td>
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<tr>
<td>ycgK</td>
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<tr>
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<td>b1973</td>
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<td>b2018</td>
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<tr>
<td>renD</td>
<td>b0542</td>
</tr>
</tbody>
</table>

Table 6.2. Verification of ygiS expression via qRT-PCR. The cycle number (C_t) for each sample is indicated for the target genes (ygiS, katE, and osmY) as well as for the housekeeping gene, rrsG, which was used to normalize the data. Fold changes in transcription were calculated using the formula:

\[2^{-(C_{t\text{target}}} - C_{t\text{rrsG}}) - 2^{-(C_{t\text{target, wild-type}}} - C_{t\text{rrsG, wild-type}}\]

The specificity of the qRT-PCR products were verified by melting curve analysis. Means and standard deviations are indicated (n=2).

<table>
<thead>
<tr>
<th>Strain</th>
<th>C_t (target gene)</th>
<th>C_t (housekeeping gene)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW25113 wild-type</td>
<td>23.99 ± 0.07</td>
<td>10.87 ± 0.19</td>
<td>3.6 ± 0.4</td>
</tr>
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<td>NW25113 ΔmqsRA</td>
<td>21.96 ± 0.10</td>
<td>10.67 ± 0.18</td>
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<tr>
<td>NW25113 wild-type</td>
<td>25.14 ± 0.26</td>
<td>11.80 ± 0.01</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>NW25113 ΔmqsRA</td>
<td>24.74 ± 0.16</td>
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<tr>
<td>NW25113 wild-type</td>
<td>21.30 ± 0.16</td>
<td>11.80 ± 0.01</td>
<td>2.0 ± 1.6</td>
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<tr>
<td>NW25113 ΔmqsRA</td>
<td>20.39 ± 0.76</td>
<td>11.89 ± 0.16</td>
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</table>

6.4.4 MqsR degrades YgiS mRNA

As confirmation that YgiS mRNA is degraded by MqsR endonuclease activity, we performed an in vitro cleavage assay. The 5’-GCU selectivity of MqsR was previously demonstrated via lack of degradation of GhoT mRNA, with no cleavage sites, and degradation of GhoS mRNA and mutated GhoT mRNA, both containing 5’-GCU cleavage sites. Here, we synthesized in vitro a 192 nt segment of the YgiS mRNA transcript containing six 5’-GCU sites; this size fragment was more convenient to synthesize than the whole-length transcript. We found that purified MqsR readily degraded the YgiS mRNA similarly to the positive controls, OmpA and GhoS mRNAs (each containing three cleavage sites). As a
negative control, GhoT mRNA lacking cleavage sites had almost no degradation (Fig. 6.3). Thus, YgiS mRNA is a substrate for MqsR-specific cleavage, which is likely well regulated due to the chromosomal proximity of ygiS and mqsR.

Figure 6.3. MqsR cleaves YgiS mRNA in vitro. Two micrograms of in vitro synthesized OmpA mRNA (211 nt, three 5'-GCU sites), YgiS mRNA (192 nt, six 5'-GCU sites), GhoT mRNA (180 nt, no 5'-GCU sites), and GhoS mRNA (278 nt, three 5'-GCU sites) were incubated at 37°C with (+) or without (-) 30 ng of purified MqsR for 15 min. M, low-range ssRNA ladder (sizes indicated in nt on the left).

6.4.5 YgiS reduces growth during deoxycholate stress

In order to test whether regulation of ygiS is involved in the deoxycholate growth phenotype, we removed the antibiotic selection marker in the BW25113 ΔygiS strain (used for all subsequent experiments) from the Keio single-gene knock-out collection55, eliminating any confounding effects of the antibiotic resistance cassette. We then compared growth of the ΔygiS mutant to the wild type in the presence of 4.5% deoxycholate. Critically, the ΔygiS mutant consistently grew better than the wild type (Fig. 6.2A). Due to the sequence homology with oppA, we also included a ΔoppA mutant, which we found grew similarly to the wild type, thus confirming that the growth phenotype was specific to ygiS.
Additionally, we confirmed that this improved growth is specific to deoxycholate stress, since the ΔygiS mutant grew the same as the wild type in media lacking deoxycholate (Fig. 6.2B). This suggests that YgiS reduces the ability of cells to grow in the presence of deoxycholate stress.

We sought to further study the role of YgiS during growth with deoxycholate by producing YgiS from an IPTG-inducible plasmid. Based on the primary protein sequence, YgiS is predicted to be a periplasmic protein, and the widely used ASKA collection of single-gene expression vectors includes additional N-terminal amino acid residues (e.g., 6× His-tag) which likely affects protein translocation via the altered signal peptide. Therefore, we cloned the chromosomal copy of ygiS from BW25113 (including the signal sequence) into the pCA24N IPTG-inducible expression vector under the control of the P_tac promoter with the strong, synthetic ribosome binding site used to make the ASKA clones to form pCA24N-ygiS_{native} that produces full-length YgiS including its signal peptide.

By using our native ygiS expression vector in a ΔygiS host, we found that production of YgiS complemented the improved deoxycholate growth phenotype of the ΔygiS mutant, reducing growth at low induction (0.05 mM IPTG) and completely inhibiting growth at high induction (0.5 mM IPTG) (Fig. 6.4A). We also measured growth without deoxycholate and observed that YgiS production caused a slight reduction in growth (Fig. 6.4B); however, the growth effect was consistent between 0.05 mM and 0.5 mM IPTG induction, suggesting that the slight growth reduction is not due to YgiS production. Additionally, this effect was minor in comparison to the significantly reduced growth with observed for YgiS production with deoxycholate stress. These results demonstrate that there is a clear growth inhibitory phenotype for producing YgiS specifically in conjunction with deoxycholate stress, indicating that YgiS reduces cell fitness against deoxycholate.
**Figure 6.4.** Complementation of ΔygiS growth phenotype. Comparison of growth in HEPES-glucose medium measured by turbidity at 600 nm for BW25113 ΔygiS/pCA24N (○), BW25113 ΔygiS/pCA24N-ygiS native (▼), and BW25113/pCA24N (●) with 0.05 mM and 0.5 mM IPTG induction during exposure to (A) 4.5% deoxycholate or (B) no deoxycholate. Data are averaged from three independent cultures and one standard deviation is shown.

6.4.6 YgiS is a periplasmic protein that increases deoxycholate uptake

Since bioinformatics analysis of the primary sequence suggests that YgiS is a periplasmic protein with a structure somewhat similar to that of OppA, a periplasmic-binding protein, we produced YgiS via our native ygiS expression vector, visualized the separate protein fractions using SDS-PAGE, and identified protein bands via mass spectrometry to confirm that YgiS is in fact a periplasmic protein (Fig. 6.5). Since OppA is an ABC membrane transporter, we investigated the effect of YgiS on deoxycholate transport. We grew the ΔmqsRA mutant (i.e., increased YgiS) and the ΔygiS mutant (i.e., abolished YgiS) in the presence of 4.5% deoxycholate and isolated the cell lysate in order to quantify intracellular
deoxycholate via HPLC analysis. The ΔmqsRA mutant accumulated 37 ± 12% more deoxycholate and the ΔygiS mutant accumulated 21 ± 6% less deoxycholate in comparison to the wild type (Fig. 6.6A). This indicates the MqsR/MqsA TA system reduces intracellular deoxycholate and that YgiS increases intracellular deoxycholate concentrations either by increasing import or decreasing export. For comparison, a similar magnitude change was reported for the deletion of two bile acid efflux pumps (AcrAB and EmrAB), which caused cells to accumulate 68% more chenodeoxycholate, another bile acid. Therefore, YgiS increases intracellular deoxycholate concentrations, which likely increases cell stress.

**Figure 6.5.** YgiS is localized in the periplasm. Cytoplasmic (lanes 2-4) and periplasmic (lanes 5-7) protein fractions from BW25113 ΔygiS/pCA24N-ygiS native (lanes 3 and 6), BW25113 ΔygiS/pCA24N (empty vector control, lanes 2 and 5), and BW25113/pCA24N-oxIR (cytoplasmic protein control, lanes 4 and 7) were visualized by SDS-PAGE. Lane 1 is the protein ladder with standards covering a molecular weight range from 10 to 170 kDa. Arrows indicate YgiS (lane 6) and OxyR (lane 4).
Figure 6.6. MqsR/MqsA increases deoxycholate tolerance via YgiS. (A) Fold change of normalized intracellular deoxycholate concentrations for BW25113 ΔmqsRA, ΔygiS, and wild-type grown in HEPES-glucose medium with 4.5% deoxycholate to a turbidity of ~0.2-0.5 at 600 nm. (B) Cell survival for BW25113 ΔmqsRA, ΔygiS, and wild-type grown in LB medium to a turbidity of 1.0 at 600 nm and resuspended in HEPES-glucose medium with 20% deoxycholate for 30 min. (C) Leakage of 260-nm-absorbing material from BW25113 ΔmqsRA, ΔygiS, and wild-type grown in HEPES-glucose with 4.5% deoxycholate to a turbidity of ~0.2 to 0.6 at 600 nm and incubated in HEPES-buffered saline for 2 h. (D) Leakage of 260-nm-absorbing material from BW25113 ΔygiS/pCA24N, BW25113 ΔygiS/pCA24N-ygiSnative, and BW25113/pCA24N grown in HEPES-glucose with 4.5% deoxycholate to a turbidity of ~0.2 to 0.6 at 600 nm, induced with 0.5 mM IPTG for 4 h, and incubated in HEPES-buffered saline for 2 h. Data are averaged from three independent cultures and one standard deviation is shown. The asterisk indicates statistical significance as determined using a Student’s t-test (*: P < 0.05, **: P < 0.025, ***: P < 0.01).

6.4.7 MqsR and YgiS influence deoxycholate tolerance

Since YgiS affects deoxycholate transport, we hypothesized that the altered intracellular deoxycholate
concentrations should affect the ability of *E. coli* to tolerate high concentrations and maintain membrane integrity. We exposed cells to 20% deoxycholate and consistently found that the Δ*mqsRA* mutant had fewer survivors (-2.5 ± 0.3 fold reduced survival) while the Δ*ygiS* mutant had more survivors (2.3 ± 0.2 fold increased survival) in comparison to the wild type (Fig. 6.6B). Thus, the MqsR/MqsA TA system increases tolerance to deoxycholate and YgiS reduces tolerance of *E. coli* to deoxycholate, mediated by an adverse effect on transport.

To provide additional evidence of the effect of increased deoxycholate uptake on cell physiology, we took cells grown with 4.5% deoxycholate, resuspended them in a buffered saline solution, and monitored leakage of 260-nm-absorbing material as an indicator of membrane integrity (i.e., more leakage means more membrane damage). Although both the Δ*mqsRA* mutant and the wild type had similar membrane integrity, the Δ*ygiS* mutant exhibited significantly less leakage of 260-nm-absorbing material (47 ± 1% reduced) (Fig. 6.6C), suggesting higher membrane integrity. Additionally, producing YgiS in the Δ*ygiS* mutant complemented the membrane integrity phenotype by increasing leakage of 260-nm-absorbing material (53 ± 6% increased) in comparison to the Δ*ygiS* mutant with the empty vector (Fig. 6.6D). Therefore, YgiS causes cells to sustain more membrane damage from deoxycholate stress. This corroborates the reduced growth phenotype observed in the Δ*mqsRA* mutants which are unable to regulate *ygiS* expression (Fig. 6.2A). Therefore, MqsR reduces production of YgiS to improve growth during deoxycholate stress.

### 6.5 Conclusions

Here we demonstrated that the MqsR/MqsA type II TA system is physiologically important for the growth of *E. coli* during exposure to deoxycholate stress. We found that MqsR degrades YgiS mRNA, and YgiS is a periplasmic protein that increases the uptake of deoxycholate. High intracellular concentrations of the detergent deoxycholate cause the cells to sustain more membrane damage, thus reducing growth and tolerance. Therefore, MqsR/MqsA mediates cell growth with deoxycholate stress by reducing YgiS; a schematic of our understanding of the mechanism is shown in Fig. 6.7.
Figure 6.7. Schematic for regulation of bile acid tolerance by MqsR/MqsA. Bile acid stress induces an oxidative stress response, activating Lon protease which degrades antitoxin MqsA. MqsA binds to toxin MqsR, preventing its endonuclease activity toward mRNA containing 5’-GCU sites (e.g., YgiS mRNA). YgiS is a periplasmic protein which facilitates bile acid uptake. → indicates induction and ⊥ indicates inhibition.

Bile is stored in the gallbladder and secreted into the duodenum (i.e., upper small intestine) during the digestive process, and contains multiple bile salts that are highly similar in structure, including deoxycholate. Deoxycholate is by far the most active bile salt, demonstrated in mammalian studies; hence it was the focus of our study. The physiological relevance of the 4.5% deoxycholate concentration used throughout the majority of this study is substantiated by previous work which demonstrated that bile typically contains ~2% bile salts and is concentrated to ~15% in the gallbladder. Since E. coli is an enteric bacterial species that also resides in the gallbladder, our experimental conditions are consistent with the environmental conditions which could be encountered in the human gut. Therefore, our findings
suggest that MqsR/MqsA mediates growth and survival of *E. coli*, particularly located near the upper intestinal tract.

Bile salt stress causes lipid peroxidation\(^{57}\) and induces an oxidative stress response, based on activation of oxidative stress genes and DNA damage-related genes in both *E. coli*\(^ {40}\) and mammalian cells\(^ {58}\). Importantly, our lab demonstrated that MqsA is degraded by Lon protease in the presence of oxidative stress\(^ {6, 14}\). Therefore, MqsA is likely degraded by bile salt stress to activate MqsR, thus improving bile salt tolerance. While there are instances in which inducing the chromosomal copy of a TA system reduces viability (e.g., reduced translation\(^ {24}\) or increased cell death\(^ {23}\)), here we found a case in which the TA system is beneficial; i.e., inactivation of MqsR/MqsA leads to a clear phenotype (decrease in growth) under physiological conditions (presence of bile acid). Hence, our results elucidate the importance of this oxidative stress response for *E. coli*, which typically resides under highly anaerobic conditions in the GI tract (i.e., an environment with few reactive oxygen species).

Bile salts also induce bacterial adhesion and biofilm formation\(^ {59-62}\), and MqsA inhibits biofilm formation\(^ {14}\) via CsgD regulation\(^ {15}\). Therefore, deactivation of MqsA from bile stress should increase biofilm formation in the GI tract, corroborating that bile salts induce biofilm formation. We posit that bile causes degradation of MqsA via oxidative stress, activating both MqsR and CsgD. Activation of MqsR improves growth and tolerance to bile salts, while simultaneously activation of CsgD promotes adhesion to establish a population and promotes biofilm formation to protect cells against stress from a fluctuating environment (e.g., temporal excretion of bile). Therefore, the type II TA system MqsR/MqsA is a multifaceted regulator that facilitates growth of *E. coli* populations residing in the gut during exposure to bile stress. Since bile plays an important role as an interkingdom signal in the GI tract\(^ {63}\), our results also illustrate how a TA system can play an important role in host-microbe interactions by ensuring the survival of a commensal bacterium.

### 6.6 Materials and Methods

#### 6.6.1 Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 6.3. Overnight cultures were
grown in lysogeny broth (LB) medium at 37°C unless otherwise indicated, washed, and inoculated in HEPES-glucose medium (79.6 mM NaCl, 20.1 mM KCl, 20.3 mM NH₄Cl, 3.0 mM Na₂SO₄, 100 mM HEPES, 1.0 mM MgCl₂, 0.2 mM CaCl₂, 0.66 mM K₂HPO₄, and 0.4% glucose) at 37°C with shaking, unless otherwise indicated. Chloramphenicol (30 µg/mL) was utilized to maintain the pCA24N-based plasmids. The kanamycin resistance cassette from BW25113 ΔygiS and BW25113 ΔoppA was removed by expressing FLP recombinase from pCP20. Gene deletions and removal of kanamycin resistance cassettes were verified by sequencing (primers listed in Table 6.4). Strains with kanamycin resistance cassettes removed were used for all experiments of this study.

**Table 6.3.** Bacterial strains and plasmids used in Chapter 6.

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Cm<sup>R</sup>, Km<sup>R</sup>, and Ap<sup>R</sup> are chloramphenicol, kanamycin, and ampicillin resistance, respectively.

### 6.6.2 Metabolic activity assay

Overnight cultures of BW25113 ΔmqsRA and BW25113 wild-type were inoculated in LB medium, grown to a turbidity of 1.0 at 600 nm, and resuspended in IF-10 (BioLog, Hayward, CA, USA). Samples were diluted in IF-10 to a turbidity of 0.07 at 600 nm and further diluted 200-fold (turbidity of 0.00035 at 600 nm) into a medium containing IF-10, BioLog Redox Dye D (BioLog), rich medium (0.1% yeast extract, 0.2% tryptone, and 0.1% NaCl), and either fusidic acid (0, 10, 50, and 250 µg/mL), radicicol (0, 2, 10, and 50 µM), ampicillin (0, 0.2, 1.0, and 5.0 µg/mL), deoxycholate (0, 0.4, 2.0, 3.0, 4.0, 4.5, and
5.0%), or Triton-X100 (0, 0.5, 2.0, and 5.0%). Cultures were grown at 37°C in 96-well microtiter plates (100 µL per well) and metabolic activity was monitored by measuring optical density at 590 nm, indicating both reduction of tetrazolium dye to formazan and sample turbidity. Experiments were performed with at least two independent cultures.

Table 6.4. Oligonucleotides used in Chapter 6 for cloning ygiS expression vector, qRT-PCR, verification of KanR excision, and verification of plasmid construct. “F” indicates forward primers and “R” indicates reverse primers.

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6.6.3 DNA microarrays and qRT-PCR

Overnight cultures of BW25113 ΔmqsRA and BW25113 wild-type were washed, inoculated in HEPES-glucose medium, grown to a turbidity of 0.6 at 600 nm, and exposed to 4.5% deoxycholate for 30 min. Cell pellets were collected with RNALater buffer (Applied Biosystems, Foster City, CA, USA), to stabilize RNA, and rapidly frozen in ethanol/dry ice. Cells were lysed using 0.1 mm zirconia/silica beads and a bead beater (Biospec, Bartlesville, OK, USA) and total RNA was isolated using an RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA synthesis, fragmentation, and hybridization to E. coli GeneChip Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA) were performed as described previously. Genes were identified as differentially expressed if the expression signal ratio was higher than the standard deviation (1.64) and the P-value for comparing two chips was less than 0.05. The gene expression dataset is available through NCBI GEO accession number GSE59441.

qRT-PCR was performed to verify differential gene expression from the microarray study following the manufacturer’s instructions for the Power SYBR Green RNA-to-Ct 1-Step kit (Life Technologies, Carlsbad, CA, USA) using 100 ng of total RNA as the template. Primers were annealed at 60°C, and data was normalized against the housekeeping gene rrsG. The specificity of qRT-PCR primers (Table 6.4) was verified via standard PCR, and fold changes were calculated using the $2^{-\Delta\Delta C_T}$ formula.

6.6.4 MqsR endoribonuclease assay

MqsR was produced from pET30a-mqsR and purified/refolded as described previously. PCR was performed on BW25113 chromosomal DNA using primers listed in Table 6.4, with the T7 RNA polymerase promoter sequence included in the forward primers. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and 0.5-1.0 µg were used as DNA templates for in vitro transcription of YgiS, OmpA, GhoS, and GhoT mRNAs with the AmpliScribe T7-Flash Transcription Kit (Epicentre, Madison, WI, USA). The MqsR endoribonuclease cleavage assay was performed in a reaction mixture (10 µL) containing 2 µg of mRNA, 100 mM KCl, 2.5 mM MgCl₂, and either 30 ng of purified MqsR in MqsR buffer (10 mM Tris pH 7.0, 250 mM NaCl, and 0.5 mM TCEP) or an equivalent volume of MqsR buffer without MqsR. The reaction mixture was incubated at
37°C for 15 min and then quenched by addition of an equal volume of 2x sample loading buffer (Invitrogen, Carlsbad, CA, USA), addition of RNase Inhibitor (New England Biolabs, Ipswich, MA, USA) to a concentration of 2.5%, and heating at 65°C for 5 min. The reaction products were resolved via electrophoresis with a 15% TBE-Urea denaturing gel and stained with ethidium bromide.

**6.6.5 Construction of the ygiS expression vector with an intact signal sequence**

To construct a plasmid for producing native YgiS including its signal sequence, genomic DNA from BW25113 was amplified for ygiS by PCR using primers containing SalI and HindIII restriction sites and the strong synthetic ribosome binding site used in constructing the ASKA collection\(^6\). The amplified product was cloned into pCA24N under the control of the P\(_{T5-lac}\) promoter to form pCA24N-ygi\(_S\)\(_{n}\)ative and the cloned vector was confirmed by DNA sequencing. Primer sequences are listed in Table 6.4.

**6.6.6 Verification of YgiS in the periplasm**

Cultures of BW25113 ΔygiS/pCA24N-ygi\(_S\)\(_{n}\)atives, BW25113 ΔygiS/pCA24N (empty vector control), and BW25113/pCA24N-\(_{o}\)xyR (cytoplasmic protein control) were grown in LB medium to a turbidity of 0.5 at 600 nm and induced with 1 mM IPTG for 6 h. Cells were pelleted, resuspended in osmotic shock buffer (200 mM Tris, 20% sucrose, and 1 mM EDTA at pH 7.5), and incubated at 30°C for 15 min. Cells were pelleted, resuspended in chilled deionized water, and incubated on ice for 15 min. Cells were centrifuged and the supernatant was collected (i.e., periplasmic protein fraction). Cells were resuspended in lysis buffer (25 mM Tris at pH 8.0) and sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific, Hampton, NH, USA). Samples were pelleted and the supernatant was collected (i.e., cytoplasmic protein fraction). An EDTA-free protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) was used to stabilize protein fractions. This modified procedure was based on previous periplasmic protein isolation protocols\(^6\) (PeriPreps Periplasting kit; Epicentre. Samples were visualized by SDS-PAGE with Coomassie blue staining and the identity of protein bands was verified via mass spectrometry.

**6.6.7 Intracellular deoxycholate assay**

Overnight cultures were washed, inoculated into HEPES-glucose medium containing 4.5% deoxycholate, and grown until reaching a turbidity of ~0.2-0.5 at 600 nm. Cells were pelleted,
resuspended in HEPES-glucose medium, sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific), and centrifuged to remove cell debris. Deoxycholate in the supernatant was purified with a BakerBond SPE reversed-phase (C18) disposable column (Avantor Performance Materials, Center Valley, PA, USA)\textsuperscript{70}, using a MasterFlex peristaltic pump (Cole-Parmer, Vernon Hills, Illinois, USA) to maintain a flow rate of 5 mL/min. The column was washed with methanol and conditioned with deionized water, the supernatant was applied to the column, the column was washed with deionized water, and the pump was run to remove remaining solvent from the column. Deoxycholate was eluted from the column in methanol and quantified by comparison to a standard curve via HPLC analysis. HPLC analysis was performed on a reversed-phase (C18) column (Waters Spherisorb ODS2, 5 µm, 4.6 mm x 250 mm; Waters, Milford, MA, USA) at a flow rate of 1 mL/min with a mobile phase consisting of methanol:acetonitrile:0.02 M sodium acetate (37.5:37.5:25) adjusted to pH 4.3\textsuperscript{70} with HCl. Peak absorbance for deoxycholate was analyzed at 210 nm with a retention time of ~14-16 min and the results were normalized against turbidity at 600 nm (Fig. 6.8). Samples were spiked with deoxycholate solubilized in methanol to verify that the correct peak was analyzed. Experiments were performed with at least three independent cultures.

6.6.8 Deoxycholate tolerance assay

Overnight cultures were inoculated into LB medium and grown until reaching a turbidity of 1.0 at 600 nm. Cells were pelleted, resuspended in HEPES-glucose medium with 20% deoxycholate, and incubated for 30 min. To determine survival, cell viability was measured for samples taken before and after exposure to deoxycholate. Samples were serially diluted in 0.85% NaCl solution, plated on LB agar, and grown overnight at 37°C to determine CFU/mL\textsuperscript{71}. Experiments were performed with at least three independent cultures.
Figure 6.8. Comparison of deoxycholate uptake via HPLC. (A, B, and C) BW25113 wild-type, (D, E, and F) ΔmqsRA, and (G, H, and I) ΔygiS were grown with 4.5% deoxycholate and HPLC was used to analyze intracellular deoxycholate via a reversed-phase (C18) column and absorbance at 210 nm. Deoxycholate levels were compared based on integration of the peak with a retention time of ~14.3 min, and this peak was verified by spiking samples.

6.6.9 Leakage assay of 260-nm-absorbing material

Overnight cultures of were washed, inoculated into HEPES-glucose containing 4.5% deoxycholate, and grown until reaching a turbidity of ~0.2 to 0.6 at 600 nm. For strains with expression vectors, cultures were incubated for an additional 4 h with 0.5 mM IPTG induction. Cultures were pelleted and resuspended in HEPES-buffered saline (140 mM NaCl, 1.5 mM Na₂HPO₄, and 50 mM HEPES). Samples were taken after 2 h and centrifuged, the supernatant was filtered (0.2 µm pore size), the absorbance was measured at 260 nm, and the results were normalized against turbidity at 600 nm.
Experiments were performed with at least three independent cultures.

6.7 Acknowledgements

This work was supported by the ARO (W911NF-14-1-0279) to T.K.W. and an NSF-CAREER award (MCB0952550) to R.P., and T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University. We are also grateful for the Keio and ASKA strains provided by the National Institute of Genetics of Japan.

6.8 References


Chapter 7. Combatting Bacterial Infections by Killing Persister Cells with Mitomycin C

7.1 Preface

This chapter is adapted from the following manuscript that has been submitted for publication: Kwan, B.W., Chowdhury, N., Wood, T.K. Combatting bacterial infections by killing persister cells with mitomycin C. In this study, the anticancer drug mitomycin C is found to potently eradicate both growing and non-growing cells, including persister cells. These results demonstrate the efficacy of mitomycin C against several species (e.g., pathogenic strains of Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa) in clinically relevant growth states (e.g., biofilm cultures, an in vitro wound model, and an in vivo infection of Caenorhabditis elegans). This finding suggests that mitomycin C is a paradigm for repurposing anticancer drugs to combat recalcitrant bacterial infections through eradication of persister cells.

7.2 Abstract

Persister cells are a multi-drug tolerant subpopulation of bacteria that contribute to chronic and recalcitrant clinical infections such as cystic fibrosis and tuberculosis. Persisters are metabolically dormant, so they are highly tolerant to all traditional antibiotics which are mainly effective against actively-growing cells. Here, we show that the FDA-approved anti-cancer drug mitomycin C (MMC) eradicates persister cells through a growth-independent mechanism. MMC is passively transported and bioreductively activated, leading to spontaneous crosslinking of DNA, which we verify in both active and dormant cells. We find MMC effectively eradicates cells grown in numerous different growth states (e.g., planktonic cultures and highly robust biofilm cultures) in both rich and minimal media. Additionally, MMC is a potent bactericide for a broad range of bacterial persisters, including commensal Escherichia coli K-12 as well as pathogenic species of E. coli, Staphylococcus aureus, and Pseudomonas aeruginosa. We also demonstrate the efficacy of MMC in an animal model and a wound model, substantiating the clinical applicability of MMC against bacterial infections. Therefore, MMC is the first broad-spectrum
compound capable of eliminating persister cells, meriting investigation as a new approach for the
treatment of recalcitrant infections.

7.3 Introduction

There are 17 million new biofilm infections every year in the U.S.A., which lead to 550,000
fatalities\(^1\), and biofilms are difficult to treat due to the presence of persister cells\(^2\). Persisters arise due to
metabolic inactivity\(^3-5\) and are highly tolerant against all traditional antibiotic classes, which are primarily
effective against actively growing cells. Bacterial persistence is a non-hereditary phenotype\(^6\) which occurs
both stochastically\(^7\) or through environmental influence\(^4, 8-12\) in a small sub-population of all tested
bacterial species\(^2\) (~1% during stationary phase and in biofilm cultures\(^2-3\)).

Few distinctly new antibiotics have been discovered recently\(^13\), and current antibiotics are ineffective
against persister cells. Thus, we searched for a compound which could eradicate persister cells. Mitomycin C (MMC) is used as an FDA-approved\(^14\) chemotherapeutic agent for a wide range of cancer
treatments (e.g., bladder, gastric, and pancreatic)\(^15\). As an amphipathic molecule, MMC passively diffuses
into cells\(^16\). Bacterial cytoplasm is a reducing environment\(^17\), so after entering into cells, the quinone
functional group of MMC is reduced spontaneously, initiating crosslinking of adjacent guanine residues
in 5′-CG sequences to join two opposing strands of DNA\(^18\). Because transport is passive and the reaction
is spontaneous, we reasoned that MMC would be effective against cells in the persister state, a state of
metabolic dormancy, since MMC activity would not require active metabolism.

We found that MMC is effective against persister cells in a broad range of bacteria including
commensal *Escherichia coli* K-12 as well as pathogenic strains of *E. coli*, *Staphylococcus aureus*
(frequently found in wounds), and *Pseudomonas aeruginosa*. We also demonstrated that MMC eradicates
bacteria in biofilms, communities of notoriously difficult to treat cells present in a majority of infections.
Furthermore, we verified that MMC kills persister cells by crosslinking DNA, and we demonstrated the
efficacy of MMC in an animal model and in a wound model. Therefore, MMC has broad-spectrum
activity against growing, non-growing, and persister cells, and should be used for the treatment of
recalcitrant infections.
7.4 Results and Discussion

7.4.1 MMC kills active and persister cells in rich medium

MMC activity is decreased at high pH\textsuperscript{19}; hence, we buffered the medium to avoid high pH fluctuations and to match the physiological resting pH of \textasciitilde7.4 and exercising pH of \textasciitilde7.1 and \textasciitilde6.4\textsuperscript{20}. For this work, we compared MMC with ciprofloxacin, a fluoroquinolone that inhibits DNA replication and kills both growing and non-growing cells but not persister cells\textsuperscript{21} and which is commonly used in persister studies\textsuperscript{22}. Therefore, throughout this work, ciprofloxacin tolerance represents the baseline level of persistence. Additionally, antibiotic treatments were generally at least 5x the minimum inhibitory concentration (MIC) (Table 1) to ensure eradication of non-persisters and to minimize the survival of potential spontaneous resistant mutants.

**Table 7.1. MICs for antibiotics used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MMC</th>
<th>Ciprofloxacin</th>
<th>Ampicillin</th>
<th>Gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-12 BW25113</td>
<td>2 μg/mL</td>
<td>0.05 μg/mL</td>
<td>10 μg/mL</td>
<td>5 μg/mL</td>
</tr>
<tr>
<td>EHEC 86-24</td>
<td>1 μg/mL</td>
<td>0.05 μg/mL</td>
<td>5 μg/mL</td>
<td></td>
</tr>
<tr>
<td>S. aureus ATCC29213</td>
<td>0.2 μg/mL</td>
<td>0.5 μg/mL</td>
<td>2 μg/mL</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa PAO1</td>
<td>15 μg/mL</td>
<td>2 μg/mL</td>
<td>400 μg/mL</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa PA14</td>
<td>2 μg/mL</td>
<td>0.1 μg/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As evidence that ciprofloxacin tolerance is due to persistence rather than spontaneous genetic resistance, we measured the tolerance of *E. coli* K-12 cultures after three rounds of ciprofloxacin treatment (5 μg/mL for 3 h) and subsequent regrowth of persisters in fresh media. There was no observable increase in ciprofloxacin survival after each round of regrowth (survival at 0.026 ± 0.007%, 0.048 ± 0.002%, and 0.044 ± 0.001% for rounds 1, 2 and 3, respectively). In addition, no colonies were detectable with 5 μg/mL ciprofloxacin, showing absolutely that there were no resistant strains. These results confirm the reliability of using ciprofloxacin tolerance as an indicator of persistence.

We initially evaluated MMC with *E. coli* K-12 and found, compared to ciprofloxacin, that MMC was 2,300-fold more effective against exponentially-growing cells (Fig. 7.1A) and 150,000-fold more effective against mid-stationary-phase cells in buffered lysogeny broth (LB) medium (Fig. 7.1B). As
evidence of the ability of MMC to kill persister cells, we found that treatment of a late-stationary phase culture with MMC does not show the bi-phasic death curve that is characteristic of a persister population (Fig. 7.1C). We then utilized a rifampicin pretreatment which we previously demonstrated to induce high levels of persistence (~10-100%)\(^4\) and found that MMC was highly effective against rifampicin-induced persister cells, in stark contrast to ciprofloxacin (Fig. 7.1D). Therefore, MMC kills non-persister cells and dormant persister cells.

**Figure 7.1.** MMC eradicates metabolically dormant *E. coli* K-12 cells in suspension and in biofilms. Cell viability for exponential (turbidity of 2 at 600 nm) (A) and mid-stationary phase (turbidity of 4 at 600 nm) (B) cultures in buffered LB. (C) Time course of killing of late stationary-phase cells (16 h of growth) in buffered LB. (D) Cell viability of rifampicin-induced persister cultures in buffered LB (30 min pretreatment with 100 μg/mL rifampicin followed by resuspension in fresh media). Cell viability of exponential (turbidity of 0.4 at 600 nm) (E) and late-stationary phase (24 h of growth) (F) cultures in M9-glucose. Cell viability of biofilm cultures (24 h of growth) in M9-glucose (G), anaerobic late-stationary phase cultures (16 h of growth) in LB (H), anaerobic biofilm cultures (24 h of growth) in M9-glucose (I), and VBNC cultures (36 days of starvation in saline) (J). Cell viability is shown before (black) and after treatment (3 h for planktonic cultures, 24 h for biofilm cultures, and 16 h for VBNC cultures) with 5 μg/mL ciprofloxacin (green), 10 μg/mL gentamicin (purple), and 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown throughout (n ≥ 2). MMC is mitomycin C and CIP is ciprofloxacin.

### 7.4.2 MMC kills active and persister cells in minimal medium, in anaerobic cultures, and in biofilms

The previous assays were performed in planktonic cultures grown in rich medium; however, these growth conditions are a poor representation of ecological bacterial growth. Thus, we investigated MMC activity against cultures grown in minimal medium, in biofilms, and in anaerobic cultures. Exponential-
phase cultures in M9-glucose were similarly susceptible to MMC and ciprofloxacin (Fig. 7.1E); however, during late-stationary phase in M9-glucose, we found that while the population was highly persistent against ciprofloxacin (10 ± 1 %) and the aminoglycoside gentamicin (44 ± 5 %), MMC eradicated cells (Fig. 7.1F).

Biofilms more accurately model clinical bacteria growth with a high population of persisters, and we found that MMC was effective against biofilms in M9-glucose, killing 100,000-fold more cells than ciprofloxacin and nearly eliminating cells after 24 h of treatment (Fig. 7.1G). Additionally, MMC did not cause biofilm dispersal, confirming that efficacy against biofilms was in fact due to eradication of cells (Fig. 7.2). Bacterial infections have a propensity to exist under anaerobic conditions, and we found that MMC eradicated anaerobic, late-stationary phase cells in rich medium beyond the limit of detection, in comparison to 0.44 ± 0.08 % survival against ciprofloxacin treatment (Fig. 7.1H). Furthermore, anaerobic biofilm cultures in M9-glucose were 2,500-fold more susceptible to MMC than ciprofloxacin (Fig. 7.1I).

Figure 7.2. MMC does not cause biofilm dispersal. Biofilm formation for E. coli K-12 BW25113 wild-type cultures after 24 h of static growth and after an additional 24 h of static growth with no treatment (NT) or with 10 μg/mL MMC treatment (MMC) at 30°C in M9-glucose. Means ± s.d. are shown throughout (n ≥ 2).

7.4.3 MMC kills viable but non-culturable cells

Numerous species of bacteria enter the viable but non-culturable (VBNC) state, another state of metabolic dormancy closely related to persistence, as a survival response to environmental stresses, and
these cells do not resuscitate and become culturable unless exposed to suitable stimuli\textsuperscript{24}. VBNC cells exhibit high antibiotic tolerance, similarly to persisters, and pose a risk to human health because they can avoid detection in goods, leading to infection\textsuperscript{24}. Hence, we generated VBNC cultures by starving cells in saline solution for 36 days until there were \~1,000-fold more VBNC cells than culturable cells.

Respiratory activity is a commonly used criterion for viability\textsuperscript{25}, so RedoxSensor Green, a fluorescent dye for detection of actively respiring cells, was used to enumerate the VBNC population. Upon antibiotic treatment of these cultures, we found that MMC was 7-fold more effective at killing VBNC cells than ciprofloxacin (Fig. 7.1J), while also eradicating the culturable population (Fig. 7.3), unlike ciprofloxacin (0.40 \pm 0.05 \% survival). Therefore, we have demonstrated that MMC is highly effective against metabolically dormant cells in both the persister and VBNC states.

**Figure 7.3.** MMC eradicates the culturable population in VBNC cultures. Cell viability of the culturable population of *E. coli* K-12 BW25113 VBNC cultures. Cell viability is shown before (black) and after 16 h treatment with 5 μg/mL ciprofloxacin (green) and 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown throughout (n ≥ 2).

### 7.4.4 MMC kills persister cells by crosslinking DNA

To verify that MMC kills bacteria via DNA crosslinks, we investigated MMC activity against single-gene deletion mutants lacking *uvrA*, *uvrB*, and *uvrC*. The UvrABC complex is part of the bacterial SOS response in *E. coli\textsuperscript{26} to repair DNA crosslinks\textsuperscript{27}, and contributes to MMC tolerance\textsuperscript{28}. We found that the Δ*uvrA*, Δ*uvrB*, and Δ*uvrC* mutants were much more sensitive to MMC than the wild-type strain, and were
rapidly eradicated (beyond the limit of detection) within less than 30 min of treatment (Fig. 7.4A). Additionally, we were able to complement the high sensitivity to MMC of a ΔuvrA mutant with production of UvrA via plasmid (Fig. 7.4B). These results confirm that DNA crosslinking is the basis for MMC bactericidal activity in actively-growing cells.

**Figure 7.4.** MMC crosslinks DNA in *E. coli* K-12 persister cells. (A) Cell viability of *E. coli* K-12 wild-type, Δ*uvrA*, Δ*uvrB*, and Δ*uvrC* mid-stationary phase cultures (turbidity of 3 at 600 nm) in buffered LB treated for 30 min with MMC. (B) Cell viability of *E. coli* K-12/pCA24N, Δ*uvrA*/pCA24N, and Δ*uvrA*/pCA24N-Δ*uvrA* exponential phase cultures (turbidity of 2 at 600 nm) in buffered LB treated for 1 h with MMC. Cell viability is shown before (black) and after treatment with 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown for A and B (n ≥ 2). (C) Denaturing gel electrophoresis for pDNA (4,518 nt) from *E. coli* K-12/pCA24N non-persisters (lanes 2 and 3) and rifampicin-induced persisters (lanes 4 and 5) before (lanes 2 and 4) and after (lanes 3 and 5) MMC treatment. Lane 1 is a positive control with *in vitro* crosslinked pDNA. “M” indicates the DNA ladder, the red arrow indicates migration as double-stranded DNA, and the blue arrow indicates migration as single-stranded DNA.

However, persister cells are dormant, thus having a different metabolic state than non-persisters. Therefore, we sought to verify that MMC was in fact forming DNA crosslinks within persisters, rather than killing persisters through an unknown mechanism. Genomic DNA (gDNA) was isolated from both exponential phase cells (i.e., non-persisters) and rifampicin-induced persisters before and after MMC treatment. We hypothesized that crosslinks within DNA should inhibit amplification via quantitative PCR (qPCR). qPCR was performed with primers designed to amplify a 234 nt region of *rrsG* and a 302 nt region of *murB*, containing 18 and 10 potential MMC crosslinking sites, respectively. As a positive control, qPCR was performed on gDNA crosslinked by MMC *in vitro*, verifying that DNA crosslinking inhibited amplification dramatically (*rrsG*: -1,456 ± 1 fold and *murB*: -1,621 ± 28 fold). Our *in vivo*
results revealed the presence of gDNA crosslinks based on reduced quantities of PCR-amplifiable DNA after MMC treatment for non-persisters (rrsG: -5.95 ± 0.20 fold and murB: -5.13 ± 0.14 fold) and persisters (rrsG: -5.01 ± 0.51 fold and murB: -5.99 ± 0.50 fold) (Table 7.2).

**Table 7.2.** Verification of DNA crosslinking via qPCR. The cycle number (Ct) for each sample is indicated for the target genes (rrsG and murB) for samples before and after MMC crosslinking, performed in vivo (persisters and non-persisters) and in vitro. Fold changes in amplifiable DNA were calculated using:

\[ 2^{-(C_{t\text{after MMC}} - C_{t\text{before MMC}})} \]

The specificity of the qPCR products were verified by melting curve analysis.29 Means and standard deviations are indicated (n = 2).

<table>
<thead>
<tr>
<th>Condition</th>
<th>C&lt;sub&gt;t&lt;/sub&gt; (before MMC)</th>
<th>C&lt;sub&gt;t&lt;/sub&gt; (after MMC)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Persisters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rrsG</td>
<td>8.41 ± 0.72</td>
<td>10.74 ± 1.10</td>
<td>-5.01 ± 0.51</td>
</tr>
<tr>
<td>murB</td>
<td>12.41 ± 1.14</td>
<td>15.00 ± 1.24</td>
<td>-5.99 ± 0.50</td>
</tr>
<tr>
<td><strong>Non-persisters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rrsG</td>
<td>8.00 ± 0.87</td>
<td>10.57 ± 0.35</td>
<td>-5.95 ± 0.20</td>
</tr>
<tr>
<td>murB</td>
<td>11.85 ± 0.90</td>
<td>14.20 ± 0.39</td>
<td>-5.13 ± 0.14</td>
</tr>
<tr>
<td><strong>In vitro crosslinked</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rrsG</td>
<td>6.98 ± 0.08</td>
<td>17.48 ± 0.01</td>
<td>-1456 ± 1</td>
</tr>
<tr>
<td>murB</td>
<td>10.52 ± 0.41</td>
<td>21.18 ± 0.37</td>
<td>-1621 ± 28</td>
</tr>
</tbody>
</table>

Crosslinked DNA runs differently from non-crosslinked DNA after denaturation because the crosslinks covalently bind the two strands, preventing separation.30 As confirmation of DNA crosslinking within persister cells found with qPCR, we isolated plasmid DNA (pDNA) from both exponential-phase cells (i.e., non-persisters) and rifampicin-induced persisters before and after MMC treatment and performed agarose gel electrophoresis under denaturing conditions to allow uncrosslinked DNA to migrate as single strands. Under denaturing conditions, pDNA samples from cells without MMC treatment migrated as single-stranded DNA, while samples for both non-persisters and persisters treated with MMC showed a high percentage of crosslinking, based on migration as double-stranded DNA (Fig. 7.4C). DNA treated in vitro with MMC migrated in the expected manner as double-stranded DNA. Therefore, we have demonstrated by two independent means that MMC does in fact crosslink the DNA of persister cells.
7.4.5 MMC kills persister cells of pathogens

Clinical application of MMC is dependent on efficacy against pathogenic bacteria. Therefore, we tested the ability of MMC to kill *E. coli* O157:H7 (EHEC) a common pathogenic strain of Gram-negative *E. coli*. MMC was substantially more effective than ciprofloxacin against EHEC, eradicating both exponential (Fig. 7.5A) and mid-stationary phase cells (Fig. 7.5B) in rich medium beyond the limit of detection. Additionally, MMC eradicated late-stationary phase EHEC cells in M9-glucose beyond the limit of detection, while 1.2 ± 0.1 % of cells survived against ciprofloxacin (Fig. 7.5C). Biofilm cultures of EHEC were also eradicated (beyond the limit of detection) after 24 h of MMC treatment, in comparison to 3 ± 1 % of cells surviving ciprofloxacin (Fig. 7.5D).

**Figure 7.5.** MMC eradicates pathogens in suspension and in biofilms. Cell viability of EHEC exponential (turbidity of 2 at 600 nm) (A) and mid-stationary phase (turbidity of 4 at 600 nm) (B) cultures in buffered LB, late-stationary phase cultures (24 h of growth) in M9-glucose (C), and biofilm cultures (24 h of growth) in M9-glucose (D). Cell viability of *S. aureus* exponential (turbidity of 0.8 at 600 nm) (E) and mid-stationary phase (turbidity of 3 at 600 nm) cultures in tryptic soy broth (TSB) (F), and biofilm cultures (24 h of growth) in modified M9-glucose (G). Cell viability of *P. aeruginosa* PA14 exponential phase cultures (turbidity of 2 at 600 nm) in buffered LB (H) and late-stationary phase cultures (24 h of growth) in M9-glucose (I). Cell viability is shown before (black) and after treatment (3 h for planktonic cultures and 24 h for biofilm cultures) with 5 μg/mL ciprofloxacin (green) and 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown throughout (*n* ≥ 2).

MMC was also tested against Gram-positive *S. aureus* (methicillin sensitive) and Gram-negative *P. aeruginosa*, two other common species of human pathogens. Against planktonic cultures of *S. aureus* grown in rich medium, MMC was highly effective, eradicating both exponential (Fig. 7.5E) and mid-
stationary phase cultures (Fig. 7.5F), in comparison to ciprofloxacin (0.55 ± 0.04 % survival and 2.2 ± 0.1 % survival, respectively). MMC was also highly effective against biofilm cultures of *S. aureus* grown in minimal medium, eradicating cells beyond the limit of detection after 24 of treatment, compared with 18 ± 2 % survival against ciprofloxacin (Fig. 7.5G). MMC also killed cultures of *P. aeruginosa* PA14 grown planktonically in rich medium to exponential (Fig. 7.5H) and mid-stationary phase (0.0038 ± 0.0005 % survival) and in minimal medium to late-stationary phase (Fig. 7.5I), although the extent of killing activity was similar to that of ciprofloxacin. Therefore, MMC is significantly more effective in eradicating EHEC and *S. aureus* and is similar to other potent antibiotics against *P. aeruginosa*, demonstrating the efficacy of MMC against several species of human pathogens.

### 7.4.6 MMC kills persister cells in a wound model

In clinical infections, bacteria are exposed to drastically different growth conditions from those generally used within laboratory cultures. The *in vitro* Lubbock chronic wound pathogenic biofilm model was previously developed to closely represent growth conditions of polymicrobial infections\(^{31}\). We used this *in vitro* wound model to test MMC activity against cultures of EHEC, *S. aureus*, and *P. aeruginosa* PAO1 as well as a co-culture of *S. aureus* and *P. aeruginosa* PAO1. Our strain of *S. aureus* is coagulase-positive\(^{32}\), causing the medium to form a jelly-like mass consisting of insoluble fibrin\(^{33}\). Cultures were grown statically so that coagulated plasma served as a scaffold for bacterial growth\(^{34}\) in cultures containing *S. aureus*, while biofilms formed at surface interfaces served as scaffolds for bacterial growth in cultures without *S. aureus*. We found that MMC was more effective than ciprofloxacin and ampicillin against all three species under wound-like conditions in mono- and co-cultures (Fig. 7.6A-D). These results show that MMC is a significantly more effective treatment than other antibiotics against pathogenic strains of several species (e.g., EHEC, *S. aureus*, and *P. aeruginosa*) grown using an *in vitro* wound model. This substantiates the efficacy of MMC as a clinical treatment for clearing infections.
Figure 7.6. MMC eradicates pathogens in clinically relevant wound and animal models. Cell viability of EHEC (A), S. aureus (B), and P. aeruginosa PAO1 (C) mono-cultures and S. aureus and P. aeruginosa PAO1 co-cultures (D) in an *in vitro* wound model (24 h of growth). Cell viability is shown before (black) and after 5 h treatment with ciprofloxacin (blue; 5 µg/mL for EHEC and S. aureus or 10 µg/mL for PAO1 mono- and co-cultures), ampicillin (green; 100 µg/mL for EHEC and S. aureus or 2 mg/mL for PAO1 mono- and co-cultures), and MMC (red; 10 µg/mL for EHEC and S. aureus or 15 µg/mL for P. aeruginosa PAO1 mono- and co-cultures). * represents eradication beyond the limit of detection. (E) Survival of *C. elegans* after infection with EHEC (days -2 to 0) followed by 6 h exposure to 5 µg/mL ciprofloxacin (green), 100 µg/mL ampicillin (blue), 10 µg/mL MMC (red), or no treatment (white). As a negative control, *C. elegans* was grown on OP50 without antibiotic treatment (black). Means ± s.d. are shown throughout (*n* ≥ 2).

### 7.4.7 MMC is effective in an animal model

In order to test the efficacy of MMC treatment *in vivo*, we used an EHEC infection within the nematode *Caenorhabditis elegans*. *C. elegans* was fed on lawns of EHEC on nematode growth media (NGM) agar plates for 2 days in order to establish an infection. Nematodes were then exposed to MMC, ciprofloxacin, ampicillin, or no treatment, transferred to lawns of avirulent *E. coli* OP50, and monitored for viability. All three antibiotic treatments enhanced the survival of the EHEC-infected worms; however,
survival with MMC was higher than with either ciprofloxacin or ampicillin based on four experimental replicates (10 worms per replicate) (Fig. 7.6E), likely because MMC eradicates persisters that can reestablish infection. Of note, we obtained similar results from four additional replicates performed with different antibiotic treatments and EHEC infection conditions. Therefore, MMC is consistently more effective than other antibiotics at clearing EHEC infection within an animal model.

7.5 Conclusions

Traditional antibiotics (e.g., fluoroquinolones, aminoglycosides, and β-lactams) are ineffective against persister cells due to their mechanisms which rely on cellular activity. Here, we found that MMC is highly effective because of its unique mechanism of action, which is independent of the metabolic state, by demonstrating its activity against slow-growing, non-growing, and dormant (e.g., persister and VBNC) cells, as well as its activity on cells grown planktonically, in biofilms, in an in vitro wound model, and in an in vivo animal model. In comparison, several methods have been proposed for eradicating persister cells, including increasing aminoglycoside uptake via glycolysis intermediates35, altering membranes via Trp/Arg-containing antimicrobial peptides36, activating ClpP-mediated self-digestion via rifampicin and ADEP422, and converting persisters to non-persisters via cis-2-decenoic acid37. However, the potential application of these treatments against clinical infections is distant due to limited levels of in vivo testing. These treatments are also likely limited to a small range of species that are susceptible to the compounds. In contrast, MMC has been an FDA-approved chemotherapeutic cancer drug for over forty years14 with a well-characterized biochemical mechanism18. Additionally, MMC passively diffuses into cells, and the DNA crosslinking activity of MMC is spontaneous, so MMC treatment should be effective against many bacterial species which cannot be fully cleared with traditional antibiotics such as recalcitrant internal and external (wound) infections.

Throughout the majority of this study, MMC was effectively applied at a concentration of 10 μg/mL, although the effective concentration should be much lower against strains with low MICs (e.g., EHEC and S. aureus; Table 7.1). For various cancer treatments, intravenously infused MMC dosages are often administered at concentrations between 0.5-2.0 μg/mL (20-80 mg/m²)15 and topical dosages have been
safely applied at concentrations up to 400 μg/mL. Therefore, the bactericidal concentrations of MMC are similar to the therapeutic concentrations that have been established for cancer treatments, which validates MMC as a readily applicable treatment for clinical infections.

7.6 Materials and Methods

7.6.1 Bacterial strains and growth media

Bacterial strains and plasmids used are listed in Table 7.3. Experiments were conducted at 37°C with shaking at 250 rpm unless otherwise indicated. E. coli and P. aeruginosa strains were grown in unbuffered lysogeny broth (LB) medium at pH 6.9, LB buffered with 100 mM KPO₄ at pH 7.0 or M9-glucose (0.4%) at pH 7.0 and S. aureus was grown in tryptic soy broth (TSB) at pH 6.8 or modified M9-glucose (0.4%) at pH 7.0 [M9-glucose (0.4%) supplemented with 0.5 mg/mL Drop-out Mix Complete w/o Yeast Nitrogen Base (USBiological, Salem, MA, USA), 0.2 μg/mL nicotinic acid, and 0.2 μg/mL thiamine] unless otherwise indicated. Chloramphenicol (30 μg/mL) was utilized to maintain the pCA24N-based plasmids.

Table 7.3. Bacterial strains and plasmids used in Chapter 7.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-12 BW25113</td>
<td>rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1</td>
<td>42</td>
</tr>
<tr>
<td>E. coli K-12 BW25113 ΔuvrA</td>
<td>BW25113 ΔuvrA Ω KmR</td>
<td>42</td>
</tr>
<tr>
<td>E. coli K-12 BW25113 ΔuvrB</td>
<td>BW25113 ΔuvrB Ω KmR</td>
<td>42</td>
</tr>
<tr>
<td>E. coli K-12 BW25113 ΔuvrC</td>
<td>BW25113 ΔuvrC Ω KmR</td>
<td>42</td>
</tr>
<tr>
<td>EHEC 86-24</td>
<td>EHEC O157:H7 Stx2⁺</td>
<td>43</td>
</tr>
<tr>
<td>E. coli OP50</td>
<td>E. coli B strain (uracil auxotroph)</td>
<td>44</td>
</tr>
<tr>
<td>S. aureus ATCC 29213</td>
<td>Antibiotic-susceptible reference strain</td>
<td>ATCC</td>
</tr>
<tr>
<td>P. aeruginosa PAO1</td>
<td>Wild-type strain</td>
<td>45</td>
</tr>
<tr>
<td>P. aeruginosa PA14</td>
<td>Wild-type strain</td>
<td>46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCA24N</td>
<td>CmR; lacF, pCA24N</td>
<td>41</td>
</tr>
<tr>
<td>pCA24N-uvrA</td>
<td>CmR; lacF, pCA24N P₅₅lac::uvrA</td>
<td>41</td>
</tr>
</tbody>
</table>

CmR and KmR are chloramphenicol and kanamycin resistance, respectively.
7.6.2 Minimum inhibitory concentration (MIC) assay

The MICs of MMC, ciprofloxacin, ampicillin, and gentamicin for *E. coli* K-12, EHEC, *S. aureus*, and *P. aeruginosa* (PA14 and PAO1) were determined by incubating freshly inoculated cultures in unbuffered LB (buffered LB for MMC and TSB for *S. aureus*) for 16 h with varying concentrations of each antibiotic and observing inhibition of growth based on lack of turbidity. Experiments were performed with at least two independent cultures.

7.6.3 Planktonic antibiotic killing assays

In order to obtain exponential and mid-stationary phase cultures in LB, buffered LB or TSB, overnight cultures (16 h) were diluted 1:1000 in fresh medium and grown to the desired turbidity (i.e., a turbidity below 2 at 600 nm for exponential phase and a turbidity of 3 to 4 at 600 nm for mid-stationary phase). Late-stationary phase cultures in LB or buffered LB were obtained by growing overnight cultures (16 h). In order to obtain exponential and late-stationary phase cultures in M9-glucose, overnight cultures (16 h) were washed diluted 1:1000 in M9-glucose and grown for the desired period of time (i.e., a turbidity of 0.4 at 600 nm for exponential phase and 24 h of growth for late-stationary phase). Anaerobically grown cultures were maintained with ~20% CO₂, ~79% N₂, and ~1% H₂. In order to obtain rifampicin-induced persister cultures in buffered LB, overnight cultures (16 h) were diluted 1:1000 in fresh medium and grown to a turbidity of 0.8 at 600 nm. Cultures were exposed to rifampicin (100 µg/mL) for 30 min, centrifuged, and resuspended in fresh medium to remove the rifampicin. Cultures were treated with MMC (10 µg/mL for *E. coli* K-12, EHEC, *S. aureus*, and PA14), ciprofloxacin (5 µg/mL for *E. coli* K-12, EHEC, *S. aureus*, and PA14), or gentamicin (10 µg/mL for *E. coli* K-12) for 3 h (unless otherwise indicated). These concentrations are at least 5x MIC to minimize survival of potential spontaneous resistant mutants. Cell viability was determined before and after antibiotic treatments by serially diluting cultures in 0.85% NaCl solution, plating 10 µL drops on LB agar, and counting colonies. Experiments were performed with at least two independent cultures.

7.6.4 Biofilm antibiotic killing assays

Overnight cultures (16 h) grown in LB (TSB for *S. aureus*) were washed and diluted to a turbidity of
0.05 at 600 nm in M9-glucose (modified M9-glucose for *S. aureus*) and cultures were grown for 24 h at 30°C in 96-well plates (300 µL/well). Biofilm cultures were treated with MMC (10 µg/mL for *E. coli* K-12, EHEC, and *S. aureus*) or ciprofloxacin (5 µg/mL for *E. coli* K-12, EHEC, and *S. aureus*) for 24 h. Samples were assayed before and after antibiotic treatments by carefully removing the supernatant from a well, resuspending the biofilm in 0.85% NaCl solution (adapted from Bernier et al.\(^48\)), triturating to break apart the biofilm, and combining the suspensions from multiple wells to obtain an averaged sample. Then cell viability was determined by serially diluting cells in 0.85% NaCl solution, plating 10 µL drops on LB agar, and counting colonies\(^47\). Biofilm formation was assayed using crystal violet staining as described previously\(^49\), confirming the presence of biofilms for all tested conditions. Experiments were performed with at least two independent cultures.

7.6.5 **Biofilm dispersal assay**

Overnight cultures (16 h) grown in LB were washed and diluted to a turbidity of 0.05 at 600 nm in M9-glucose and cultures were grown for 24 h at 30°C in 96-well plates (300 µL/well). Cultures were incubated for an additional 24 h with or without MMC. Biofilm levels were assayed using crystal violet staining as described previously\(^49\). Cell growth (turbidity at 620 nm) was used to normalize the total biofilm formation (absorbance at 540 nm). Data points were averaged from 15 replicate wells using at least two independent cultures.

7.6.6 **VBNC antibiotic killing assay**

Overnight cultures (16 h) of *E. coli* K-12 BW25113 grown in LB were diluted 1:1000 in LB and grown to a turbidity of 3.0 at 600 nm. Cells were washed three times with 0.85% NaCl to remove nutrients and incubated in 0.85% NaCl for 36 days until the VBNC population was ~1,000-fold higher than the culturable population. VBNC cultures were treated with MMC (10 µg/mL) or ciprofloxacin (5 µg/mL) for 16 h. Samples were assayed before and after antibiotic treatments for viability of both cultivable cells, determined by cell viability when plated on LB agar, and VBNC cells, determined by staining with the BacLight RedoxSensor Green Vitality Kit (Life Technologies, Carlsbad, CA, USA). Samples (0.5 mL) were diluted 1:1 with LB to stimulate cellular respiration for 10 min prior to staining.
with 1 µL of 1 mM RedoxSensor™ Green dye and 1 µL of 20 mM propidium iodide. Samples were visualized in a hemocytometer (Hausser Scientific, Horsham, PA, USA) using an Olympus BX61 confocal microscope (Olympus, Tokyo, Japan) at 200× magnification in order to determine viability of the VBNC population. Cell counting data was averaged from four images and experiment was performed with at least two independent cultures.

7.6.7 Quantitative real time polymerase chain reaction (qPCR)

Overnight cultures (16 h) of *E. coli* K-12 BW25113/pCA24N grown in LB were diluted 1:1000 in buffered LB and grown to the desired turbidity. For exponential phase cells (i.e., non-persisters), cultures were grown to a turbidity of 2.0 at 600 nm. For rifampicin-induced persisters, cultures were grown to a turbidity of 0.8 at 600 nm, exposed to rifampicin (100 µg/mL) for 30 min, centrifuged, and resuspended in fresh medium to remove the rifampicin. Non-persister and rifampicin-induced persister cultures were treated with MMC (100 µg/mL) for 1 h. gDNA was extracted, using an UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), from samples taken before and after MMC treatment and 100 ng of DNA was used for qPCR of *rrsG* and *murB* using the GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and the StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (primers are shown in Table 7.4). As a positive control, 3 µg of gDNA from BW25113 non-persisters was crosslinked *in vitro* with 500 µg/mL (1.5 mM) MMC in 0.2 mM EDTA, 50 mM NaCl, and 5 mM Tris (pH = 7.6) with Na$_2$S$_2$O$_4$ added six times at 10 min intervals to reach a final concentration of 2.25 mM (1.5× molarity of MMC)$^{50}$. The *in vitro* crosslinking reaction was performed anaerobically on ice and the reaction was stopped by exposure to air.$^{50}$ Experiments were performed with at least two independent cultures.

Table 7.4. Oligonucleotides used in Chapter 7 for qPCR. “F” indicates forward primers and “R” indicates reverse primers.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rrsG</em>-RT-F</td>
<td>TATTGCACAATGGGCGCAAG</td>
</tr>
<tr>
<td><em>rrsG</em>-RT-R</td>
<td>ACTTAACAAACCGCCTGCGT</td>
</tr>
<tr>
<td><em>murB</em>-RT-F</td>
<td>GAACAACAATTACTCAATGCGCTGGCCAGTGATGC</td>
</tr>
<tr>
<td><em>murB</em>-RT-R</td>
<td>CCATAAGCACCAATATTCTGGATAGGTGATGAGC</td>
</tr>
</tbody>
</table>
7.6.8 Denaturing agarose gel electrophoresis

 Overnight cultures (16 h) of *E. coli* K-12 BW25113/pCA24N grown in LB were diluted 1:1000 in buffered LB and grown to the desired turbidity. For exponential phase cells (i.e., non-persisters), cultures were grown to a turbidity of 2.0 at 600 nm. For rifampicin-induced persisters, cultures were grown to a turbidity of 0.8 at 600 nm, exposed to rifampicin (100 µg/mL) for 30 min, centrifuged, and resuspended in fresh medium to remove the rifampicin. Non-persister and rifampicin-induced persister cultures were treated with MMC (100 µg/mL) for 1 h. pDNA was extracted from samples taken before and after MMC treatment and linearized by *Hind*III digestion. As a positive control, 2 µg of linearized pDNA from non-persisters was crosslinked *in vitro* with 500 µg/mL (1.5 mM) MMC in 0.2 mM EDTA, 50 mM NaCl, and 5 mM Tris (pH = 7.6) with Na₂S₂O₄ added six times at 10 min intervals to reach a final concentration of 2.25 mM (1.5× molarity of MMC)⁵⁰. The *in vitro* crosslinking reaction was performed anaerobically on ice and the reaction was stopped by exposure to air⁵⁰. Linearized pDNA was visualized after electrophoresis through a TAE agarose gel and an alkaline denaturing agarose gel. Experiment was performed with at least two independent cultures.

7.6.9 *In vitro* wound model

 Overnight cultures (16 h) of EHEC, *S. aureus*, and *P. aeruginosa* PAO1 were grown in LB (TSB for *S. aureus*), washed with 0.85% NaCl solution, and diluted 1:1000 (as mono-cultures or co-cultures) in wound-like medium (WLM) (45% Bolton broth, 50% heparinized bovine plasma, and 5% laked horse blood)³¹. Cultures were grown for at 37°C without shaking for 24 h and then treated with MMC (10 µg/mL for EHEC and *S. aureus* or 15 µg/mL for *P. aeruginosa* PAO1 mono- and co-cultures), ciprofloxacin (5 µg/mL for EHEC and *S. aureus* or 10 µg/mL for PAO1 mono- and co-cultures), or ampicillin (100 µg/mL for EHEC and *S. aureus* or 2 mg/mL for PAO1 mono- and co-cultures) for 5 h. Cell viability was determined before and after antibiotic treatments by serially diluting cultures in 0.85% NaCl solution, plating 10 µL drops on LB agar, and counting colonies⁴⁷. Samples containing *S. aureus* were coagulated, so sections of coagulated WLM were cut and weighed add appropriate amounts of antibiotics. Coagulated samples were triturated and vortexed to release cells prior to measuring cell
viability. Experiments were performed with at least two independent cultures.

### 7.6.10 C. elegans animal model

*C. elegans* Bristol N2 was maintained using standard practices. Growth of *C. elegans* was synchronized by isolating eggs from gravid adults, hatching the eggs overnight in M9-buffer, and plating the L1-stage worms onto lawns of *E. coli* OP50 on nematode growth medium (NGM) agar plates. Nematodes were grown to young adults at 25°C and transferred (10 worms per plate) to lawns of EHEC or *E. coli* OP50 as a negative control. After feeding on EHEC for 2 days, nematodes were suspended in 20% NGM and 80% M9-buffer at pH 6.0 and exposed to MMC (10 µg/mL), ciprofloxacin (5 µg/mL), ampicillin (100 µg/mL) or no treatment for 6 h. After antibiotic treatment, worms were plated on lawns of *E. coli* OP50 and viability was scored daily. Experiment was performed with at least four replicate plates.

### 7.7 Acknowledgements

This work was supported by the Army Research Office (W911NF-14-1-0279) and the Grace Woodward Foundation. T.K.W. is the Biotechnology Endowed Professor at the Pennsylvania State University. We are grateful for the Keio and ASKA strains provided by the National Institute of Genetics of Japan.

### 7.8 References


8.1 Summary

The success rate for clinical treatment of bacterial infections has drastically improved since the advent of antibiotic use in modern medicine\(^1\). However, wide-spread use of antibiotics has revealed the existence of antibiotic tolerant bacterial populations, including cells with a genetically inherited resistance and the metabolically dormant, isogenic sub-population of persister cells\(^2\). Although mechanisms of resistance are well-characterized, there is a dearth of information regarding persister cells, owing to the low frequency of occurrence. Therefore, a crucial step for improving treatments of bacterial infections is to better understand the basis for these persister cells.

Persister cells have frequently been characterized as dormant cells in a state of low metabolism\(^3\)-\(^5\), although few experiments have been performed to verify this. Several toxin/antitoxin (TA) systems have been suggested as the basis of persister cell formation\(^4\),\(^6\). Using bacteriostatic compounds to mimic toxins MqsR and TisB, we found that a high level of persistence (10-100%) can be induced with pre-treatments that directly (e.g., inhibition of transcription) or indirectly (e.g., inhibition of ATP synthesis) inhibit protein synthesis. The high correlation with these pre-treatments and the induction of persistence suggests that persisters are cells exhibiting essentially a complete lack of protein synthesis. Additionally, these results demonstrate that persistence can also arise as a result of environmental factors because many bacteriostatic antibiotics are produced naturally by bacteria to provide a competitive advantage against neighboring species\(^7\). Combination therapy with multiple antibiotics is often used to treat clinical infections\(^8\), so these results suggest that antibiotics such as rifampicin and tetracycline may in fact reduce efficacy of partnered antibiotics. Furthermore, this newfound technique for inducing a high population of persister cells can be used in future studies of persistence.

Bacterial persistence is often attributed to toxin/antitoxin (TA) systems, which are perceived as a redundant, genetic basis for the formation of persisters from normal cells\(^4\),\(^9\). TA systems are prime models for persistence because continuous expression of the antitoxin is necessary to mediate the toxin,
that can be triggered by environmental cues\textsuperscript{10} or stochastic fluctuations that affect the relative level of toxin or antitoxin\textsuperscript{11}. Therefore, we investigated the persister cell formation mechanism for toxin YafQ of the YafQ/DinJ TA system. Our results demonstrated that YafQ reduces indole levels through down-regulation of tryptophanase, and that indole inversely influences persistence (i.e., reducing indole levels leads to increased persistence). Moreover, indole reduces biofilm formation in \textit{Escherichia coli}\textsuperscript{12-16} and is reduced in biofilms\textsuperscript{12}, suggesting that the intercellular signal indole links levels of persistence and biofilm, since persister cells are more prevalent in biofilms\textsuperscript{17}.

In an attempt to further explore the relationship between persistence and biofilm, we investigated the phosphodiesterase DosP which cleaves cyclic diguanylate (c-di-GMP), a biofilm regulatory signal\textsuperscript{18}. Contrary to our initial hypothesis, we found that DosP increases persistence via cleavage of another regulatory molecule, cyclic adenosine monophosphate (cAMP). cAMP is a direct regulator for tryptophanase, so these results corroborated our study of YafQ by indicating that reduced indole levels yielded increased persistence. The additional involvement of cAMP suggests that regulation of persistence is complex, since cAMP is a global regulatory molecule that fluctuates based on carbon metabolism\textsuperscript{19}.

MqsR/MqsA is a well-characterized TA system\textsuperscript{10, 20-25} that influences persister cell formation\textsuperscript{3, 6, 26}, so we investigated physiological conditions for which the system is important. We found that MqsR/MqsA is important for growth of \textit{E. coli} during exposure to deoxycholate, a component of bile. We found that MqsR degrades YgiS mRNA, and YgiS is a periplasmic protein that increases the uptake of deoxycholate. High intracellular concentrations of the detergent deoxycholate cause the cells to sustain more membrane damage, thus reducing growth and tolerance. Bile is stored in the gallbladder and secreted into the duodenum (i.e., upper small intestine) during the digestive process, and contains multiple bile salts that are highly similar in structure, including deoxycholate\textsuperscript{27}. Therefore, MqsR/MqsA is physiologically important for \textit{E. coli} to thrive in the gallbladder and upper intestinal tract, where high bile concentrations are prominent. This finding suggests that bile likely influences persistence because there is a strong relationship between the MqsR/MqsA TA system and persistence.
While a majority of persister research focuses on understanding the basis for persistence, there are only a few studies which have demonstrated clinically applicable methods for tackling the problem that persisters pose to public health. The persister cell population clearly represents an inability of traditional antibiotics to kill metabolically dormant bacteria. Thus, we investigated mitomycin C (MMC), an anti-cancer drug that should be effective in persister cells through a unique mechanism of action that is independent of the metabolic state within a cell. We found MMC to be significantly more effective than traditional antibiotics against slow-growing, non-growing, and dormant (e.g., persister and VBNC) cells, as well against cells grown planktonically, in biofilms, in an in vitro wound model, and in an in vivo animal model. Additionally, we demonstrated the efficacy of MMC across several pathogenic species, including *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. MMC has been an FDA-approved chemotherapeutic cancer drug for over forty years with a well-characterized biochemical mechanism, so it is readily situated for clinical use against recalcitrant infections.

The work presented in this dissertation has contributed to the understanding of the persister phenotype by demonstrating that arrested protein synthesis as the key to persistence and by elucidating the complexity of persister cell formation through characterization of new persister mechanisms. Additionally, this work has developed a readily applicable clinical treatment to improve treatment of recalcitrant infections by eradicating persisters via repurposing the anti-cancer drug MMC.

8.2 Recommendations for Future Work

8.2.1 Determine essential biomechanics in persisters

Currently, there is a wealth of literature characterizing numerous different persister cell formation mechanisms (e.g., TA systems and ppGpp). More importantly with regards to public health, there are several studies which have determined methods for potentially eradicating persisters (e.g., metabolic stimulation and unique drugs). It has long been established that persister cells are metabolically dormant, and we have demonstrated that the key to persistence is the lack of protein synthesis. However, there is large gap in knowledge regarding the biomechanics of cell maintenance that is likely required maintain viability of persisters. Persister cells are capable of surviving extended periods of time (>24 h) in the
presence of lethal antibiotics; however, this is significantly longer than the half-lives of many proteins\(^{30}\) and nearly all mRNAs\(^{31}\) in *E. coli*. Clearly, some low level of mRNA synthesis, protein synthesis/maintenance, and/or cell membrane maintenance must occur within persister cells in order to retain viability. Therefore, the protein landscape and enzymatic activities within persister cells should be studied to determine essential proteins for persister cell maintenance. An understanding of the essential biomechanics within persisters can be used to design targeted drugs that should be effective against persister cells.

### 8.2.2 Characterize waking of persister cells

One approach to eradicating persister cells is to wake them from their dormant state, thus restoring normal antibiotic susceptibility. Therefore, it is important to characterize the requirements and dynamics for this resumption of normal growth. Unlike the highly specific requirements for bacterial spore germination\(^{32}\), persister cells do not appear to require specific environmental factors for resumption of growth. Although persistence can be influenced by the environment, persister cell formation is purported to occur stochastically, seemingly through fluctuations of TA system components. Therefore, persisters should be investigated to determine whether waking from dormancy hinges on degradation of specific toxins to non-toxic levels. Additionally, resumption of normal growth should rely on replenishment of essential cellular components (e.g., proteins and mRNA); however, it should be determined whether this is a priority-ordered process and if so, which proteins are synthesized first. It should be noted that persisters do not wake up uniformly, so studying the kinetics for waking of persister cells would require single-cell level analysis. Nevertheless, characterizing the dynamics of growth resumption could potentially lead to development of treatments to expedite waking of persisters or, at the very least, a more complete understanding of the persister phenotype.

### 8.2.3 Characterize downstream regulatory effects of toxin MqsR

Toxin MqsR from the MqsR/MqsA is a well-characterized RNA endonuclease\(^{20,22,24,25}\) that cleaves mRNA at 5’-GCU sites present in all but 14 mRNAs in *E. coli*\(^{33,34}\). Investigation of these transcripts led to the discovery of toxin GhoT, part of the GhoS/GhoT TA system\(^{22}\) regulated downstream of
MqsR/MqsA. This demonstrated that MqsR has a regulatory role beyond general reduction of the mRNA pool. MazF, another RNA endonuclease toxin, cleaves mRNA transcripts at ACA sequences; however, selective protein synthesis regulates a downstream pathway of cell death/survival. Since the role of MqsR in persistence is well-established, translational regulation from MqsR should be investigated. As a targeted approach, MqsR endonuclease activity should be tested against various RNA secondary structures, which may reveal mRNA transcripts that are enriched by MqsR. Additionally, as a broad approach to determine regulatory roles of MqsR, shotgun proteomics and RNA sequencing techniques can be performed. Characterizing the post-transcriptional regulatory effects of MqsR may elucidate cell processes that are necessary for maintaining the dormant state of persister cells.

8.2.4 Investigate anti-cancer drugs for antibacterial properties

Several methods have been proposed for eradication of persister cells; however, the potential application of these treatments against clinical infections is distant due to limited levels of in vivo testing. We have demonstrated that the FDA-approved anti-cancer drug MMC can be repurposed for the eradication of persister cells. MMC is highly effective due to the spontaneous DNA crosslinking mechanism, which suggests that the key to eradicating persister cells is to use drugs which act independently of cellular metabolism. Many anti-cancer drugs share similar spontaneous alkylation mechanics with MMC, so there are likely other compounds that would be effective against persister cells. Therefore, MMC should be treated as a paradigm in a search through existing chemotherapeutics for those which eradicate persisters. In particular, compounds should be screened for those which enter cells through passive diffusion and spontaneously damage DNA. Identification of additional compounds for eradication of persisters is important to the improvement of clinical treatment of recalcitrant infections.

8.3 Final Statement

This dissertation focused on the clinically relevant multi-drug tolerant sub-population of persister cells with the motivation of improving clinical treatment of recalcitrant infections. This work was undertaken with the following goals: (i) review and determine the cellular basis for multi-drug tolerance of persister cells, (ii) characterize the genetic basis for persister cell formation mechanisms, and (iii)
develop a clinically applicable method to eradicate persister cells. A wide-array of scientific techniques was utilized in the process of achieving these goals, the principles of which directly contribute to the advancement of public healthcare.

8.4 References

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PRESENTATIONS


AWARDS AND HONORS
- ASM Student Travel Award for the General Meeting (ASM2013 General Meeting)
- Leighton Riess Graduate Fellowship in Engineering (2012)