THE EFFECT OF SUBINHIBITORY CONCENTRATIONS OF CIPROFLOXACIN ON *recA* EXPRESSION AND MUTATION RATES OF *AEROMONAS HYDROPHILA*

by

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ABSTRACT

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Despite antibiotic resistance threatening global health, consumption of antibiotics continues to rise. With human and agricultural overuse and improper disposal, antibiotics are found throughout aquatic environmental sources. These environmental contaminants can behave as microbial stressors inducing survival pathways as a prelude to increased mutation rates and subsequent antibiotic resistance. The bacterial SOS response is one such survival pathway, the mutational activity of which is induced by the expression of the activator RecA in response to cell stress. The activity of RecA is largely undefined in *Aeromonas hydrophila*, a human pathogen ubiquitous in waterways. In the present study, our objective was to determine the direct effect of RecA on mutagenesis in response to incubation with subinhibitory concentrations of ciprofloxacin. First, we determined the expression of *recA* in response to exposure to sub-MIC levels of ciprofloxacin using real-time PCR. We found increased expression of *recA* consistent with induction of the SOS response. Then we developed a *recA* deletion using splicing by overlap extension PCR. Briefly we amplified fragments flanking *recA*

with complementary overhangs, ligated the fused fragments on suicide vector pEX18Tc and inserted this via transformation to an intermediate *Escherichia coli* strain, S17-1. The modified vector plasmid was acquired in *A. hydrophila* ATCC 7966 via filter mating. Subsequent homologous recombination transferred the wild type *recA* onto the suicide plasmid leaving *recA* absent on the chromosome. Post-conjugation, high sucrose media counter-selected for strains without the plasmid leaving either wild type or deletions. After PCR screening of all resulting colonies and confirmation of a deletion, we analyzed the effect of sub-inhibitory concentrations of ciprofloxacin on mutation rate amongst the wild type and *recA* knock out *A. hydrophila*. Exposure to half the minimum inhibitory concentration of ciprofloxacin resulted in a statistically significant tenfold increase ($p< 0.05$) in mutation rate among the wild type. However, the equivalent conditions with the *recA* knock out exhibited attenuated mutation rates. Together, our findings highlight the dangers of low-level antibiotics in our environment and suggest targeting RecA as an alternative therapeutic approach to reduce the formations of mutations potentially leading to increased antibiotic resistance.

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LIST OF ABBREVIATIONS

ADA-VI: Ampicillin Dextrose Agar with Vancomycin and Irgasan

Ah: *Aeromonas hydrophila*

ANOVA: Analysis of variance

ARG: Antibiotic resistance genes

ATCC: American Type Culture Collection

cDNA: Complementary deoxyribonucleic acid

CFU: Colony forming unit

DNA: Deoxyribonucleic acid

Ec: *Escherichia coli*

ESBL: Extended-spectrum beta-lactamases

HAI: Hospital-acquired infection

IPTG: Isopropyl β-d-1-thiogalactopyranoside

Kb: Kilobase

LB: Luria-Bertani Broth

MCS: Multiple cloning site

MDR: Multi-drug resistance

MIC: Minimum inhibitory concentration

NCBI: National Center for Biotechnology Information

NEB: New England BioLabs

NRT: No reverse transcriptase

NTC: No template control

OD: Optical density

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

RT: Room temperature

SOE-PCR: Splicing by overlap extension polymerase chain reaction

TSA: Tryptic soy agar

WT: Wild type

WWTP: Wastewater treatment plant

X-Gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1: BACKGROUND

I: Introduction

1. The genus *Aeromonas*

Aeromonads are ubiquitous in the microbial biosphere, chiefly in aquatic environments, where they flourish in the water distribution system (Sen, *et al*., 2004). The genus *Aeromonas* can be isolated from rivers, lakes, ponds, seawater (estuaries), drinking water, groundwater, wastewater, and sewage in various stages of treatment (Senderovich, *et al*., 2012). Environmentally, aeromonads are present in a low concentration in groundwater, drinking water and seawater at 1 CFU/mL and at an average of 108 CFU/mL in crude sewage or domestic sewage sludge (Janda, *et al.,* 2010). A rod-shaped, Gram negative, facultative anaerobe, *Aeromonas* spp. were first recognized as zoonotic pathogens of poikilotherms, responsible for furunculosis in salmonids, with pathologies ranging from hemorrhage to septicemia, and accountable for massive die-offs resulting in substantial economic losses in the fish-farming industry (Senderovich, *et al*., 2012). Though initially notorious for its pathogenicity to aquatic animals, its capacity of causing devastating human diseases was seen most notably in the aftermath of the 2004 tsunami that affected Indonesia and Thailand (Batra, *et al.,* 2016). In survivors with skin or soft tissue injuries, *Aeromonas* ranked as the single most prevalent pathogen isolated, accounting for over 20% of the isolates identified (Janda, *et al.,* 2010). *Aeromonas* are also the causative agents of a variety of human diarrheal

diseases, and infections can lead to septicemia (Janda, *et al.,* 2010). The case fatality rate among patients with *Aeromonas* bacteremia ranges from 27.5 to 46% (Janda, *et al.,* 2010).

Additionally, *Aeromonas* has recently been characterized as an emerging nosocomial infection (Batra, *et al.,* 2016). Despite strict adherence to sterility measures and robust infection control practices, the significant morbidity and mortality associated with hospital associated infections (HAIs) is pervasive and costly (Munita, *et al.,* 2016). Compounding the detrimental and expensive consequences of post-surgical infection is the rising number of multidrug resistant (MDR) microorganisms present in the hospital environment. The increasing usage of antimicrobial agents in conjunction with invasive medical techniques has contributed to the rapid increase in MDR HAIs (Munita, *et al.,* 2016). These conditions can render traditional methods of treatment obsolete and contribute to problematic outbreaks, chiefly in hospital intensive care units where their capacity to remain viable despite stringent adherence to infection control measures demonstrates the pathogens' resilience to modern methods of treatment and disease eradication (Munita, *et al.,* 2016). Widespread use of broad-spectrum antibiotics is associated with the emergence of MDR strains of pathogenic bacteria now demonstrating a new and difficult-to-treat repertoire of virulence factors in these hospitalized patients that are predisposed to acquiring these infections (Munita, *et al.,* 2016).

2. Antibiotic resistance

Most *Aeromonas* strains are intrinsically resistant to penicillin and ampicillin; although susceptible to trimethoprim-sulfamethoxazole, fluoroquinolones, second and third generation cephalosporins, aminoglycosides, carbapenems, chloramphenicol, and tetracyclines (Janda, *et al.,* 2010). The antibiotic resistance characteristics of *Aeromonas* found in the environment are variable, and strains with increased resistance to antibiotics have been isolated in the waters where human sewage and agricultural runoff share pathways, culminating at wastewater treatment facilities (Igbinosa, *et al.,* 2012). In these waters, microbes share space and can exchange antibiotic resistance genes through natural processes of bacterial conjugation, transduction or transformation (Igbinosa, *et al.,* 2012). In a study identifying and characterizing conjugative and resistance plasmids isolated from Aeromonas species sampled from a wastewater treatment plant, 77% were found to contain plasmid DNA. Furthermore, the studies that followed revealed robust HGT activity and conjugative transfer of class 1 and 2 integrons and acquisition of transferred resistance (Moura, *et al*., 2012).

The levels of antibiotic resistance measured in organisms isolated from environmental water sources, especially those near hospital or clinic sewage release sites, are frequently increased when compared to laboratory standard strains (Khan, *et al*., 2013; Baquero, *et al*., 2011). Numerous studies have

exhibited the increase is consistent across various water sources in diverse areas of the world (Khan, *et al*., 2013; Baquero, *et al*., 2011). Similarly present in the majority of these water sources are stressors of variable origin, typically sub-inhibitory concentrations of antibiotics, heavy metals and biocides (Khan, *et al*., 2013). Studies have demonstrated that the presence of these stressors result in an increase in mutation rates of the organisms present in those conditions and/or environments (Andersson, *et al.,* 2014) and have even gone further to propose that the combinations of stressors can have cumulative or synergistic effects on mutation rates and subsequent increases in horizontal gene transfer (Gullberg, *et al*., 2011). In *E. coli,* this increase in mutation rates has been traced to the bacterial SOS pathway, and it's activation by these stressors (Kohanski, *et al.,* 2010; Mo, *et al*., 2016). These characteristics and the potential in *Aeromonas* species to behave similarly has not been elucidated.

Within wastewater ecosystems some of the existing health risks arising from continual exposure to a plethora of stressors (e.g., antibiotics, heavy metals, biocides) at low levels can be induction of bacterial mutagenesis leading to novel ARGs, multi-drug resistance, modified virulence factors or modification of their expression. Considering *Aeromonas* populations are ubiquitous in wastewater and natural aquatic environments, they present a health risk as a potential reservoir of antibiotic resistance genes in aquatic ecosystems. However, the effect of these stressors on the *Aeromonas* populations is ill defined.

II: Bacterial SOS Response and Mutation

1. Introduction

The bacterial SOS system is engaged in response to several varieties of DNA damage experienced by the cell in sub-prime conditions including UV light, antibiotics, reactive oxygen species and any type of nutrient stress (Miller, *et al.,* 1990). UV light and antibiotic stress particularly induce mutagenic and cytotoxic DNA lesions which in turn cause cyclobutene-pyrimidine dimers and genome integrity-related DNA strand breaks (Miller, *et al.,* 1990). In response to this damage, an emergency system in the form of a group of genes commences to induce cell cycle arrest, mutagenesis induction and DNA repair (Miller, *et al.,* 1990).

2. *recA*

RecA is a key protein involved in the SOS response, and senses DNA damage at stalled replication forks. The ssDNA collecting at these forks binds in tight clusters with RecA and form a nucleoprotein filament, and this complex then acquires an active conformation (Miller, *et al.,* 1990). The active form induces the cleavage of LexA, a dimer that acts as the repressor of about 57 SOS genes. Once cleaved, the mechanisms of repair can begin. One of these mechanisms is the recruitment of DNA polymerase V. The cell's normal high-fidelity DNA polymerase, Pol III, is unable to replicate past the DNA lesions caused at the junctions of these collected nucleoprotein filaments, and DNA pol V has a loose active site which can accommodate these areas in exchange for decreased

accuracy, as the selectivity toward normal bases is lost (Miller, *et al.,* 1990). The result of this DNA translesion synthesis is low sequence fidelity and increased mutagenic properties. This mutagenesis can not only increase antibiotic resistance but can also directly trigger the expression of integrases responsible for the transfer of antibiotic resistance genes (ARGs) (Baharoglu, *et al*., 2010).

The *recA* sequence and activity of *Escherichia coli* have been studied thoroughly, including studies characterizing mutagenesis activity in the presence of sublethal concentrations of antibiotics. These studies conclude that the SOS response in *E. coli* promotes horizontal dissemination of antibiotic resistance genes and promotes mutagenesis (Mo, *et al*., 2016). One review thoroughly analyzed sequence homology of *E. coli* with *Pseudomonas aeruginosa* (Sano, *et al.*, 1987). These findings stated shared homology except for the carboxy-terminal region. This region is of high interest, and one study performed mutation assays using numerous mutants with different residues of the C-terminal region knocked out and discovered an antibiotic sensitivity increase with deletion of 13-17 C-terminal residues (Lusetti, *et al.,* 2002). Even though *Pseudomonas aeruginosa*, a common nosocomial pathogen, shares 86% sequence homology of *recA* with *E. coli*, it has a recombination frequency that is 6.5 times higher (Bakhlanova, *et al.,* 2001). The *Pseudomonas* and *E. coli recA* study can serve as a guide to predict how *recA* of *Aeromonas* species influence mutagenesis potentially resulting in acquisition of antibiotic resistance. Multiple nucleotide sequences, specifically in the Mg^{2+} binding sites in the C-terminal domain, differ between

Aeromonas species and *Pseudomonas* species as well as *Aeromonas* species and *E. coli,* and even within *Aeromonas* species.

The expression of *recA* has been studied in many other species. One study involving the characterization of the SOS response in *S. aureus* found a 13-fold increase in *recA* expression when exposed to a subinhibitory concentration of ciprofloxacin (Schröder, *et al*., 2013). This real-time PCR result supported the hypothesis that ciprofloxacin stress could induce the SOS pathway in this species. The team additionally tested the expression of *recA* when incubated with novobiocin, which has been purported to antagonize induction of the SOS response. Likewise, the real-time PCR helped support this claim, as the expression level of *recA* with incubation with novobiocin resulted in a 17-fold reduction in expression, commensurate with the supposition about the action of that antimicrobial (Schröder, *et al*., 2013). In 2008, one study using *Mycobacterium tuberculosis* similarly found a 4-fold increase in *recA* expression after 12-hours of exposure to just one quarter of that species MIC of ciprofloxacin through real-time PCR analysis (O'Sullivan, *et al.,* 2008). Taken together, these results indicate ciprofloxacin as sufficient inducer of the bacterial SOS response in a conserved manner, and that *recA* expression increases as a result.

Very little is known about *recA* in *Aeromonas* species. The gene was first characterized in 1988 through a cloning experiment to deactivate the *recA* gene of *Aeromonas caviae* to initially characterize it and compare its function against

other more well-known *recA*s (Resnick, *et al*., 1988). The results demonstrated, among other things, a restored rate of recombination post UV-irradiation and exposure to methyl methansulfonate (MMS, a DNA-damaging compound) when *recA* isolated from *A. caviae* was used to complement *E. coli* as compared to complementation with *recA* from other species (Resnick, *et al*., 1988). In other species where the SOS pathway is well-characterized, researchers have begun looking at targeting these proteins, including RecA, to prevent the mutagenesis arising from DNA repair (Miller, *et al*., 1990).

3. Bacterial mutation in wastewater

Wastewater and wastewater treatment plants (WWTPs) act as reservoirs and environmental contributors of antibiotic resistance and serve as hotspots for horizontal gene transfer (HGT), thus facilitating the spread of antibiotic resistance genes (ARGs) intra- and interspecies (Karkman, *et al*., 2018). Sewage and agricultural runoff can also contain sub-inhibitory concentrations of antibiotics and select for resistant phenotypes. The method by which antibiotics enter wastewater is through incomplete metabolism or improper disposal, and most of the conventional WWTOs are not designed to remove these drugs, resulting in the presence of multiple antibiotics in WWTP systems, including wastewater effluents (Kulkarni, *et al*., 2017). The high densities of bacteria in WWTPs provides an environment ideal for HGT among environmental bacteria and human pathogens (Lerminiaux, *et al*., 2018). The location of ARGs on mobile elements such as plasmids, transposons and integrons makes the transfer of

resistance possible and easy to achieve among bacteria, even of different origins (Lerminiaux, *et al*., 2018). Sensitive bacteria thus can become resistant by acquiring resistance genes via transformation, transduction or conjugation, and selective pressure is mediated by elements present in wastewater such as antibiotics, heavy metals and biocides as stressors (Karkman, *et al*., 2018).

Numerous antibiotics present in wastewater behave as these stressors, and many are those known to elicit DNA damage with subsequent induction of the bacterial SOS response. The quinolone class of antibiotics inhibit the ligase domain of topoisomerase by causing a conformational change in the enzyme after binding. The DNA breaks in response to this change, and re-ligation is prevented by the complex formed from the quinolone-enzyme binding (Hawkey, 2003). The stalled replication is then sensed due to the build-up of single stranded DNA nucleoprotein filaments, which initiates the activation of *recA*.

Aminoglycosides have also been shown to induce an SOS response in certain bacterial species (Shapiro, 2015). There is a production of reactive oxygen species (ROS) in response to aminoglycoside antibiotics, and the ROS such as hydroxyl radicals will cause DNA strand breaks leading to SOS pathway activation. Gentamycin, a common aminoglycoside, is found not only in pharmaceutical effluents, but it's presence in water systems has been suggested to extend to seawater at low levels (Tahrani, *et al.* 2015). The third-generation cephalosporin ceftazidime has also been shown to promote activation of the

bacterial SOS system in *E. coli*, through induction of mutagenic DNA polymerases involving the activation of RecA (Pérez-Capilla, *et al*., 2005). Ceftazidime is present in sewage, surface and drinking waters, but most prevalently in wastewaters originating from hospital sewage (Aubertheau, *et al.*, 2017). Other commonly used antibiotics with other various targets have the ability to end up in water systems, or wastewater treatment for the same reasons: unmetabolized products in hospital sewage. For example, tetracycline, an inhibitor of protein synthesis via various methods of ribosomal binding, cefotaxime, a beta-lactam which inhibits cell wall synthesis, and trimethoprim, an inhibitor of folic acid production, all can be considered to induce mutagenesis as well (Lewin, *et al*., 1991).

Aeromonas species isolated from WWTPs have been shown to be resistant to fluoroquinolones and possess multiple mechanisms to confer resistance to these populations, including mutations in *gyrA*, efflux pumps, and plasmid-mediated quinolone resistance (PMQR) genes (Igbinosa, *et al*., 2012). Ciprofloxacin, a commonly used fluoroquinolone, has been shown to be present in domestic wastewater at varying concentrations (Batt, *et al.,* 2007; Manaia, *et al.,* 2010). Recently, our lab has found evidence of increased resistance to nalidixic acid in wastewater populations of *Aeromonas* as compared to the recipient riverine populations, over the course of three years (Skwor, *et al.,* 2020). Nalidixic acid resistance can be gained in a cell through very few (sometimes as few as one)

base-pair changes and can be an indicator of the induction of survival pathways, and expression of the activator, *recA*.

III. Hypothesis and specific aims

Very little is known about RecA in *Aeromonas* species. The *recA* gene was first characterized in 1988 through a cloning experiment to deactivate the *recA* gene of *A. caviae* to initially characterize it and compare its function against other more well-known *recA*s (Resnick, *et al*., 1988). The results demonstrated, among other things, the very low sequence homology between *recA* of *A. caviae* to *E. coli* K-12, and a higher rate of recombination post UV-irradiation and other select DNA-damaging events when compared to various strains of *E. coli* (Resnick, *et al*., 1988). In other species where the SOS pathway is well-characterized, researchers have begun looking at targeting these proteins, including RecA, to prevent the mutagenesis arising from DNA repair (Miller, *et al*., 1990). In the face of increasing antibiotic resistance and the prevalence of *Aeromonas* as pathogens causing increasingly resistant infections, nosocomial or otherwise, the need to elucidate the impact of subinhibitory concentrations of antibiotics and genes associated with mutation rates is critical. There is data to suggest that wastewater isolates of *Aeromonas* are undergoing mutagenesis at a rate higher than their recipient river counterparts, which if supported here, could suggest that subinhibitory concentrations of antimicrobials present in those waters increases

mutagenesis which leads to enhanced resistance to antimicrobials. This study seeks to uncover if incubation in sub-inhibitory concentrations of antibiotics triggers overexpression of *recA* and additionally, if mutagenic activity of *Aeromonas hydrophila* is attenuated without a functional *recA* gene.

The hypothesis of this thesis is: recA *expression is associated with increased mutagenesis in* Aeromonas hydrophila *in the presence of sub-inhibitory concentrations of antibiotics, and deletion of* recA *will attenuate the mutation rate.*

To test this hypothesis, the following specific aims are pursued:

1. **Determine the effect of sub-inhibitory concentrations of ciprofloxacin on *recA* expression in *Aeromonas hydrophila.*** The working hypothesis of this aim is*: the incubation of A. hydrophila with sub-inhibitory concentrations of ciprofloxacin will increase the expression of recA.*

2. Determine the impact of RecA production on mutation rate of *Aeromonas hydrophila* exposed to sub-inhibitory concentrations of ciprofloxacin. The working hypothesis of this aim is: *mutation rate of A. hydrophila decreases without RecA production.*

Chapter 3: Material and Methods

I: Real-time Polymerase Chain Reaction

1. MIC determination

To determine the minimum inhibitory concentrations of antibiotics of all species and strains used in analysis, a minimum inhibitory concentration determination was performed using ciprofloxacin. Overnight cultures were grown in 5 mL LB, diluted to a 0.5 MacFarland standard in PBS, and further diluted 1:100 (5 mcL into 495 mcL) into Mueller-Hinton (MH) broth. A stock culture is prepared of antibiotics at 2x the starting highest concentration (1 mcg/mL). One hundred mcL of 1 mcg/mL antibiotic stock is added to row B of a 96-well plate, and 50 mcL of H_2O added to the rest of the wells in that row. The antibiotic was then serially diluted through the rows, creating serial half-dilutions for a wide antibiotic concentration range (0.5 mcg/mL- 0.00195 mcg/mL). Fifty mcL of the 1:100 bacterial suspension in MH is added to each associated well, and the plate is then incubated at 30° C overnight. The OD_{600} of the plate is measured on a BioTek Spectrophotometric Plate Reader and the MIC is the lowest concentration of antibiotics that yields no bacterial growth, as noted by wells with OD_{600} taken by the spectrophotometer equal to the negative control wells (no bacterial culture). This determination, used in the following assays, is hereafter described as the MIC.

2. Bacterial cultures with antibiotic stressor

To prepare cultures with different stress conditions for expression analysis, an antibiotic stress assay was performed. An overnight culture of *A. hydrophila* ATCC 7966 was grown at 35° C, then diluted 1:100 into 50 mL room temperature (RT) Luria-Bertani broth (LB). Shaken at 250 rpm, this culture was grown to an optical density $(OD)_{600}$ of 0.4-0.6 to assure mid-log growth. A 24-well plate was prepared with 8 wells containing 2 mL LB and 8 wells containing 2 mL LB supplemented with ciprofloxacin at a concentration of 0.5 the MIC of *A. hydrophila*. Ten mcL of the 0.4-0.6 OD_{600} culture was placed into each well. The plate was incubated at 30° C, shaken at 250 rpm, with samples being drawn from the control LB and the LB with ciprofloxacin at the starting time point (initial sample- 0 hour), 30 minutes, 60 minutes and 2 hours.

3. Pellet preparation with RNAProtect

A 500 mcL sample was taken at each time point (0 hour (initial sample), 30 minutes, 60 minutes, 2 hours) and added to 1000 mcL of RNAProtect (QIAGEN) and vortexed. After RT incubation for 5 minutes, the suspension was centrifuged and the supernatant decanted. The resulting pellet was dried and stored at -80° C for future use.

4. RNA extraction

RNA extraction was performed using RNeasy (QIAGEN) according to manufacturer's instructions. An on-column Dnase digestion was added to the

protocol using Dnase MiniKit (Promega) to assure degradation of any existing contaminating DNA. RNA quantity and purity were assessed using NanoDrop One UV-Vis Spectrophotometer (ThermoFisher Scientific).

5. cDNA synthesis

cDNA synthesis was performed using Promega GOScript Master Mix with reverse transcriptase and random oligonucleotide primers. Controls for synthesis include No Reverse Transcriptase (NRT), which omits the reverse transcriptase, and No Template Control (NTC) which omits the RNA template. The reaction mix was aliquoted into PCR reaction tubes and RNA template added to all except the NTC, and the following temperature and time parameters were undertaken using Peltier PTC-100 ThermalCycler: 25° C for 10 minutes, 42° C for 50 minutes, and then 70° C for 15 minutes for enzyme inactivation. cDNA concentration and quality were analyzed via spectrophotometry of NanoDrop One UV-Vis Spectrophotometer (ThermoFisher Scientific). Additional characterization of the quality of synthesis was undertaken via agarose gel electrophoresis. Further expression analysis was performed using real-time PCR with primer sets.

6. Preliminary assays

i. Standard curves

Standard curves were performed prior to real-time PCR assays to determine appropriate concentrations of template and primer. PCR master mixes were made for each gene (*recA*, *rpoB* and 16S) containing 337.5

mcL of 2X PowerUp SYBR Green Master Mix (ThermoFisher Scientific), 33.75 mcL of 10 mcM working stocks of each forward and reverse primer, 232 mcL of nuclease-free H_2O for a final primer concentration of 500 nM. cDNA was serially diluted 3-fold to create stocks of 100 ng/mcL- 0.13 ng/mcL. A 96-well plate was set up for triplicates of each primer and concentration, with triplicate controls of NRT and NTC for each primer. Each well contained 23 mcL of primer in triplicate and 2 mcL of each cDNA concentration. The real-time PCR was performed on an Applied Biosystems StepOne Plus Real-time PCR System (ThermoFisher Scientific) using the New StepOne Software for output analysis.

ii. Primer optimization

An optimization protocol for the *recA* primer was performed to optimize the concentration. Three master mixes were made for final primer concentrations of 50 nM, 300 nM and 900 nM. Each mix contained 287.5 mcL of 2x PowerUp SYBR Green Master Mix, 10 mcL of each forward and reverse *recA* primer (volume dependent on concentration) and nuclease-free H_2O (volume dependent on concentration). The mixes were added in triplicate to a 96-well plate to which serial dilutions of cDNA were added. The dilutions were 3-fold dilutions of a 100 ng/mcL solution to result in 33.3 ng/mcL – 0.41 ng/mcL added to each primer concentration in triplicate. Controls included triplicates of NRT and NTC. The real-time

PCR was performed on an Applied Biosystems StepOne Plus Real-time PCR System using the New StepOne Software for output analysis.

7. Real-time PCR

i. Real-time PCR of treated and untreated samples of A. hydrophila *with 16S, rpoB and recA primers.*

To assess the expression of *recA* in samples exposed to subinhibitory concentrations of ciprofloxacin, a real-time PCR was performed. Master mixes for *recA* and two housekeeping genes were prepared as follows: *rpoB* and 16S, 275 mcL of 2X PowerUp SYBR Green Master Mix, 27.5 mcL of 10uM forward and reverse primers, 176 mcL nuclease-free H2O. For *recA*, 275 mcL of 2X PowerUp SYBR Green master mix, 16.5 mcL of 10uM forward and reverse primers and 261 mcL nuclease-free H2O. Each primer mix was aliquot to 21 wells of a 96-well qPCR reaction plate. To each respective well, synthesized cDNA was added in triplicate, of the following: zero-hour (initial) samples with and without exposure to ciprofloxacin, one-hour samples with and without exposure to ciprofloxacin, and two-hour samples with and without exposure to ciprofloxacin. Control included 9 wells of NTC and NRT. qPCR was performed on Applied Biosystems OneStepPlus Real-time PCR system (ThermoFischer Scientific) using New StepOne Software for output analysis.

ii. Calculation of relative fold gene expression

For calculation of relative fold gene expression, the delta delta Ct method was used. This calculation is presented as $2^{-\Delta\Delta Ct}$. Ct, the cycle threshold of the sample, is the cycle number where the fluorescence provided by the binding of SYBR green to double-stranded DNA is distinguishable from background noise of the sample. From the output of Applied BioSystems StepOnePlus, the Ct values for all replicates (triplicates of each time point, treated and untreated, for each gene) was averaged. The first ΔCt was then calculated using the averages, the difference in the Ct of the gene of interest and each housekeeping gene, to normalize the gene of interest to a housekeeping gene reported to not be affected by the experiment. The treated samples additionally needed to be compared in Ct values to the untreated samples at zero time point to remove the baseline expression levels as well. Here the difference in Ct values was determined for each average Ct. Then the ΔΔCt was determined as the ΔCt (*recA* minus housekeeping gene) - ΔCt (change compared to zero hour). Finally, to determine the fold gene expression, 2 to the power of negative ΔΔCt was calculated. The resulting values were charted in Microsoft Excel software.

II. SOE-PCR protocol for generation of AHΔ*recA* mutant:

The mutant strain was produced following adapted protocols from Heckman, *et al.,* 2007 and from protocols gifted from Dr. Sonia Bardy (Department of Biological Sciences, University of Wisconsin-Milwaukee).

1. Primer design

The gene of interest was downloaded from NCBI, and the initial sequences were selected flanking this section, with triplets of nucleotides kept intact to retain the integrity of the amino acids within that area. Using NCBI PrimerBuilder (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) complementary sections upstream and downstream were selected for low self-complementarity, similar GC percentage, and similar Tms. Primers A and D were given restriction endonuclease sites, Bam-HI-HF and Sac-I-HF, respectively on their 5' ends, with a 10 bp GC tag preceding the site to maximize enzyme binding efficiency. Primers B and C are given the reverse complement of each other on their 5' ends. Primers were resuspended to 100 nM, and working stocks made at 10 uM concentration using 10 mcL of each 100 nm primer and 90 mcL of nuclease-free water.

2. DNA Extraction

Overnight cultures of *A. hydrophila* ATCC 7966 were grown in LB from existing laboratory frozen stocks. DNA was extracted using Wizard Genomic DNA Purification Kit (Promega) according to manufacturer's instructions. DNA quantity

and purity were assessed using NanoDrop One UV-Vis Spectrophotometer and stored at -20° C.

3. PCR 1

The first PCR performed is divided into two separate reactions to generate template DNA for the subsequent PCR. One reaction used Primers A & B to generate a ~1Kb segment upstream of *recA* and the second used Primers C & D to generate a ~1Kb segment downstream of *recA* both with restriction enzyme sites on the outside ends and complementary overhangs in the middle. The volumes for PCR were 15 mcL 2X Master Mix (Promega), 0.5 mcL each of primer A and B (or C and D in the second), and 1 mcL of *A. hydrophila* DNA. A negative control contained 1 mcL of nuclease-free H_2O in the place of DNA. These reactions were placed in Peltier PTC-100 thermocycler (MJ Research) with the following conditions: 5 minutes at 95° C, 35 cycles at 94° C for 30 seconds, 55° C for 30 seconds and 72° C for 2 minutes, followed by a final 7 minutes at 72° C. The resulting fragments were verified via 1% agarose gel electrophoresis and purified using PCR Clean-Up Kit (QIAGEN) according to manufacturer's instructions.

4. PCR 2

The second PCR uses DNA from PCR 1 as template. The same conditions are followed, except only primers A & D are used to generate a 2K bp final product "AD" which will only contain DNA upstream and downstream of *recA*, as the

complementary overhangs have brought the two 1K bp segments together. The size of product “AD” is verified by 1% agarose gel electrophoresis.

5. Gel Extraction

To isolate the 2K bp band from PCR 2, an agarose gel extraction procedure is utilized. Using a UV lamp and razor blade, the 2K bp band is selected, excised and placed into a clean 1.5 mL Eppendorf tube, and Agarose Gel Purification Kit (QIAGEN) was used according to manufacturer’s instructions. The quantities and purities of the extractions were verified by NanoDrop One UV-Vis Spectrophotometer and stored at -20° C.

6. Plasmid MidiPrep

Escherichia coli DH5α containing the plasmid pEX18Tc (gift of Dr. Sonia Bardy, Department of Biological Sciences, University of Wisconsin- Milwaukee) were maintained in LB supplemented with 10 mcg/mL tetracycline. To obtain enough pEX18Tc for all future experiments, a plasmid midiprep protocol was used according to manufacturer’s instructions (QIAGEN). The quantities and purities of the extraction were verified by NanoDrop One UV-Vis Spectrophotometer and stored at -20° C.

7. Restriction Enzyme Digest

To create complementary overhangs of both fragment AD and plasmid pEX18Tc, a restriction enzyme digest is performed. Various DNA concentrations and

digestion times were tested for optimization of efficiency. A double restriction enzyme digest was performed simultaneously on pEX18Tc and fragment AD. One mcL each of restriction enzymes Bam-HI-HF (New England BioLabs) and Sac-I-HF (New England BioLabs) was added to 5 mcL of NEB 10X CutSmart Buffer (New England BioLabs), 100 ng of DNA, and brought to 50 mcL with nuclease-free H_2O. The digest incubated overnight at 37°C and was heat-inactivated by incubating in a 65°C water bath for 20 minutes. Successful digestion of linearized plasmid of pEX18Tc was confirmed via 1% agarose gel electrophoresis.

8. Ligation

To ligate the digested ends of pEX18Tc and fragment AD, appropriate ratios of insert: vector DNA volumes were calculated using NEBiocalculator web tool (https://nebiocalculator.neb.com/#!/ligation). Volumes of each vector (pEX18Tc) and insert (fragment AD) were added in a 20 mcL reaction with 1 mcL of T4 Ligase (New England BioLabs) and 2 mcL of 10X T4 Ligase Buffer (New England BioLabs), and brought to 20 mcL with nuclease-free water. The ligation incubated at room temperature for 20 minutes and then was heat-inactivated at 65°C for ten minutes. The resulting ligation reaction was purified via QIAquick PCR Clean-Up Kit (QIAGEN) according to manufacturer's instructions. Controls included one reaction with digested pEX18Tc without AD insert.

9. Competent Cell Creation

To make *E. coli* S17-1 cells (gift of Dr. Sonia Bardy, Department of Biological Sciences, University of Wisconsin- Milwaukee) competent for transformation, a competent cell creation protocol was followed. Overnight cultures of *E. coli* S17-1 grown in 100 mcg/mL streptomycin were diluted 1:100 into 100 mL LB and grown to OD_{600} of 0.4-0.6. Fifty mL of culture is incubated on ice for 20 minutes and centrifuged to pellet. Each pellet was resuspended in ice cold 100 mM $CaCl_2$ and incubated on ice again for 60 minutes. After a second centrifugation, the resulting pellet was suspended in 2 mL 85 mM $CaCl_2$ and 15% glycerol, aliquot into cryovials, and stored at -80° C until used.

10. Transformation

To transform competent *E. coli* S17-1 with pEX18Tc::AD, a heat-shock transformation protocol was followed. Competent cells were thawed on ice and divided into 50 mcL aliquots in sterile 1.5 mL Eppendorf tubes. One mcL of thawed pEX18Tc::AD was added to each and incubated on ice for 30 minutes. The tubes were incubated at 42° C for exactly 30 seconds and returned to ice for an additional 5 minutes. Room temperature LB (500 mcL) was added and mixed via pipette. The tubes were incubated at 37° C while shaking at 250 rpm for 60 minutes. Serial dilutions were plated on TSA with 10 mcL/mL tetracycline and incubated overnight at 35° C. IPTG and X-Gal were included in the media (25 mg/mL X-Gal, 100 mM IPTG) to assist with blue and white screening for successful transformants. Screening was performed using restriction enzyme

digest and gel electrophoresis following a plasmid miniprep, and an additional colony PCR.

11. Conjugation

To mate *E. coli* S17-1 pEX18Tc::AD with wild type *A. hydrophila* ATCC 7966, a conjugation protocol was followed. Overnight cultures of donor and recipient cells were grown in 3 mL LB with appropriate antibiotics (10 mcg/mL tetracycline for *E. coli* with pEX18Tc::AD) at 37° C. The cultures were diluted 1:100 into fresh media with appropriate antibiotics and shaken at 37°C until OD_{600} readings reveal late-log stage. Ratios of 2:1 and 4:1 donor: recipient cultures were added to Eppendorf tubes, and centrifuged. The pellet was resuspended in 100 mcL LB without antibiotics. Sterile 0.45 um nitrocellulose filters were placed on TSA and 100 mcL of donor and recipient suspension were pipetted onto the same spot on the filter. The agar was incubated filter-side up at 37° C overnight. After incubation, the filters were placed into a sterile tube containing 1.5 mL sterile PBS and vortexed to remove bacterial cells from the filter. The resulting suspension was serially diluted and plated on selective agar (ADA-VI with 10 mcg/mL tetracycline).

12. Counter-Selection

Any colonies appearing on selective agar, phenotypically positive for *Aeromonas* with plasmid-induced resistance to tetracycline, were incubated overnight in 5 mL LB with 10 mcg/mL tetracycline to facilitate homologous recombination and

integration of the plasmid with fragment AD into the *A. hydrophila* genome. The next day, the cultures were passed to 5 mL LB without tetracycline to encourage the exit of pEX18Tc which then contained the working copy of the *recA* gene. Serial dilutions of each culture were plated on TSA with 10% sucrose, which inhibited the growth of *A. hydrophila* still containing the plasmid, as sucrose acts as a counterselection agent due to a *sacB* gene encoded on the plasmid which when translated in the presence of sucrose activates to produce a toxic metabolite. Colonies which grew on 10% sucrose were then patched onto both TSA with 10 mcg/mL tetracycline and TSA with 10% sucrose. Colonies that grow on the sucrose plates and not the tetracycline will be wild-type or successful deletion. These colonies were selected for screening via colony PCR (cPCR: touch of colony with sterile pipette tip transferred into PCR reaction tube with Master Mix) with original forward and reverse primers (A and D) for confirmation. Successful deletions will amplify a band matching the pEX18Tc::AD control, and wild-type will amplify a band matching control DNA from wild type *A. hydrophila*.

III. Mutation Analysis

1. MIC determination

To determine the minimum inhibitory concentrations of antibiotics for the mutation assay following, a minimum inhibitory concentration determination was performed on *A. hydrophila* and *A. hydrophila* Δ*recA* as described above, using

nalidixic acid at a range of concentrations from 16 mcg/mL-0.5 mcg/mL) and ciprofloxacin at a range of concentrations from (0.5 mcg/mL- 0.00195 mcg/mL).

2. Mutation Assay

The mutation frequency analysis was adapted from Krašovec, *et al*., 2019 . Overnight cultures were grown in 5 mL LB at 30° C, shaking at 250 rpm. Culture conditions for each individual treatment group were created in sterile 15 mL conical tubes containing 10 mL LB with appropriate concentrations of ciprofloxacin at half the MIC (0.0039 mcg/mL). Overnight cultures are diluted twice, once at 1:100 in LB and then again at 1:200 into each antibiotic culture condition, for a final dilution of 1:20,000. A grid of 19 randomly selected wells per treatment group was created for a 96-deep-well plate, and 500 mcL of the corresponding treatment group was pipetted into each corresponding well. The deep well plate was covered and incubated for 24-26 hours at 30° C with shaking at 250 rpm.

Select cultures representing each treatment group was measured for optical density to ensure similar stationary stage. Three of the 19 wells of each group were serially diluted ten-fold, and 50 mcL of the resulting 10^{-7} and 10^{-8} suspensions were plated on TSA to calculate CFUs. The remaining 16 wells of each group were poured in their entirety on TSA containing nalidixic acid at four times their MIC and allowed to dry on the media. All plates were incubated at 30° C.

At 24 hours, the resulting colonies on the TSA plates were counted to calculate CFUs per group. At 48 hours the colonies on nalidixic acid TSA were counted to quantify mutants per well per treatment group. The resulting mutant number was divided by the CFU per 500 mcL of the same treatment group to calculate the mutation rate. Each CFU count was an average of triplicate wells.

Statistical analysis included an one-way ANOVA with values of $p < 0.05$ considered significant.

Chapter 3: Results

I. Specific Aim I:

1. Preliminary Assays

i. Amplification parameters determined by standard curve analyses

The primers selected for real-time PCR were referenced from at least two separate manuscripts each, to increase likelihood that their efficiencies were tested (Table 1). However, not all protocols include this step, so it was performed prior to any data collection to ensure that gene expression data collected was reliable, and outcomes were not due to different primer binding strengths or amplification rates per template. Additionally, two housekeeping genes were selected, as housekeeping genes for prokaryotic cells can have additional variability in amplification with greater irregularities of expression rates instead of steady constituent production compared to eukaryotic standards like GADPH. It is recommended that two (or more) be selected and both be used in the real-time PCR assay to procure a more reliable quantification of relative expression (Fey, *et al*., 2004; Rivera, *et al*., 2015). Primers are listed in Table 1.

Name	Sequence
16S-F 16S-R$_{(1,2)}$	5'- GCCTAACACATGCAAGTCGAG 5' - GCGGTATTAGCAGTCGTTTCC
rpoB-F *rpoB*-R $_{(3,4)}$	5' - GGATCACGGTGCCTACAT 5' - TAACGCTCGGAAGAGAAGA
recA-F *recA*-R $_{(5,6)}$	5' - CGACCCCATCTATGCCGC 5' - CCATCTCACCTTCGATTTCCG

Table 1. Primers used in Real-time PCR. Primers taken from the following publications 1. Vilches, *et al*., 2009 2. Griffin, *et al*., 2013 3. Teng, *et al*., 2018 4. Navarro, *et al*., 2018 5. Liu, *et al*., 2019 6. Dong, *et al*., 2018.

Standard curves were performed with three-fold serial dilutions of template (100 ng- 0.1ng). The log of template concentration plotted versus the average critical threshold value should have an equation with an R^2 very close to 1, according to the qPCR Assay Design Guide supplied by the manufacturer (Applied Biosystems). The R^2, or linearity of the resulting equation, demonstrates how the amplification changes based on the different starting template copy numbers. Being close to 1 numerically establishes the near-linear progression of amplification per template. The R^2 for *recA,* 16S and *rpoB* was 0.9996, 0.9994 and 0.9991 respectively. Additionally, the efficiency for each run should be assessed. This is done by first determining PCR efficiency (E) by calculating $E = 10^{(-1/\text{-slope})}$. Next the efficiency percentage is determined by subtracting 1 from E, and multiplying by 100% ((E-1) x 100%). The efficiency should be in the range of 90-110%, again according to the manufacturer. The efficiencies for *recA*, 16S and *rpoB* are 71.59, 105 and 110% respectively.

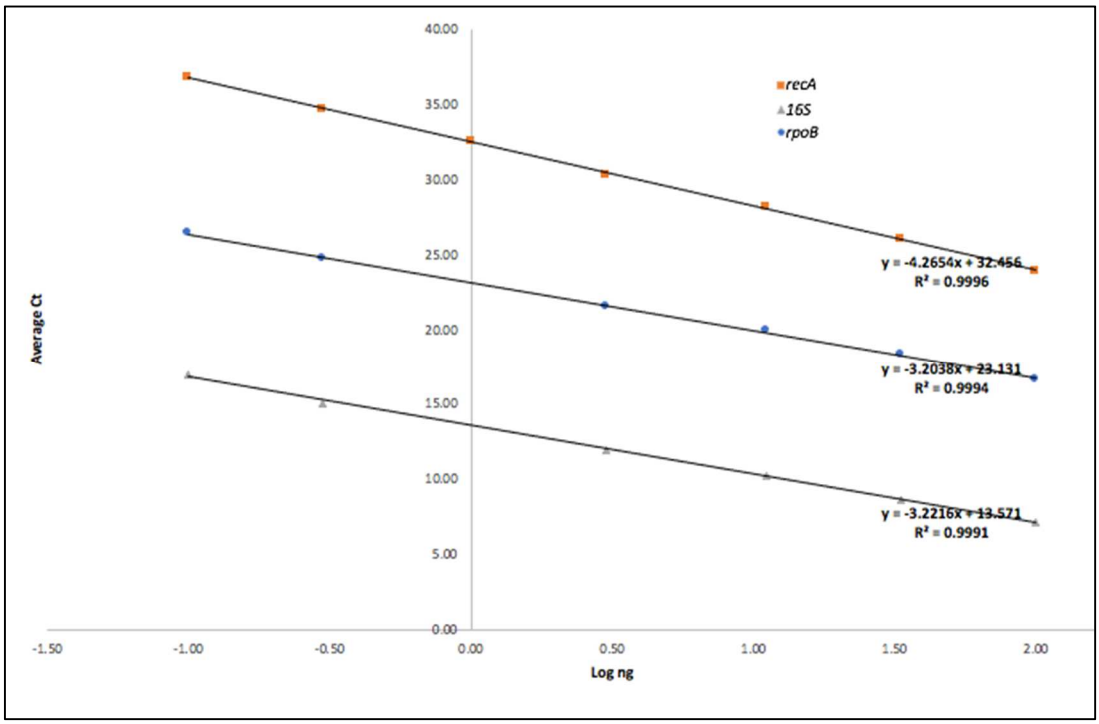

Figure 1. Plot of standard curves for each primer show appropriate R^2 value. Negative natural log of each concentration vs. average Ct. Samples run in triplicate.

ii. Determining relative primer efficiency

The initial relative primer efficiency standards demonstrated a problem, likely inherent in the design of the primer for *recA.* In testing relative primer efficiency, the differences in Ct values per template concentration are calculated and plotted against the negative natural log of template concentration to ensure that as the value of the Ct of one primer decreases, the value of the Ct of the second is decreasing at relatively the same rate. In the resulting graph from the relative efficiency of the two housekeeping genes, *rpoB* and 16S, these data show an acceptable

relative efficiency as determined by having a slope equal to or less than 0.03 (m= 0.0178) (Figure 2). In both of these primer sets tested with *recA* however, the resulting slope is very low (Figures 3, 4). In real-time PCR where the data is being generated from a comparative value, it is imperative that there are no variables in amplification which would give unreliable Ct values. Therefore, the relative primer efficiency taken with the overall efficiency of the *recA* alone (see above) indicated that the *recA* primer set needed further optimization before continuing.

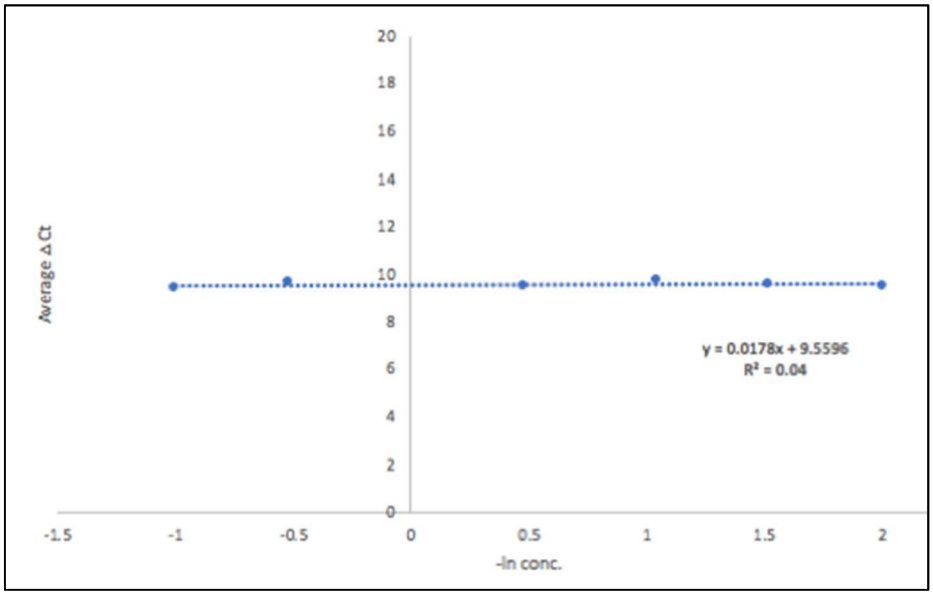

Figure 2. Plot of relative efficiency of 16S and *rpoB* primer pairs. Plot shows average difference in Ct value against negative natural log of template concentration. Samples ran in triplicate.

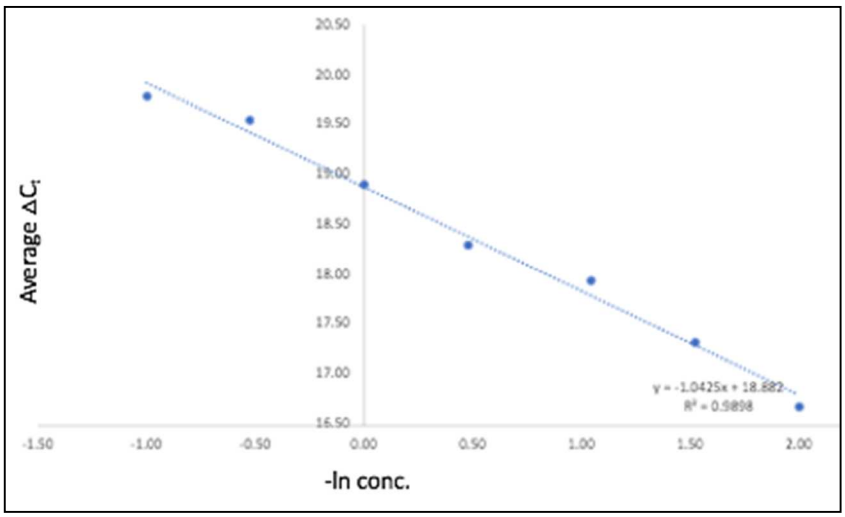

Figure 3. Plot of relative efficiency of *recA* primer and 16S primer. Plot shows average difference in Ct value against negative natural log of template concentration. Samples ran in triplicate.

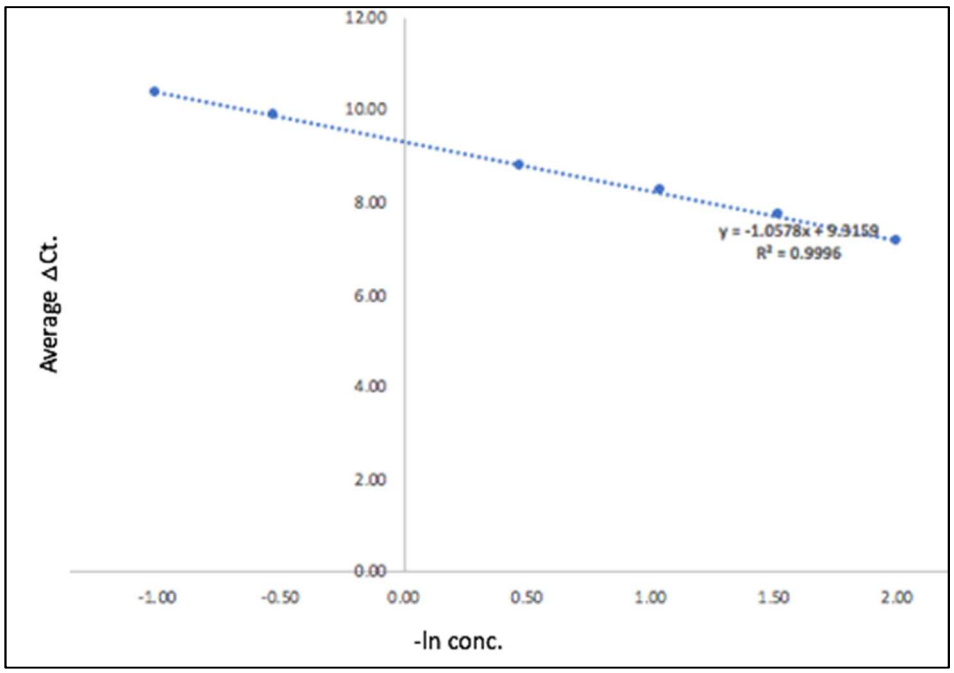

Figure 4. Plot of relative efficiency of *recA* and *rpoB* primer pairs. Plot shows average difference in Ct value against negative natural log of template concentration. Samples ran in triplicate.

iii. Determination of recA *primer concentration via primer optimization*

Due to the results of the overall primer optimization assay, a second optimization directed at the recA primer alone was performed. Different concentrations (50 nm, 300 nm, and 900 nm) of the primers were tested with a three-fold serial dilution of cDNA to determine the concentration with the greatest reproducibility and a slope similar to that of the two housekeeping gene primer sets. The log of each concentration was plotted against each average Ct value, and the resulting graph is displayed (Figure 5). The 300 and 900 nm primer equation best fits and 900 nm *recA* primers are used moving forward with the assay for real-time PCR (Figure 6).

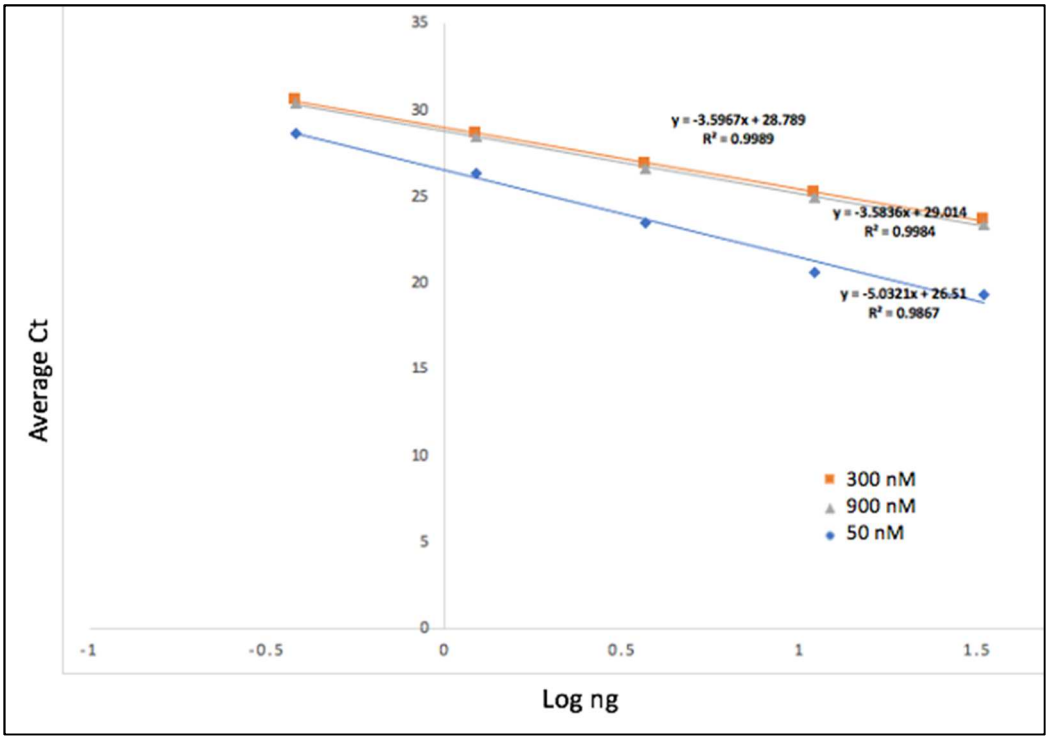

Figure 5. Standard curves for *recA* primer optimization. Plot shows concentration of the log of cDNA (ng/mcL) concentration vs. average Ct for each concentration of primer. R^2 values for all within acceptable limits, samples ran in triplicate.

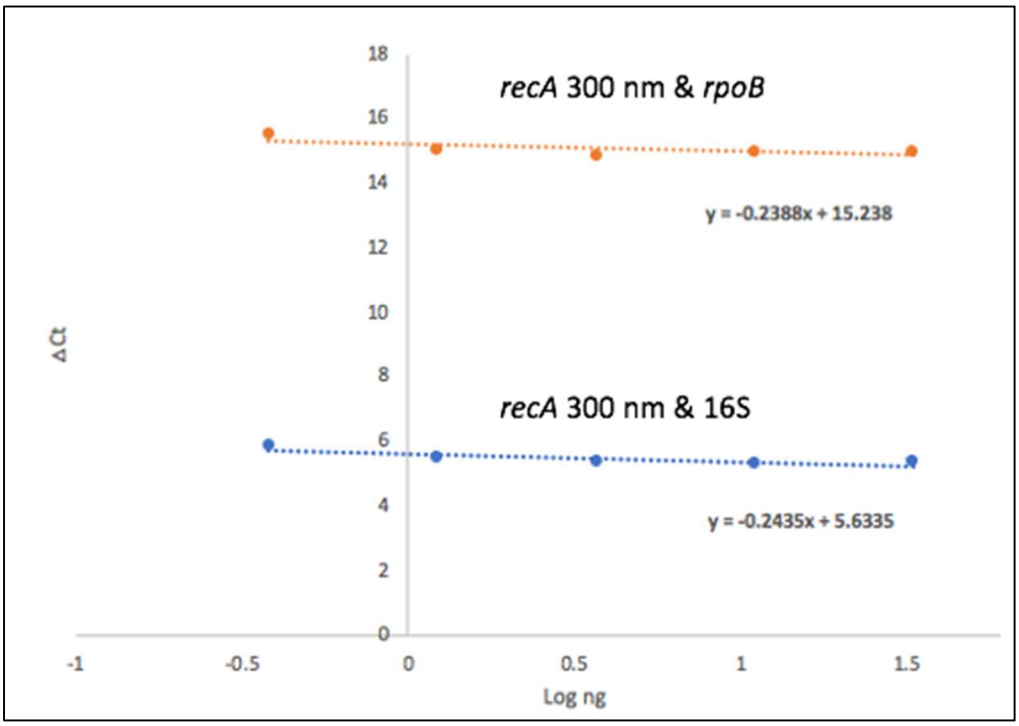

Figure 6. Plot of relative efficiency of *recA* primer (300nm) and *rpoB* primers (top) and 16S primers (bottom). Plot shows average difference in Ct value against negative natural log of template concentration. Samples ran in triplicate.

2. *RecA* expression in *A. hydrophila* under stress by subinhibitory concentrations of ciprofloxacin

The expression of *recA* in response to incubation with subinhibitory concentrations of ciprofloxacin (0.5x MIC, 0.0039 mcg/mL) was determined using real-time PCR. The one hour and two-hour samples Ct results were both normalized to the zero-hour sample used as baseline expression level. Then the difference in Ct values for each sample were determined, and the results are the averages of triplicates. There was no measurable expression in either the NTC or the NRT. All relative fold expression changes are expressed as a result of the ΔΔCt method of evaluation.

Using the *rpoB* as housekeeping gene with the samples normalized to the baseline expression, revealed a 6.188-fold increase in *recA* expression in *A. hydrophila* exposed to 0.5x MIC ciprofloxacin for one hour. This expression increase was continued in the two-hour samples, showing an 8.114-fold increase in *recA* expression under antibiotic stress. These increases stand out in comparison to the samples of *A. hydrophila* not exposed to ciprofloxacin stress, as those demonstrated only a slight increase in expression for both the one hour and the two-hour samples, at 1.237- and 2.803-fold increases, respectively (Figure 7).

Similarly, using the 16S as housekeeping demonstrated expression increases in *recA* as well. When normalized to the zero hour as baseline, the one-hour sample showed a 7.81-fold increase in *recA* expression when incubated with 0.5x subinhibitory concentrations of ciprofloxacin. A 4.94-fold increase was seen at the two-hour time point samples (Figure 7). The one-hour sample without the stressor increased only slightly compared to baseline levels, only a 1.576-fold increase, and then decreased at the two-hour timepoint, with a 1.219-fold increase.

Overall, these data do demonstrate an increase in expression level of *recA* in response to 0.5x subinhibitory concentrations of ciprofloxacin.

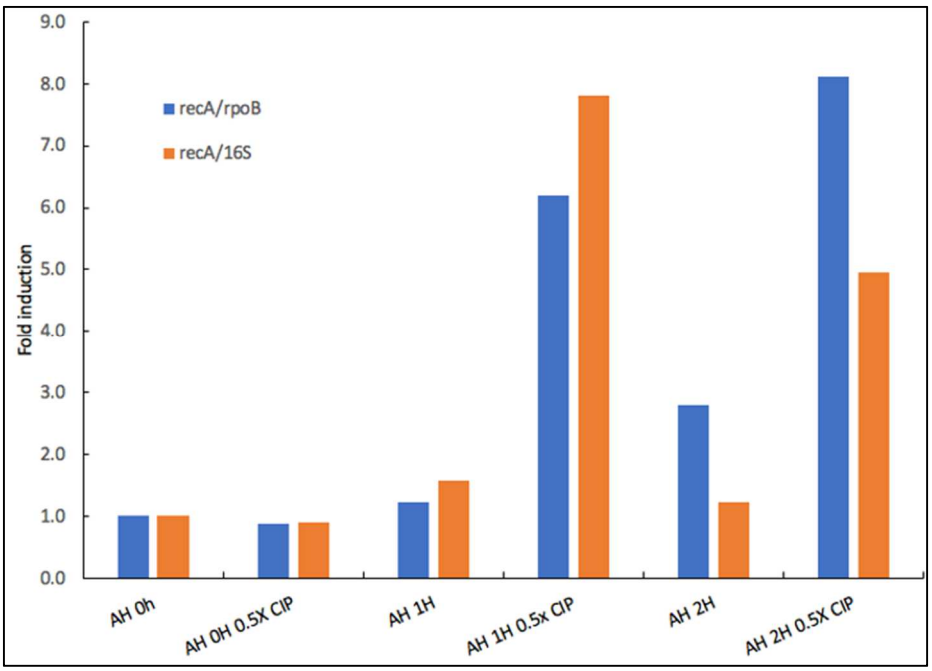

Figure 7. *recA* expression in *A. hydrophila* with and without exposure to subinhibitory concentrations of ciprofloxacin at 0h, 1 hour and 2 hours. Relative fold induction of r*ecA* as compared to the housekeeping gene *rpoB* (blue) or 16S (orange). Results represent average of triplicate samples.

II. Specific Aim II:

1. Deletion of *recA* from *Aeromonas hydrophila* ATCC 7966

i. Amplification of up- and downstream portions of recA *created AD fragment*

The initial genomic DNA extraction of *A. hydrophila* yielded 1427 ng/mcL DNA with OD_{600} ratios in range acceptable for purity standards (1.8-2.0). This DNA was the template for two separate PCR reactions to amplify regions of DNA sequences upstream and downstream of *recA* while generating complementary overhangs in the resulting fragments called "AB" and "CD." Primers A and B were used in one reaction and primers C

and D (Table 2) were used in a separate reaction to be run concurrently. The agarose gel ran of these two reactions showed two distinct bands of 1000 bp when compared to the DNA ladder (Figure 8). These PCR reactions were purified using QIAGEN PCR Clean-Up kit and analyzed via NanoDrop One UV-Vis Spectrophotometer (ThermoFisher Scientific) to calculate volume needed for second PCR reaction.

Name	Location	Sequence
A	Upstream Forward	5'- gtagcGGATCCGCGAATCTCTGACGTGGA
B	Upstream Reverse	5'- *AAACAGAAGGCACTGGCG*GCCGAAAGCGAACAAGAG
C	Downstream Forward	5'- CTCTTGTTCGCTTTCGGC*CGCCAGTGCCTTCTGTTT*
D	Downstream Reverse	5'- cgccgGAGCTCTGGCGTGATAGGGAACGC

Table 2. Primers used in initial PCR. Lowercase characters are GC tag. Underlined characters are restriction enzyme digest sequences. Italicized characters are reverse complement of each other to generate complementary overhangs.

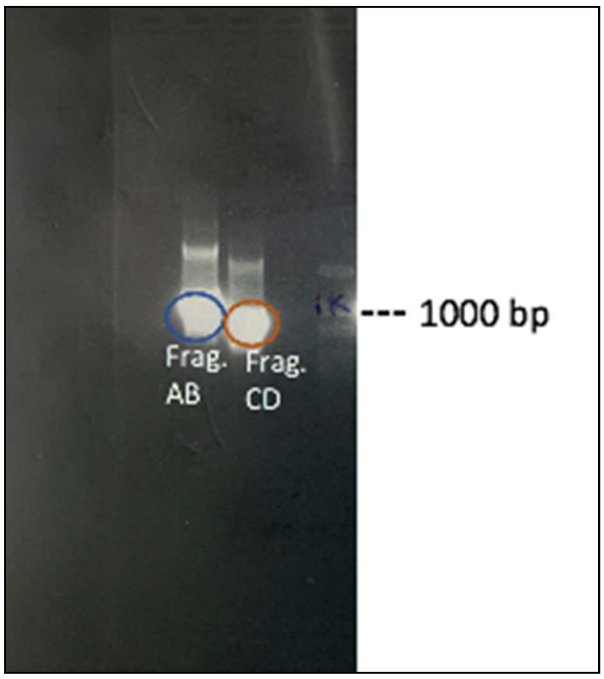

Figure 8. Agarose gel of PCR I. Extracted *A. hydrophila* DNA with primers A and B and extracted *A. hydrophila* DNA with primers C and D, ran on 1% agarose gel.

The second PCR reaction uses the amplicons from previous PCR as template, with primers A and D, to anneal the resulting fragments into product “AD”, at a length of approximately 2000 base pairs. The agarose gel showed an “unclean” band of the correct size (which was alluded to in the protocol supplied by Heckman, *et al.,* 2007) therefore the band was excised and purified. QIAGEN Agarose Gel Excision kit was used to purify the 2000 bp band (Figure 9) and the result was 30.1 ng/mcL and within correct purity ratios for use.

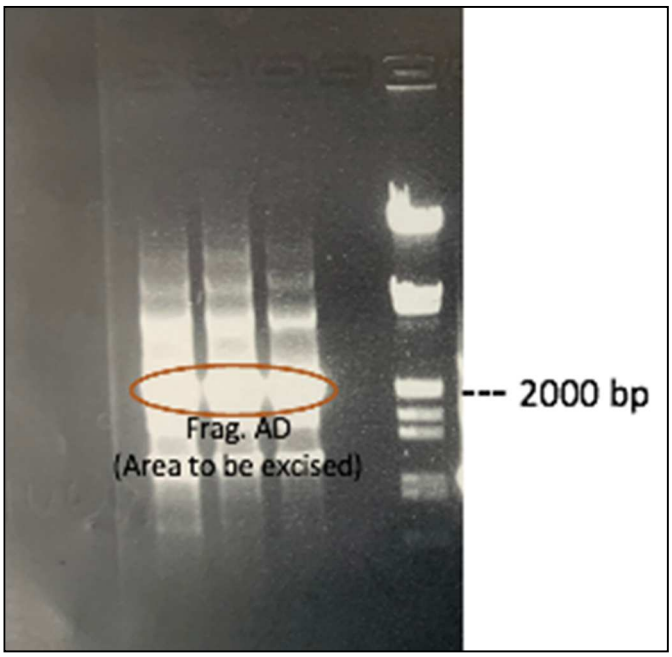

Figure 9. Agarose gel with PCR of fragment AB and fragment CD with primers A and D. Template fragments AB and CD are amplified with primers A & D and ran in triplicate on 1% agarose gel. The resulting 2Kbp bands were excised for clean-up.

ii. *Ligating fragment AD into pEX18Tc plasmid*

Escherichia coli DH5-α cells containing plasmid pEX18Tc were maintained with media supplied with 10 mcg/mL tetracycline for maximum plasmid retainment. The plasmid was isolated through a midiprep procedure which yielded 188.6 ng/mcL. The DNA was read spectrophotometrically for quantity and purity. A restriction enzyme digest was performed on both pEX18Tc and fragment AD to cut the ends with matching overhangs in preparation for ligation.

The efficiency of the restriction enzyme digest was questioned due to variable agarose gel results. New primer sets were ordered which

included a longer GC tag (Table 3). Enzymes cleave DNA much less efficiently towards the end of a fragment, so the extension of the fragment by the addition of nucleotides on the 5' end increases the cleavage efficiency. Our original primers had 5 base pairs, so we ordered new ones with ten base pairs at this site for increased digestion efficiency. Additionally, different lengths of time and different experimental temperatures and times were attempted to maximize digest efficiency. The combination of new primers and an overnight digest at 35° C yielded a clean cut of fragment AD and pEX18Tc as demonstrated by agarose gel electrophoresis (Figure 10). To inactivate the enzymes, the products were applied to QIAGEN PCR Clean-Up kit and analyzed via NanoDrop One UV-Vis Spectrophotometer. The digest of pEX18Tc yielded a cut product at 27.4 ng/mcL and fragment AD resulted in a concentration of 16.8 ng/mcL. Both products were within recommended purity ratios.

Name	Location	Sequence
A- ExtraGC Tag	Upstream Forward	5' - cggtagcagcGGATCCGCGAATCTCTGACGTGGA
D- ExtraGC Tag	Downstream Reverse	5' - cgattcgctgGAGCTCTGGCGTGATAGGGAACGC
M-13	Flanking MCS of pEX18Tc	F: 5' – CGCCAGGGTTTTCCCAGTCACGAC R: 5' – AGCGGATAACAATTTCACACAGGA

Table 3. Primers with extra restriction enzyme binding site. Lowercase characters are binding site for restriction enzyme (GC tag). Underlined characters are restriction site sequences. Universal M13 Primers for screening transformants.

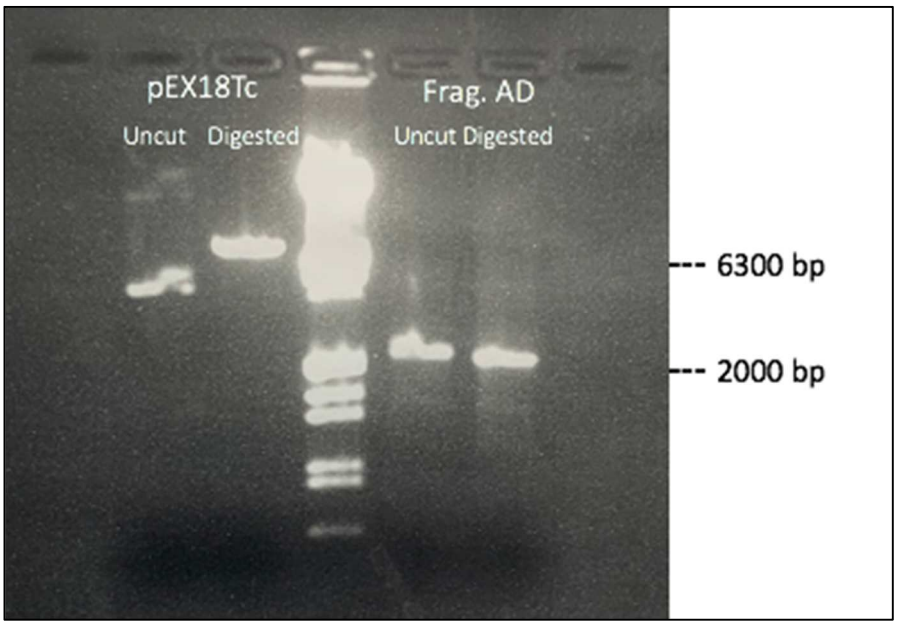

Figure 10. Post-digest agarose gel of uncut and digested pEX18Tc and fragment AD. Samples were digested overnight at 35° C and ran on 1% agarose gel.

Per manufacturer's recommendations, three ratios of vector: insert (pEX18Tc: AD) were calculated to use for ligation (Table 4). All three ratios of resulting ligation were used in the transformation to determine the most efficient ratio that resulted in ligated product pEX18Tc::AD.

		NEB 1:1	NEB 3:1	NEB 5:1
	T4 Buffer	1	2	2
	Vector (pEX)	3.86	3.86	2
	Insert (AD)	2.01	6.03	10.04
	H20	2.13	7.11	4.96
*Add last	T4 Ligase*	1	1	1
	total (mcl)	10	20	20

Table 4. Volumes calculated to test three ratios of vector: insert DNA for ligation. Ratios and recommended volumes were collected from https://nebiocalculator.neb.com/#!/ligation

iii.. *Transformation of* E. coli S17-1 *with pEX18Tc::AD*

To select for successful transformants, TSA media is supplied with 10 mcg/mL tetracycline. Initial transformation confirmation was achieved by colony PCR with universal M13 primers (Table 3). pEX18Tc has built-in M13 sites (Figure 11) up and downstream of the multiple cloning site (MCS) to indicate by an increase in band size if the plasmid incorporated into the cell contains the added fragment AD, as resistance to tetracycline would be conferred by the plasmid both with and without successful ligation. Initial screenings of successful transformants did not indicate a successful ligation.

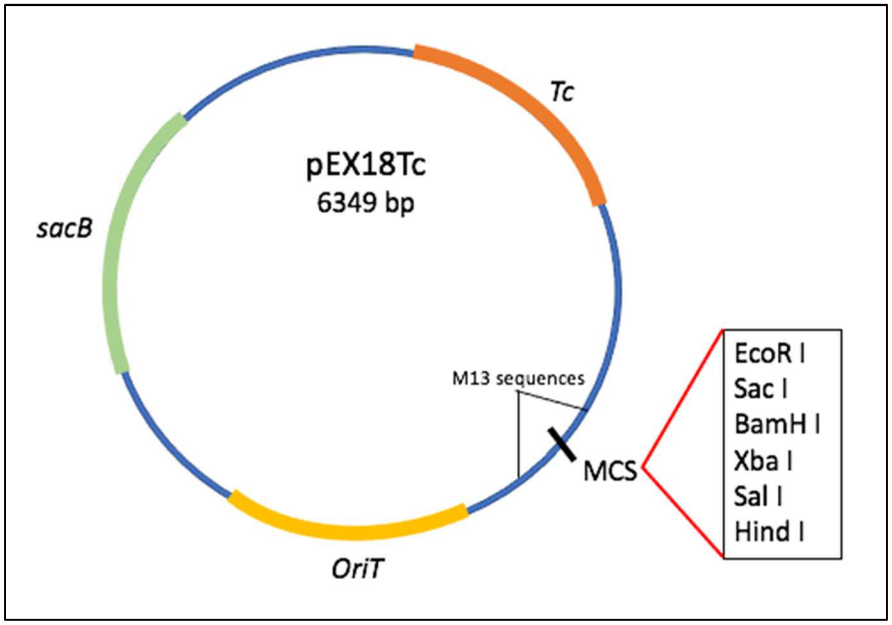

Figure 11. Map of pEX18Tc, 6349 bp. Selectable marker, *Tc*, codes for resistance to tetracycline. *sacB* provides the plasmid suicide characteristic, self-replication possible through inclusion of *oriT*. The multiple cloning site shows the restriction enzyme sequences included, and M13 sequences flanking MCS are indicated.

To address this concern, chromogenic media was added to the protocol to color screen for successful transformants. The reagents X-Gal and IPTG assist in this by indicating functional β-galactosidase activity. Plasmid pEX18Tc contains a functional *lacZ* gene spanning the MCS. Upon successful uptake of ligated DNA, the β-galactosidase activity at this site is disrupted. Competent cells containing a fragmented deletion in *lacZ* can be complemented by transformation with a plasmid containing the required sequence. The β-galactosidase activity cleaves lactose to form galactose and glucose. This is mimicked in this media as β-galactosidase activity can be induced by IPTG and can use X-gal as a substitute substrate. A functionally complete metabolism of X-gal will result in blue colored colonies due to chromogenic properties of the reagent. Therefore, the media supplied with X-gal and IPTG should yield white colonies to indicate successful transformation of a correctly ligated plasmid through indication of mutant β-galactosidase.

Numerous attempts at transformation yielded only blue colonies. To address this concern, the competency of *E. coli* S17-1 was questioned and NEB DH5-α β-10 cells were used instead, which are designed specifically for large inserts and inserts that are possibly toxic to the vector. Transformation with NEB DH5-α β-10 was successful; however, these cells do not contain conjugation machinery required for the next

step. However, at this point the plasmids from six selected isolates were harvested and isolated, so that they may be used in future transformations with full confidence that their ligation contains fragment AD. The resulting plasmids were placed in a double digest with restriction enzymes overnight at 35° C and then run on an agarose gel to confirm composition of both components, pEX18Tc and fragment AD (Figure 12). Six digested plasmids were run from six transformant colonies, called M1-M6, and the ligated plasmid M3 was sequenced to confirm the correct orientation of the insert and was used moving forward in the protocol.

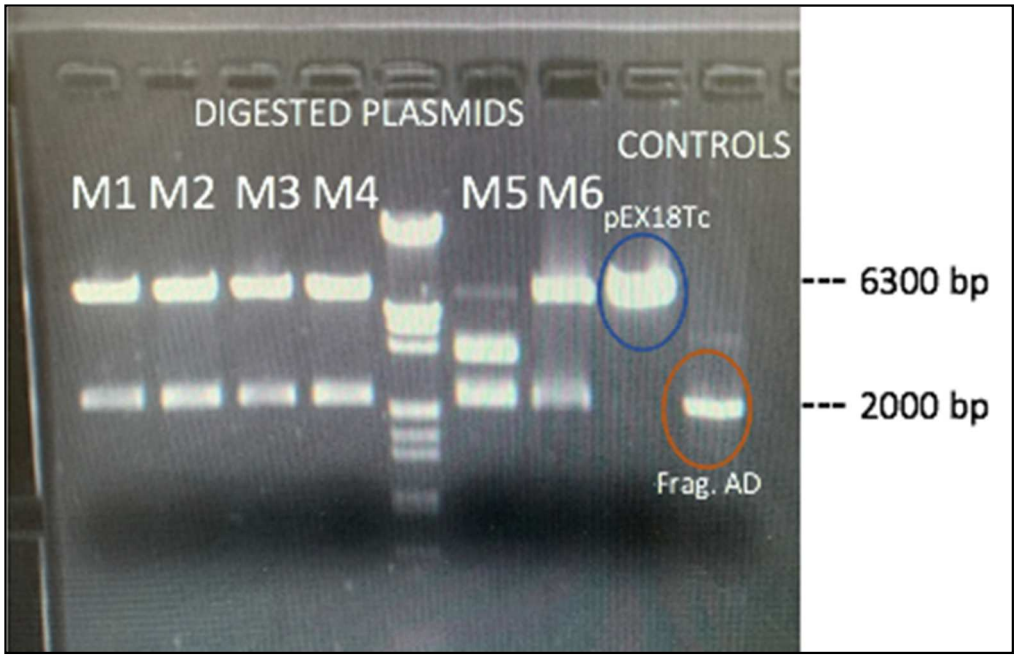

Figure 12. Agarose gel of double restriction enzyme digest of plasmids resulting from miniprep isolation of possible transformants. M1-4 and M6 contained the required fragments post-digestion. All future experiments used M3.

At this point, blue/white screening techniques were surrendered in favor of screening via plasmid isolation followed by a double restriction enzyme digest and agarose gel electrophoresis as previously described, to confirm successful transformation. Once an isolate was confirmed, it was grown overnight in LB with 10 mcg/mL tetracycline and frozen as a glycerol stock until the next steps.

iv. *Conjugation of* E. coli *S17-1 with pEX18Tc::AD with* Aeromonas hydrophila *ATCC7966 yields merodiploids*

A filter-mating conjugation protocol was followed to mate *E. coli* S17-1 containing pEX18Tc::AD with *A. hydrophila* ATCC 7966. For this protocol, media supplemented with 10 mcg/mL of tetracycline at 37° C was used to grow *E. coli* S17-1 to retain the plasmid and to express the genes for the conjugative pilus. Both cultures are grown to late-log phase, as read by spectrophotometric determination (OD_{600} of 1.4-1.6), and plated together on a 0.4 nm nitrocellulose filter on TSA overnight. The suspension resulting from vortexing the filter in PBS is serially diluted and plated on selective agar. ADA-VI with 10 mcg/mL tetracycline was initially used in the screening, however when the control *E. coli* S17-1 strain was plated on this media, it produced colonies. *Aeromonas* will have a phenotypic difference on this media, but it was determined to be too difficult to ensure

that there would be significant enough isolation surrounding colonies to rely on this difference.

An MIC protocol was performed at this point on *E. coli* S17-1 and *A. hydrophila* ATCC 7966 using the antibiotics ampicillin and irgasan in ADA-VI media to determine a concentration of either that would inhibit *E. coli* S17-1 yet allow for the growth of *A. hydrophila* post-conjugation. During the experiments, control strain of *E. coli* S17-1 with pEX18Tc::AD never had growth, so it was with confidence that the resulting colonies produced from the conjugation were *A. hydrophila* with pEX18Tc::AD, with additional confirmation due to the phenotypic difference provided from fermentation of the ADA-VI media, indicated by a yellow halo on the media, and growth when supplemented with tetracycline.

v. Selection and screening of possible isolates yields successful deletion

Selected isolates from the ADA-VI with 10 mcg/mL tetracycline post-conjugation were re-plated on another ADA-VI with 10 mcg/mL tetracycline plate and grown overnight to confirm their resistance to tetracycline. About half of the isolates tested grew, thus merodiploids, having parts of their DNA sequence on their chromosome matching sequences on a plasmid. Two of the resulting isolates were selected to inoculate LB without antibiotics. Growing in LB without antibiotics serves two purposes: 1) Gives the cells ample opportunity to undergo

homologous recombination, which is possible due to the merodiploidy of the cells (having long segments of identical DNA sequence); 2) The absence of the antibiotic stressor encourages the cells to get rid of the plasmid, because without the selective antibiotic pressure the bacteria might not retain it. When the plasmid is kicked post-homologous recombination, it should contain the working copy of *recA* due to having those common sequences up and downstream of the gene.

The method to ensure that the protocol only continues with cells containing no plasmid involves plating on TSA with 10% sucrose. pEX18Tc encodes a *sacB* gene which codes for toxic metabolites in the presence of sucrose and kills the cells. Therefore, the colonies on 10% sucrose are assured to not have the plasmid pEX18Tc and likely got rid of it during the period without antibiotic stress and the resulting selective pressure while it perhaps contained the working *recA*. Matching isolates from this sucrose plating are transferred via patching to three separate plates: another 10% sucrose TSA, a TSA with 10 mcg/mL tetracycline, and an ADA-VI (no tetracycline). Colonies which grow on 10% sucrose, do not grow on 10 mcg/mL tetracycline TSA, and have a typical *Aeromonas* phenotypic appearance on ADA-VI can be a possible successful *recA* deletion, or revertants to wild-type.

To screen for successful deletion, multiple rounds of PCR on colonies were performed. Controls in this PCR include *A. hydrophila* wild type and pEX18Tc::AD. Amplicons of these two controls yield bp lengths of ~3000 and ~2000 respectively, the latter indicative of successful deletion. Colonies were selected until a PCR with original primers A and D yielded an amplicon matching the relative size of the AD fragment as amplified from pEX18Tc::AD control. A concurrent PCR using primers specific to *recA* using real-time primers (see Table 1) was performed as well. If a colony yielded a band with primers A and D which matched the plasmid with insert band size (2000 bp) and did not amplify with the *recA* specific primers, then it was confidently identified as a successful *recA* deletion mutant. Of the ~200 isolates screened post-conjugation selection and one was found that yielded these results (Figures 13, 14) on 10/15/2021.

Figure 13. PCR of final round of screening with primers A and D. Controls included wild-type *A. hydrophila* DNA (AH-WT), and pEX18Tc with AD insert. Colony 10-C3 yielded an amplicon of correct size (2000bp) matching the control pEX18Tc::AD. Samples ran on 0.9% agarose gel at 100 v for 55 minutes.

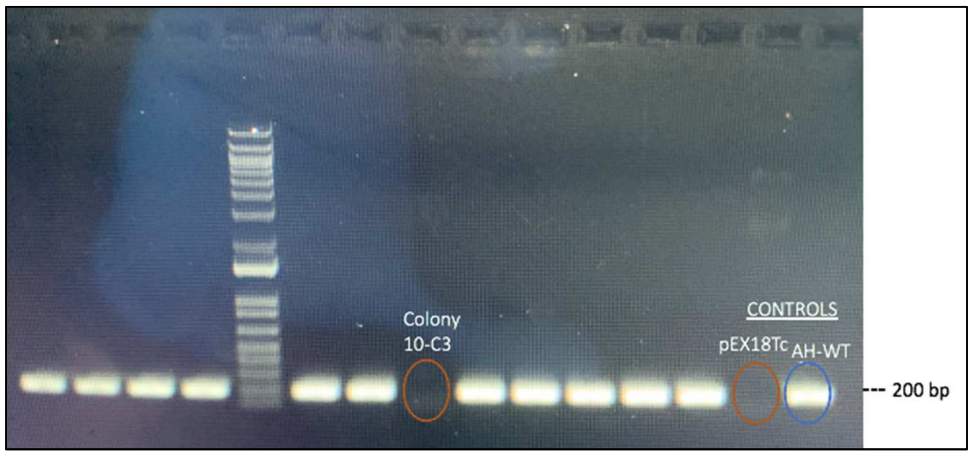

Figure 14. Agarose gel of PCR using real-time primers specific to *recA*. Controls include pEX18Tc with AD insert and wild type *A. hydrophila*. A positive result is absence of an amplicon. Colony 10-C3 additionally amplified with primers A and D at correct band size, indicative of deletion. Samples ran on 0.9% agarose gel at 100v for 55 minutes.

2. Mutation Analysis

i. Effect of 0.5x MIC of ciprofloxacin on A. hydrophila *mutation rate*

The effect of subinhibitory concentrations of ciprofloxacin revealed a significant increase in mutation rate in wild type *A. hydrophila*. The initial MIC revealed that *A. hydrophila* is inhibited at a concentration of 0.0078 mcg/mL of ciprofloxacin, and the antibiotic stressed used for this assay was therefore a concentration of 0.0039 mcg/mL. Using 19 wells of each, treated and untreated wild type, were incubated for 26 hours at 30C shaking at 250 rpm. Three wells were chosen at random for CFU determination. These wells were additionally analyzed for turbidity via optical density measurement, with the average of triplicates for wild type untreated at an OD_{600} of 1.915, and the stressed wild type at an OD_{600} of 1.841. These numbers reflect appropriate advancement of the cultures into stationary stage which is the ideal timepoint for determination of

mutation rate. The same triplicates were serially diluted to 10^{-7} and 10^{-8} with 50 mcL of each dilution plated on TSA. The CFU results revealed the cultures had an average of 6.73 x 10^{9} CFU/mL for wild type unstressed control and 1.11 x 10^{10} CFU/mL for the wild type incubated with ciprofloxacin. These values are halved in the equation for mutation frequency, due to the mutants counted being a total out of 500 mcL, not 1 mL. The results for CFU determination are found in Table 5.

	CFU/mL (1)	CFU/mL (2)	CFU/mL (3)
Control (WT)	7.40E+09	6.80E+09	6.00E+09
0.5X cipro (WT)	1.26E+10	1.00E+10	1.08E+10

	Average CFU/mL	Average /2
Control (WT)	6.73E+09	3.37E+09
0.5X cipro (WT)	1.11E+10	5.57E+09

Table 5. The CFU determination of *A. hydrophila* wild type with 0.5 times MIC of ciprofloxacin (green) and unstressed control (grey). The resulting CFU is the average of triplicates of 50 mcL of 10^{-7} dilutions.

The remaining 16 wells per group are used for the mutant count. The wells were plated in their entirety on TSA containing four times their minimum inhibitory concentration of nalidixic acid. The MIC for this antibiotic was determined to be 1.0375 mcg/mL for both wild type and the deletion of A. hydrophila, so the same concentration of nalidixic acid was used to plate both (4.15 mcg/mL). The stressed wild type is split into two wells of 450 mcL and 50 mcL to prevent lawn growth resulting in an inability to determine a colony count, as it has been found to result in such high mutation rate as to cover the surface of the agar. The mutant colonies in

the two wells are either added together, or if the 450 mcL well has resulted in lawn growth, then the 50 mcL well is used to the power of ten. This is used to give an approximation of mutant colony count if the result of the mutant count in the well is higher than 150. A well containing lawn growth was considered more than 150 colonies. Any wells resulting in zero colonies was given a “m” value as determined by the negative natural log of the number of wells resulting in zeros divided by the total number of well, if “m” is a value between 0.3 and 2.3 (Rosche, *et al*., 2000). The mutant counts of these wells are shown in Table 6.

Mutant CFU (48-hr) (450uL)								
Control (WT) (500uL)	13	8	0	0	20	6	40	33
	26	0	1	3	33	5	29	8
0.5X cipro (WT)	150	150	150	150	150	150	150	150
	150	150	150	150	150	150	150	150
Mutant CFU (48-hr) (50uL)								
0.5X cipro (WT)	0	0	1	3	1	2	0	0
	0	0	1	4	150	12	23	27
Mutant CFU (48-hr) (TOTALS)								
Control (WT) (500uL)	13	8	1.673976434	1.673976434	20	6	40	33
	26	1.673976434	1	3	33	5	29	8
0.5X cipro (WT)	150	150	150	150	150	150	150	150
	150	150	150	150	1500	150	230	270

Table 6. The mutant colonies on 16 wells 4X MIC of NA of *A. hydrophila* unstressed control (grey) and *A. hydrophila* incubated in 0.5 MIC of ciprofloxacin (green). The counts are determined after 48-hours of incubation. The totals are the 450 mcL well colonies added to the 50 mcL well colonies or tenfold the 50 mcL well colonies, whichever is higher.

Calculation of mutation rate results from dividing the total mutant count for each well by the CFU/500 mL and taking the average of all 16 wells. Each individual mutation rate is shown (Table 7). The results show that *A. hydrophila* wild type when incubated with a subinhibitory concentration of ciprofloxacin results in a mutation frequency ten-fold higher than the

unstressed control. One-way ANOVA analysis determined significance at *p*-value of 0.0303, below the threshold of a *p*-value of 0.05 (Figure 15).

Individual mutation frequencies 48-hour (500mcL)								
Control (WT) (500uL)	3.86E-09	2.37E-09	4.97E-10	4.97E-10	5.93E-09	1.78E-09	1.19E-08	9.79E-09
	7.72E-09	4.97E-10	2.97E-10	8.90E-10	9.79E-09	1.48E-09	8.61E-09	2.37E-09
0.5X cipro (WT)	2.69E-08	2.69E-08	2.69E-08	2.69E-08	2.69E-08	2.69E-08	2.69E-08	2.69E-08
	2.69E-08	2.69E-08	2.69E-08	2.69E-08	2.69E-07	2.69E-08	4.13E-08	4.85E-08

Table 7. Individual mutation frequencies of each well of *A. hydrophila* wild type unstressed control (grey) and *A. hydrophila* wild type incubated in 0.5xMIC of ciprofloxacin (green). The mutation frequency is determined by the individual mutant colonies divided by the total CFU of that same culture condition.

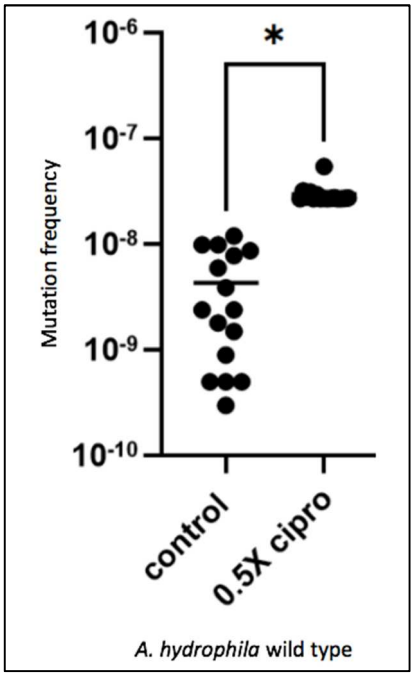

Figure 15. Mutation frequencies of *A. hydrophila* wild type. Each point represents the mutation frequency of individual wells (total of 16). Data analyzed via one-way ANOVA, $p < 0.05$.

ii. Mutation frequency of ΔrecA *compared to wild type incubated with subinhibitory concentrations of ciprofloxacin.*

The MIC of the deletion for both ciprofloxacin and nalidixic acid were determined to be the same as the wild type, 0.0078 and 1.0375 mcg/mL

respectively, so the same concentrations of antimicrobials were used for the cell stress conditions and the determination of mutants via colonies on antibiotic media.. The overnight growth of both the stressed and unstressed deletion resembled that of the wild type, as measured by spectrophotometry. The 26-hour OD_{600} of unstressed deletion was 1.86 and the OD_{600} of the stressed deletion was 1.839, again indicating stationary stage and acceptable for analysis.

The determination of CFUs for the deletion was performed the same as described above for the wild type, and the results are shown in Table 8. The CFUs for both samples were reduced compared to the wild type, though their overnight ODs were the same. The plating for mutants again used 16 wells of 500 mcL each on TSA supplemented with nalidixic acid at four times the MIC, and the resulting mutant counts are shown (Table 9) again with “m” replacing zeros where applicable, and the stressed group divided into 450 mcL and 50 mcL samples in the event of lawn growth disallowing an accurate colony count. This problem was not encountered with the deletion strain.

	CFU/mL (1)	CFU/mL (2)	CFU/mL (3)
Control (Deletion)	1.60E+09	1.60E+09	2.20E+09
0.5X cipro (Deletion)	2.00E+09	0.00E+00	2.00E+09

	Average CFU/mL	Average /2
Control (Deletion)	1.80E+09	9.00E+08
0.5X cipro (Deletion)	1.33E+09	6.67E+08

Table 8. The CFU determination of *A. hydrophila recA* deletion with 0.5x MIC of ciprofloxacin (blue) and deletion unstressed (red). The resulting CFU is the average of triplicates, determined from 50 mcL of a 10^{-7} plating of serial dilutions on TSA.

Mutant CFU (48-hr) (450uL)								
ontrol (Deletion) (500u	3	1	0	0	18	150	32	1
	26	3	4	0	3	0	2	6
0.5X cipro (deletion)	0	0	3	2	10	20	1	0
	52	3	0	48	7	1	2	12
Mutant CFU (48-hr) (50uL)								
0.5X cipro (deletion)	0	0	0	0	1	4	0	0
	2	0	0	0	0	0	0	1
Mutant CFU (48-hr) (TOTALS)								
ontrol (Deletion) (500u	3	1	1.386294361	1.386294361	18	150	32	1
	26	3	4	1.386294361	3	1.386294361	2	6
0.5X cipro (deletion)	1.386294361	1.386294361	3	2	11	24	1	1.386294361
	54	3	1.386294361	48	7	1	2	13

Table 9. The mutant colonies on 16 wells 4x MIC of nalidixic acid TSA of *A. hydrophila recA* deletion unstressed control (red) and *A. hydrophila* incubated in 0.5x MIC of ciprofloxacin (blue). The counts are determined after 48-hours of incubation, with lawn growth considered to be 150, and "m" replacing zeros where appropriate.

The individual mutation frequencies of each sample were determined again by dividing the mutant colony formation total of each well by the resulting CFUs/500 mcL of that sample group (Table 10). The 16 total frequencies were averaged to find a mutation frequency for the group as a whole (Table 11). When plotted against the data from the wild type, it is revealed that there is no significant change in mutation frequency amongst the *A. hydrophila recA* deletion with or without the presence of ciprofloxacin, nor in the difference in mutation frequencies of either of the deletion samples against the wild type samples (Figure 16). Significance was determined using a one-way ANOVA, with $p < 0.05$.

Individual mutation frequencies 48-hour (500mcL)								
ontrol (Deletion) (500u	3.33E-09	1.11E-09	1.54E-09	1.54E-09	2.00E-08	1.67E-07	3.56E-08	1.11E-09
	2.89E-08	3.33E-09	4.44E-09	1.54E-09	3.33E-09	1.54E-09	2.22E-09	6.67E-09
0.5X cipro (deletion)	2.08E-09	2.08E-09	4.50E-09	3.00E-09	1.65E-08	3.60E-08	1.50E-09	2.08E-09
	8.10E-08	4.50E-09	2.08E-09	7.20E-08	1.05E-08	1.50E-09	3.00E-09	1.95E-08

Table 10. Individual mutation frequencies of each well of *A. hydrophila recA* deletion unstressed control (red) and *A. hydrophila recA* deletion mutant incubated in 0.5x MIC of ciprofloxacin (blue). The mutation frequency is determined by the individual mutant colonies divided by the CFU/500 mcL of that group.

Average mutant CFU/ 500 mcL	
Control (WT)	4.26599E-09
0.5X cipro (WT)	4.43223E-08
Control (Deletion)	1.77E-08
0.5X cipro (Deletion)	1.63554E-08

Table 11. The average mutation frequencies for each sample. Each value is an average of 16 samples, each mutant colony total divided by CFU/500 mcL for that group.

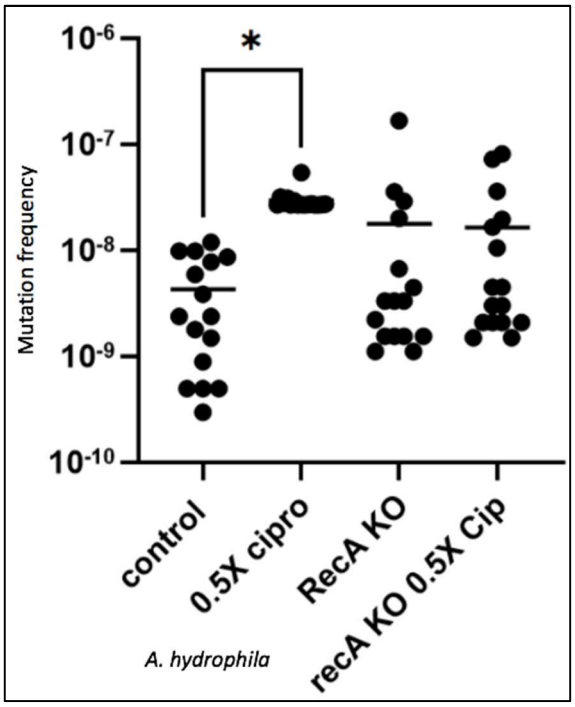

Figure 16. The effect of ciprofloxacin on mutation frequency of *A. hydrophila* wild type and *A. hydrophila recA* deletion. Data analyzed via one-way ANOVA, $p < 0.05$.

Chapter 4: Discussion

Ciprofloxacin has been shown to induce the SOS response in a variety of bacterial species; however, its effect on this survival pathway in *A. hydrophila* is unexplored. Ciprofloxacin, a fluoroquinolone, is a DNA gyrase inhibitor which works through blockage of a subunit of topoisomerase, which results in DNA double-strand breaks (Bush, *et al*., 2020). RecA is recruited to the area of these breaks as the broken double strands are processed into single-stranded DNA, and with RecA, these strands form nucleoprotein filaments. These filaments are considered to be activated RecA, which when gathered, induce the cleavage of the LexA dimer, the repressor of the SOS genes. Resulting transcription of the genes produces subunits for an error-prone polymerase, which can replicate past the strand breaks while sacrificing genetic integrity and result in mutations. This raises concern over the regularity of the usage of ciprofloxacin, as fluoroquinolones are one of the most frequently prescribed antibiotics in the United States (Linder, *et al.,* 2005).

The expression of *recA* in response to subinhibitory concentrations of ciprofloxacin has been studied in numerous species, as well as the varying effects of other gyrase inhibitors. Schröder, *et al*. discovered a 13-fold increase in *recA* expression after one hour of incubation with ciprofloxacin in the Gram-positive organism *Staphylococcus aureus* (Schröder, *et al*., 2012). Among Gram-negative bacteria, ciprofloxacin was also found to induce *recA* expression 9-fold

in *Pseudomonas aeruginosa* (Valencia, *et al*., 2017. In *E. coli*, similar investigation showed that exposure to ciprofloxacin at sublethal concentrations increased recA expression up to 4.7-fold (da Silva Ribeiro, *et al*., 2020). Our research findings agree with these increases, showing 8-fold increase in *recA* expression after exposure to half the MIC value of ciprofloxacin in *A. hydrophila.* Further analyses have demonstrated that this expression is correlated with the SOS response, whereby antagonizers of the SOS diminished *recA* expression. Amikacin, novobiocin, and small molecule inhibitors of RecA (Wigle, *et al*., 2007) have been shown to diminish *recA* expression to levels commensurate with non-stressed samples when administered with ciprofloxacin (Valencia, *et al*., 2017; Schröder, *et al*., 2012). In these and other studies, researchers have begun to target RecA in an effort to prevent the downstream effects of the increased expression level in response to antibiotic exposure (da Silva Ribeiro, *et al*., 2020; Wigle, *et al*., 2007).

The induction of the bacterial SOS response has been shown to have many downstream effects, none more so characterized than enhanced mutagenesis. The activation of RecA and derepression of LexA leads to the transcription of genes which code for DNA polymerase V, with a loose active site which facilitates the incorporation of erroneous bases during DNA synthesis, as well as an absence of 3'-5' exonuclease proofreading which prevents correction of the errors (Jaszczur, *et al*., 2016). Single nucleotide mutations in a variety of genes can lead to increased resistance to various antimicrobials. For example, single

base-pair mutation in the quinolone resistance determining region (QRDR) of *gyrA* is enough to confer resistance to fluoroquinolones (Hooper, *et al.,* 2015). Many environmental stressors have been shown to increase mutation rates in *E. coli*, such as UV-light exposure, heavy metals and low-level antibiotics present in runoff (Bates, *et al*,. 1989; Li, *et al*., 2019; Ozdemirel, *et al*., 2021), however no such investigation into these factors influence on *Aeromonas* species has been performed. Our results support increased SOS-induced mutations caused by subinhibitory concentrations of antimicrobials, however a complete characterization of mutational activity is difficult.

Proper CFU determination is integral in this experiment as a value too high or too low skews the resulting mutation rate and can give undue weight to one category of control group if their numbers don't accurately reflect the cell count. With the OD_{600} of each sample being relatively similar, the discrepancy in CFU values is concerning to the outcome of this assay. Additionally, the OD_{600} for the samples incubating in subinhibitory concentrations of ciprofloxacin could be unable to serve as a guide to cell count and could explain the differences in CFUs seen. The OD_{600} is similar between all of the samples, yet the outcome of the CFU determination doesn't reflect this and this could potentially be due to filamentation. Ciprofloxacin and other antimicrobials can induce filamentation in certain bacterial species through dysregulation of binary fission and disruption of septation (Vranes, *et al*., 1996). If ciprofloxacin induces filamentation in our wild type, the optical density readings could remain similar, though the resulting CFUs

could vary greatly because the growth could actually be slowed or stunted due to the disruption of binary fission.

Preliminary microscopy revealed filamentation in wild type *A. hydrophila* incubated overnight in a concentration of ciprofloxacin at half its MIC (data not shown) however, quantification and measurement would help support this. One study performed a centrifugation step prior to their mutation assay to resolve the filamented cells seen so they were not included (Thi, *et al*., 2011). Additionally, if filamentation is indeed seen in response to engaging the cell's SOS response, the lack of filaments visualized in the knock-out incubated with subinhibitory concentrations of antibiotics could help further support this. Concurrent with visualization of filamentation should be a growth kinetics assay to confirm the parallel growth curves of both the wild-type and the knockout. Overnight optical density readings were similar for both species, however demonstrating this experimentally could give more conclusive support for utilizing the OD readings to determine cell number and parallel growth.

An additional concern in the overall determination of mutation frequency is this assay tests for mutations that confer resistance to antimicrobials, not overall mutations. Recent whole-genome sequencing of *E. coli* under constitutive SOS-induction found base-pair substitutions at a rate 60 times higher than spontaneous mutations of the same type of wild type control (Niccum, *et al.,* 2020). SOS-induced mutagenesis results in "untargeted" mutations, that is,

spontaneous mutations not giving the cell any particular fitness benefit. In one study, placing *E. coli* under constitutive SOS activation on an unrequired carbon source showed the cells mutated to metabolize the carbon at the same rate they starved (Foster, *et al*., 2004). Our mutation frequency assay documents those mutations needed to confer resistance to antimicrobials but is not indicative of all SOS-induced mutations. Clearly, many of the induced base-pair substitutions may not have a fitness benefit, and result in cell death instead.

With the variability in the CFU determination, the physiological considerations of the cells, and frequency of mutations not conferring resistance aside, the outcome of these assays shows a 9.62-fold increase in mutation frequency among wild type *Aeromonas hydrophila* in the presence of 0.5x subinhibitory concentrations of ciprofloxacin. Together, the increased *recA* expression and mutation rate support the induction of the bacterial SOS response.

To further support our findings, we generated an *A. hydrophila recA* deletion mutant via splicing by overlap extension PCR. This method has been used previously in *A. hydrophila* ATCC 7966. To identify genes responsible for a novel outer membrane receptor and its transcriptional regulator, a series of genes were deleted and then complemented using this method (Funahashi, *et al*., 2014). The technique is more frequently seen with other *Aeromonas* species however, namely *Aeromonas caviae*. One study used SOE-PCR simply to create a site for cassette insertion for creation of kanamycin resistant mutants to provide a

selectable marker in characterization of LPS biosynthesis (Tabei, *et al*., 2009). These examples provided confidence that the deletion of *recA* would be successful.

When the *recA* deletion mutant was applied to the mutation assay, no difference in the mutation frequency was evident when exposed to subinhibitory levels of ciprofloxacin. Interestingly, the mutation frequency of the control (incubation without ciprofloxacin) was increased itself when compared to the wild type (not significant, $p > 0.05$). A few factors may have contributed to this increase such as improper determination of CFUs, or as discussed, varied growth in the culture or different physiology of the cell giving an erroneous OD reading when compared to wild type. Prior experiments with deletion of *recA* in *E. coli* demonstrated similar results though with the *recA* knockout displaying a higher base mutation frequency in absence of stressor when compared to the wild type under similar conditions (Mo, *et al.,* 2017). Reasons for this increase seen were not discussed in those results but could be attributed to the above causes.

Chapter 5: Conclusions and Future Directions

Despite growing resistance to antimicrobials continuing to be a global public health crisis, consumption of antibiotics continues to rise. Bacteria present in waterways are exposed to cell stress provoked by contaminating antibiotics present from improper exposure and human and agricultural overuse. This cell stress can result in induction of survival pathways which leads to increases in mutation frequency and acquired or increased resistance to antibiotics. Additionally, in these waters, genetic exchange can happen through conjugation, transduction and transformation, resulting in new and perhaps pathogenic species newly acquiring resistance to antibiotics to which it was previously susceptible to. *A. hydrophila* could therefore behave as a vehicle of resistance transfer while sharing these waters rife with contaminating stressors.

The objective for this thesis was to determine the impact of sublethal concentrations of ciprofloxacin on the mutation rate and expression of *recA* in *Aeromonas hydrophila*. We hypothesized that recA *expression is associated with increased mutagenesis in* Aeromonas hydrophila *in the presence of sub-inhibitory concentrations of antibiotics, and deletion of* recA *will attenuate the mutation rate.* The first aim, to establish that *recA* expression increased in response to subinhibitory concentrations of ciprofloxacin, was supported. Using two reference genes, the data presented here supported the increase in expression of *recA* at two different time points post-exposure to half the minimum inhibitory concentration of ciprofloxacin. It is likely that this increase is due to the cellular response to single-stranded DNA breaks caused by the action of the antimicrobial, and

the subsequent induction of the bacterial SOS. The second aim, to identify the impact of *recA* on mutagenesis post-ciprofloxacin exposure, was partially supported. Exposure to ciprofloxacin at half the MIC increased the mutation rate of the wild type ten-fold, and the mutation rate was attenuated in the *recA* deletion in matching conditions, but the limitations of the reliability of the quantification of cells in this assay hindered making a complete conclusion. More research into the cellular physiology is needed before ascertaining that the mutation frequency isn't increased as well in the knockout. In addition, the data suggest that the deletion mutant alone has a higher baseline mutation frequency, however non-significant, and that needs investigation as well.

Moving forward in this work first requires complementation. The laboratory has acquired and isolated a plasmid suitable for complementation, pSB109 (Dr. Sonia Bardy, University of Wisconsin-Milwaukee, Department of Biological Sciences) a derivative of pJN105. The plasmid contains a ParaBAD promoter which is in inducible with arabinose, and the primers for *A. hydrophila* complementation with *recA* have been designed (Table 12). For validation purposes, complementation will be used to restore the wild type phenotype of the mutant to further support its impact on mutation rates. In addition, we would complement *recA* genes from other bacterial species. Primers for this have been designed as well (Table 12) and include *E. coli* K-12, *P. aeruginosa* PAO1, *A. caviae*, *A. media*, and *A. veronii*. Part of the preliminary investigation for this project included sequence analysis for *recA* amongst other *Aeromonas* species, *E. coli* K-12 and *Pseudomonas aeruginosa* PA01 (Table 13). Previous publications have alluded to areas of interest in the sequence as having greater impact on mutational

activity (Ahel, *et al*., 2005; de Val, *et al.,* 2019). In particular interest is the C-terminal domain including the Mg^{2+} binding site. The C-terminal domain of RecA is the area that determines its affinity for binding dsDNA, with previous publications hypothesizing that the negative charge carried by this area repels the phosphate of the DNA backbone (de Val, *et al.,* 2019). Deletions in this area in *P. aeruginosa* increased binding affinity, and one study deleted 25 residues in this area which resulted in a phenotype resembling constitutive SOS induction (Ahel, *et al*., 2005). Within this C-terminal domain a binding site for Mg^{2+} is located, which when bound, abolishes the dsDNA binding repulsion. When a C-terminal deletion mutant of *P. aeruginosa* was exposed to mitomycin C (a known activator of the bacterial SOS response) the sensitivity to the compound was enhanced, but their sensitivity was not similarly increased when exposed to UV light or ionizing radiation, suggesting that the C-terminal domain has a role in cross-linking repair mechanisms or interaction with other proteins which induce the SOS response (de Val, *et al.,* 2019).

E. coli K-12 F: 5'- gcggcg**GAGCTC**ATGGCTATCGACGAAAAC – 3' R: 5'- gcggcgTCTAGATTAAAAATCTTCGTTAGT – 3' Bp: 1062
P. aeruginosa PAO1 PAO1F: 5' - gcggcg**GAGCTC**ATGGACGAGAACAAGAAG– 3' PAO1R: 5'- gcggcgTCTAGATCAATCGGCTTCGGCGTC – 3' Bp: 1041
A. hydrophila ATCC7966, *A. caviae* ATCC 15468, *A. media* 105A F: 5' - gcggcg**GAGCTC**ATGGATCAGAACAAACAG – 3' R: 5' – gcggcgGAGCTCTTATTCGAACTCTTGTTC – 3' Bp: 1065
A. veronii CETC7060 F: 5' - gcggcg**GAGCTC**ATGGATCAGAACAAACAG – 3' R: 5' – gcggcgGAGCTCTTACTCGAACTCTTGTTC - 3' Bp: 1065

Table 12. Primers for complementation. Bold uppercase letters indicate restriction enzyme binding site for *Sac-I*. Underlined uppercase letters indicate restriction enzyme binding site for *XbaI*.

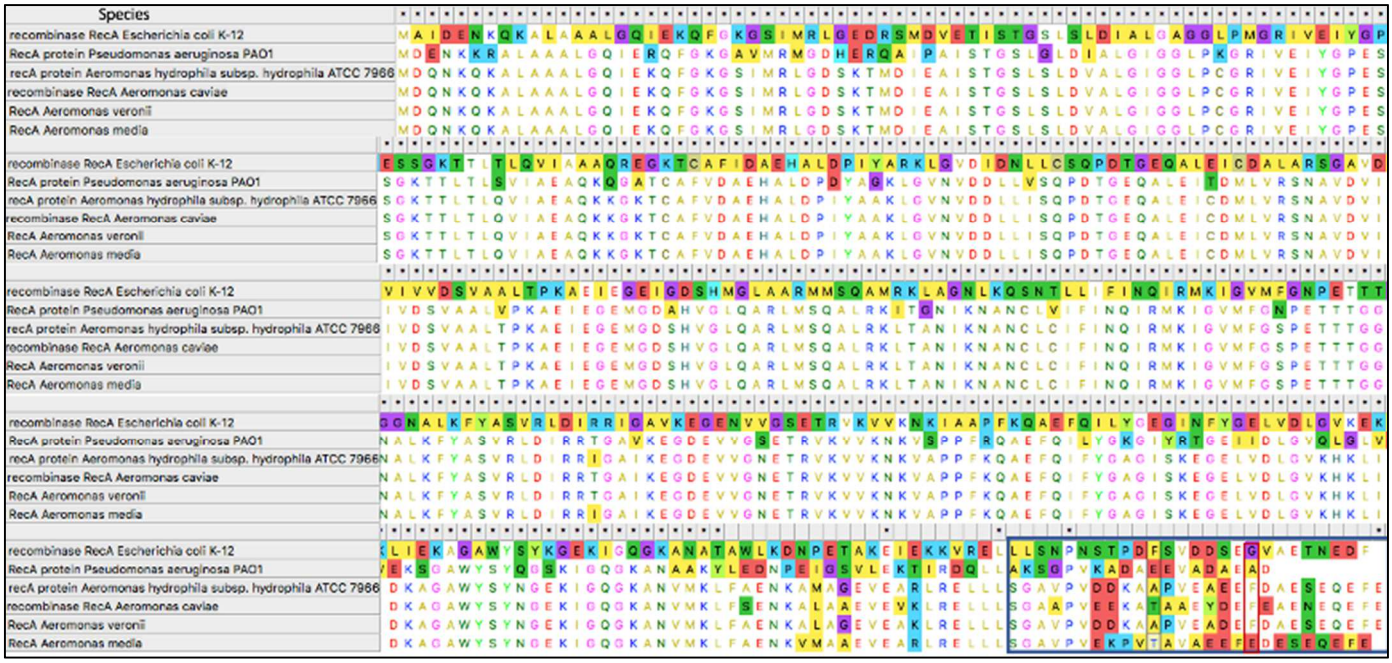

Table 13. MegaX alignment of amino acid residues of *recA*. 1. *E. coli* K-12. 2. *P. aeruginosa* PAO1. 3. *A. hydrophila* ATCC7966. 4. *A. caviae* FDA_ARGOS. 5. *A. veronii.* 6. *A. media*. Solidly colored box in a residue indicates different residue at that position for that species. No colored background indicates homology. Area of interest, Mg2+ binding site in red box and C-terminal domain in blue box

Sequence analysis among *Aeromonas* species revealed much conservation of the *recA* sequences amongst the species analyzed, with the greatest variation found in residues at the C-terminal region. To establish the residues of most importance in activation of the bacterial SOS response that could elicit a stronger response by greater binding affinities, complementing the *A. hydrophila recA* deletion with *recA* from other species and determining the differences in mutational activities may further support the hypothesis that this area is responsible for the difference in activity. Further work could additionally involve sequentially inducing point mutations in residues to further solidify this characterization. Work in our lab has previously shown *A. caviae* has experimentally been shown to have a higher mutation rate than *A. hydrophila* (data not shown), and conclusions could be drawn as to the exact residues responsible for this increase, as the sequences are homologous except for seven residues at the C-terminal binding domain, including the Mg^{2+} binding site.

Following these experiments, one of the next steps would be to characterize the effects of a wider variety of antimicrobials, on wild-type *A. hydrophila* alone and with the *recA* deletion. Because the SOS response induced by *recA* is primarily mediated by strand breaks in double-stranded DNA, ciprofloxacin, which targets the replication processes of DNA by halting replication through inhibition of supercoiling facilitated by DNA gyrase, was an exemplary first choice for preliminary assays. The mutation rate of the wild-type *A. hydrophila* in response to subinhibitory concentrations of ciprofloxacin suggests the induction of error-prone polymerases in response to the cell stress, however there are

other proteins that may modify this response as well. Though we saw attenuation of this mutation rate without the activity of *recA*, this mutagenesis increase observed in the *recA* knock-out unstressed control cannot be attributed to *recA* alone. In a search for a strategy to target the SOS response through adjuvant administration, it was found in *E. coli* that a *lexA* knockout had a similar phenotype as a *recA* deletion. The *lexA* deletion resulted in derepression of the SOS and a similarly increased mutation frequency (Mo, *et al*., 2016). Similarly, a *Clostridium difficile lexA* mutant was found to have increased virulence and enhanced resistance to metronidazole after exposure to subinhibitory concentrations of DNA-damaging antimicrobials (Walter, *et al*., 2015). Antibiotics which function by other mechanisms would perhaps not activate the bacterial SOS response or induce mutational activity, and therefore the mutagenic activity of more antibiotics should be tested.

One antibiotic of interest would be novobiocin, which inhibits DNA gyrase (like ciprofloxacin) but without inducing double-stranded DNA breaks. This would enable a better characterization of the activation of the SOS response in *A. hydrophila* and if the mutation rate of the deletion of *recA* would be attenuated here as well, perhaps this would demonstrate that *recA* is not the only gene responsible for activation of the SOS response, and the repressor *lexA* may also have a large role in instigating mutagenic activity. One study showed no change in the mutation rates of *E. coli recA* deletion mutant in response to novobiocin stress, though additionally citing that novobiocin could antagonize the SOS response (Mo, *et al.,* 2017).

Trimethoprim has been shown to induce the bacterial SOS response as well (Lewin, *et al*., 1989). This folate synthesis inhibitor prevents thymine incorporation into newly synthesized DNA through dihydrofolate reductase inhibition. The resulting DNA lesions can induce RecA activation as well as leave an imbalance in available nucleotides, the presence of which has been hypothesized to have a synergistic effect on mutational activity (Kunz, *et al*., 1991). Existing laboratory data shows an increase in mutation rate in wild-type *A. hydrophila* when incubated with subinhibitory concentrations of trimethoprim (data not shown) and if this increase is due, at least in part, to the activity of RecA and SOS induction, the mutation rate would be abolished when the *recA* knockout would be added to this assay.

Beta lactam antibiotics have been shown to activate the bacterial SOS response (Maiques, *et al*., 2006). The third-generation cephalosporin ceftazidime inhibits enzymes required for cell wall synthesis and has been shown experimentally in our laboratory to increase the mutation rate of wild type *A. hydrophila* (data not shown). Additionally, an increase in *recA* expression has been shown experimentally in *P. aeruginosa* post exposure to subinhibitory concentrations of ceftazidime (Hocquet, *et al.,* 2012), and considering the wide conservation of the bacterial SOS response, these data taken together could suggest the expression of *recA* may result in the increase in mutation rate in *A. hydrophila* as well, which would be demonstrated by testing the mutational frequency of the knockout in these same conditions.

An additional consideration may be the effect of subinhibitory concentrations of antibiotics on cell morphology and physiology. Ciprofloxacin and other antimicrobials can induce filamentation in certain bacterial species through dysregulation of binary fission and disruption of septation (Huisman, *et al*., 1984)). If ciprofloxacin induces filamentation in our wild type, the optical density readings could be similar and be giving a false idea of similar cell number and overnight growth, which would be different than reflected in the OD due to the increased size of the bacterial cells. Preliminary microscopy revealed filamentation in wild-type *A. hydrophila* incubated overnight in a concentration of ciprofloxacin at half its MIC (data not shown); however, quantification and measurement would help support this. Overnight optical density readings were similar for both species, however demonstrating this experimentally could give more conclusive support for utilizing the OD readings to determine cell number and parallel growth.

Induction of the SOS response through incubation in subinhibitory concentrations of antibiotics has been shown to have downstream effects on activities other than mutation-induced resistance to antimicrobials, such as biofilm formation and enhancement of virulence factors that contribute to pathogenicity. Preliminary data in the lab suggest an increase in biofilm formation in both *A. hydrophila* and *A. caviae* post exposure to sublethal concentrations of ciprofloxacin (data not shown). An assay involving the *recA* knockout would identify the extent of the increase seen in biofilm formation that *recA* expression is responsible for, and better characterize the impact of

SOS induction on virulent properties of *A. hydrophila* in the presence of subinhibitory concentrations of antibiotics.

Additionally, the effect of sub-inhibitory concentrations of antibiotics on virulence factors could be assessed. The laboratory possesses primers for real-time PCR to evaluate expression levels of numerous genes responsible for production of virulence factors such as the toxins aerolysin and hemolysin. The impact of various antimicrobials on virulence factor expression would give firm preliminary data to support that the induction of the SOS response could be responsible for an increase in pathogenicity. Aerolysin, AerA, is a pore-forming toxin and one of the main virulence factors produced by *A. hydrophila* (Wu, *et al.,* 2010). AerA has cytotoxic, hemolytic and enterotoxic activity, and is considered a marker for pathogenic *A. hydrophila* (Abrami, *et al.,* 2003). Aerolysin will form pores in target membranes through heptameric ring-shaped structures which will oligomerize through proteolysis of its C-terminal binding domain (Degiacomi, *et al*., 2013). HlyA is an *A. hydrophila* β-hemolysin, genetically homologous to that of *Vibrio cholerae,* in which it is essential to its intestinal virulence (Heuzenroeder, *et al.,* 1999). Research into the distribution of these genes demonstrates that 78.8% of virulent strains of *A. hydrophila* display an $aerA^{+}hlyA^{+}$ genotype (Heuzenroeder, *et al.,* 1999). Additional studies of gene expression of both *aerA* and *hlyA* in *A. hydrophila* has uncovered that inactivation of expression of one of these genes reduces, but does not eliminate, hemolytic or cytotoxic activities of *A. hydrophila* which suggests that the toxins work synergistically (Wong, *et al.,* 1998). By understanding the effect of subinhibitory concentrations of antimicrobials on virulence factor expression, we could

further enhance understanding of the effects of induction of the bacterial SOS environmentally as well as under antibiotic stress during a human infection. To further characterize the latter, assays determining direct cellular affects could be employed. For example, hemolysis, cell adhesion and cytotoxicity assays would further support if the increase of the virulence factor expression translates to direct cellular effects as well. In the cell-culture based assays, adding the *recA* knockout would further help characterize the impact of bacterial SOS induction on virulence and pathogenicity.

In conclusion, exposure to subinhibitory concentrations of antibiotics impact many cellular processes, one being the induction of the bacterial SOS response with a consequence of increased mutagenesis. Our findings provide the first characterization of *recA* in *Aeromonas hydrophila* as it pertains to the activity of the bacterial SOS and it's contribution to mutagenesis both in the presence of subinhibitory concentrations of ciprofloxacin. Our findings agree with previous research in other species demonstrating the ability of sublethal concentrations of antibiotics to increase *recA* expression leading to increased mutation frequencies. Further studies are needed for a better understanding of the consequences of subinhibitory concentrations of antibiotics present in waterways, and how the bacterial response to them leads to an increase in resistance to antimicrobials and even enhanced virulence properties and pathogenicity.

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