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OPTIMIZATION OF A GFP BIOSENSOR FOR THE DISCOVERY OF NOVEL ANTIBIOTICS

by

Drake Heinz B.S.

A Thesis Presented in Partial Fulfillment
of the Requirements of the Degree
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COLLEGE OF APPLIED AND NATURAL SCIENCES
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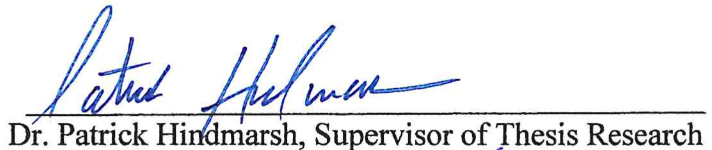

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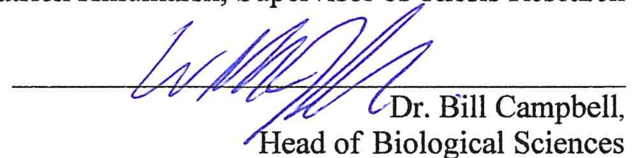

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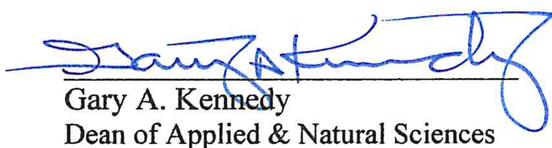

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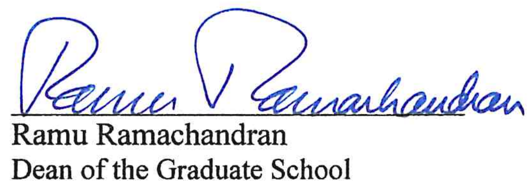

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ABSTRACT

In today's world, there is an urgent need to discover novel antibiotics due to the rise of antibiotic resistance and the lack of new ones being approved. Building off of Hanson et al. and their reduction-oxidation sensitive green fluorescent protein (roGFP)(1), that we yeast codon optimized (2); I wanted to determine if I could create a real-time biosensor that has the ability to respond to ROS produced in the stress response triggered by antibiotics as a platform to identify novel antibiotics. To achieve this, I need to know if it will oligomerize in bacterial cytoplasm due to its two cysteines at 48 and 70 and what the optimal conditions are for the biosensor. From western blot results, royGFP does not oligomerize in the cells showing that our biosensor should not lose fluorescence. royGFP had a strong sensitivity to hydrogen peroxide and ROS triggered from antibiotics. The results showed that royGFP can be used as an ROS biosensor tool to help discover novel antibiotics.

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DEDICATION

This thesis is dedicated to my parents who have supported and allowed me to get my Masters while I pursued my dream to go medical school. I also want to dedicate this to all of the professors who supported me and assisted me with my thesis, I could not have done it without them.

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CHAPTER 1

INTRODUCTION

1.1 An Urgent Need to Find Novel Antibiotics

Novel antibiotics need to be identified to combat the growing problem of antimicrobial resistance. In today's world, antibiotic resistance is on the rise, and we will soon be in a time similar to the early nineteenth century, where there were no medical therapies against bacterial infection (3). Almost all antibiotics used today were discovered in the mid-twentieth century, as these are mostly natural products. Resistance was already present in a small percentage of bacteria but was quickly enriched and widespread due to misuse and overuse of antibiotics (4). Meanwhile, newer drugs are rarely used in order to try to decrease the risk of further resistance development, as they are based on older drugs that bacteria have already shown some resistance to. The fact that fewer pharmacological companies are researching novel antibiotics exacerbates the problem (5). It is paramount that new, advanced, and unique antibiotics are continued to be discovered to combat this rise in resistance.

To combat this growing threat, my goal is to find new antibiotics. This will accomplished by quantifying the stress response of cells to known antibiotics. Cells can become easily stressed due to any number of factors such as toxic agents like antibiotics or a lack of nutrients and as a response, release massive amounts of reactive oxygen species (ROS)(6). ROS molecules like superoxide anion, hydroxyl radicals, and

hydrogen peroxide are a natural byproduct of cell respiration and metabolism and are known physiological messengers but do accumulate during times of stress(7). This stress response is strong when antibiotics are administered and can be used to determine the potential of an antibiotic's efficacy. Using roGFP from Hanson et al. (1), we can quantify the stress response by measuring the ROS triggered by the antibiotic stress. Furthermore, the presence of two natural cysteines allows GFP to oligomerize in response to ROS, a concern this research due to a loss in fluorescence.

By the end of my research, I will have determined if oligomerization is a concern for the biosensor and will have made progress on discovering the optimal conditions for a system using a redox-sensitive GFP biosensor. This biosensor will have the capability to identify a change in the oxidative environments and measure the stress response of *E. coli* based on a simple change in fluorescence.

The GFP strains used in these experiments are yeast-enhanced GFP (yEGFP) and yeast-enhanced GFP fast folder (yEGFP FF). Redox-sensitive yeast-enhanced GFP (royGFP) and redox-sensitive yeast-enhanced GFP fast folder (royGFP FF). All are yeast codon optimized that give it 75 times more fluorescence in *E. coli* (2) and contain the enhanced mutations at F64L and S65T, while royGFP and royGFP FF contains two cysteine mutations at S147C and Q204C to turn it into a redox biosensor.

1.2 Antibiotic Resistance

Antibiotics are a natural substance or pharmacologically altered substance that hinders the growth of or kills a bacterium. As a natural product, antibiotics originally evolved to reduce competition between eukaryotes and prokaryotes, as well as between bacterial species. Growth inhibiting (static) and killing (cidal) are the two main

categories of antibiotics which act by inhibiting any of three main cell functions: DNA replication, protein synthesis, and cell wall construction (6). Two examples of these categories are Kanamycin and Ampicillin. Kanamycin binds to the 30s ribosomal subunit, acting as a bacteriostatic agent, inhibiting growth by creating mistranslated proteins. Without the ability to make functional proteins for cell usage, the cell will slowly die due to a lack of functionality and growth. Ampicillin hinders the synthesis of the peptidoglycan linkages in the cell wall causing bacteriolysis and cell death (4).

Antibiotic resistance is the ability of a bacteria to resist or defend itself from a substance that kills or inhibits the growth of that bacteria. The study of antibiotic resistance is as old as the use of antibiotics themselves (4), but it wasn't until recently that we started realizing that bacterial resistance has the inevitable potential to spread faster than our present library of antibiotics creating a need to identify new classes of antibiotics. Resistance, like any other genetic advantage, is mainly created through horizontal gene transfer and point mutations. The causes of these point mutations come mostly from replication errors and mutagens in the environment. With a high enough population, millions of individual cells creating mutations greatly increases the possibility of a new antibiotic resistance gene. Once the resistance gene is in the genome of one cell, bacteria have multiple ways of spreading the resistance gene. If a population is being attacked by an antibiotic, the resistant cell will be unaffected and live on to produce more cells with the same mutation. This is natural selection; the surviving unaffected population will be able to produce more offspring carrying this gene while all the non-resistant ones perish. Bacteria can spread this gene to other bacteria through transformation, conjugation, and transduction. In transformation, if a cell releases its

contents due to it being killed or other potential factors, its genetic material can be taken up from the environment by other living bacteria, who can integrate this gene into its own genome to gain its function through homologous recombination. Conjugation is the process of sharing genes through sex pili on the bacteria's surface. And lastly, transduction is when genes are transferred virally. All three of these processes allow other bacteria to use the resistant gene for its own genetic advantage (8).

In the healthcare field, there are two main problems that are responsible for increasing antibiotic resistance. The first problem is that most antibiotic treatments are prescribed for a short period of time because it only takes a few weeks to rid the body of a bacterial infection. This is in contrast to other chronic illnesses such as Parkinson's that take years of medication to cure or just ease the symptoms. This trend in medicine really limits the amount of antibiotics sold, lowering profit margins. The second reason is more indirect; newer antibiotics are not used unless for a more serious case to prevent acquired resistance. This lowers the market demand for new antibiotics since profit cannot be made up front (9). Contributing to this issue is the influx of generic competitors, whose drugs not only reduce profit margins because they can be sold for less but flood the market helping create this natural selective pressure for resistance. Large pharmaceutical companies only allocate about 1.6% of its resources to developing new antibiotics (4). The effect of these patterns has already begun to show itself in hospitals. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been killing on average 19,000 people a year in the US and adding about 3 billion dollars' worth to healthcare cost. The rise of resistant strains slowly spread, creating even worse strains such as a vancomycin-resistant strain that will most likely wreak havoc in the near future (9)(10).

1.2.1 Reactive Oxygen Species

Bacteria, like all other cellular organisms produce and use reactive oxygen species (ROS). ROS are compounds such as hydrogen peroxide, hydroxyl radicals, and superoxide anion. They all contain oxygen and are natural by-products of an organism's metabolism and aerobic respiration. ROS has been shown to be an essential physiological messenger in cellular systems such as cancer, stem cells, and the immune system (7), but can cause damage to DNA, lipids, and proteins when accumulated (11)(12). Most cells have an organelle or an enzyme that can pump out ROS or chemically convert it to a safer substance, such as catalase that converts hydrogen peroxide to water and oxygen gas. In addition to their specific biological targets, members of the three major classes of antibiotics indirectly harm the cell by triggering the production lethal amounts of ROS, mainly hydroxyl radicals. The large amounts of hydroxyl radicals come from a metabolic deficit leading to the oxidation of iron-sulfur clusters, called the Fenton reaction, that triggers the release of these hydroxyl ions that work like a wrecking ball in the cell damaging all types of cellular structures (6)(7).

1.3 Green Fluorescent Protein

Green fluorescent protein (GFP) is a bioluminescent protein that was discovered in the jellyfish *Aequorea victoria* by Shimomura et al. in 1962 (11). The protein becomes bioluminescent by absorbing blue light at 470 nm created by the protein aequorin and emits green light at the 508 nm wavelength in the jellyfish (14). GFP consists of 238 amino acids long and is fairly stable and resistant to photobleaching and a wide range of environments due to its beta barrel structure, which is composed of 11 beta strands with 6 alpha helixes (15). The chromophore sits in the middle of the beta barrel and consists of

amino acids serine at 65, tyrosine at 66, and glycine at 67 (13). These amino acids together form a p-hydroxybenzylidene-imidazolidinone structure that requires oxygen to become fluorescent. Due to this, GFP cannot be used in anaerobic organisms, limiting its range of research (16).

1.3.1 GFP Beginnings

From the beginning, GFP showed real promise as a local reporter protein and as a fusion tag in cells due to how easily it can be transformed and its ability to be tracked in real-time with long wave UV light source and not needing a cofactor to be fluorescent (17)(13). Before more in-depth research could be done with GFP, it needed some improvements to be a better molecular tool. GFP being from a cold-water jellyfish of the north Pacific Ocean, does not fold well or fluoresce at warmer temperatures like 37°C (13). To improve this, mutations were made to and around the chromophore that consisted of serine to threonine at 65 and phenylalanine to leucine at position 64. These mutations allow for better protein synthesis, folding efficiency, and a 30-fold increase in brightness. This new version of GFP was termed enhanced GFP, EGFP, and acts very similar to the wildtype GFP but its excitation changed from two peaks to only one maximum peak at 488 nm (18). EGFP's mutations had such an improvement that it is used as the basis for almost every type of GFP research today (19)(20)(21). Dr. Hindmarsh's lab has plans to use this EGFP in the yeast *Candida albicans,* as well. *C. albicans* belongs to a group referred to as the CTG clade. For the codon CTG, most species encode leucine while *C. albicans* encodes serine instead. To further expand GFP's capabilities, it was enhanced for yeast cells and other singled celled organisms such as *E. coli* where the yeast enhancement mutations work very well. EGFP was

optimized for *C. albicans* by replacing all CTG codons for a different leucine codon and replacing a few other amino acids to optimize the coding sequence for *C. albicans*. When transformed into *E. coli,* there is 75x more fluorescence in these cells than in the original wild type GFP cells (2).

1.3.2 GFP Color Variations

Utilizing the color of proteins has high potential for research purposes. With only one or two amino acid mutations, GFP has the ability to fluoresce at multiple colors. Useful research color variants that have be used so far are blue, cyan, and yellow (22). They are used together to track multiple targets at once and each color mutation comes with its own special abilities. Mutating glutamic acid at position 69 to lysine (E69K) and threonine to tyrosine at position 203 (T203Y) changes the fluorescence yellow. Yellow fluorescent proteins are best at withstanding extreme temperatures and have the ability to refold after denaturing temperature exposure (23). Mutating tyrosine to tryptophan at position 66 (Y66W) changes the green to a cyan color. Cyan fluorescent proteins are good at withstanding wide pH ranges (13)(15). Another useful color mutation is tyrosine to histidine at position 66 (Y66H) creating a blue color. Blue fluorescent proteins are mainly used in tandem with GFP to track multiple proteins at once (13). Of the color mutants, blue tends to photo bleach and fold more poorly than the rest (24).

1.3.3 Problems with GFP

As discussed earlier, ROS are essential to some physiological processes but can be harmful in cells if it accumulates and can cause damage to DNA, lipid, and membranes (7). EGFP creates one hydrogen peroxide molecule as the chromophore matures to fluorescence using an oxygen molecule. While this process does not create

enough hydrogen peroxide to be dangerous to the cell, it is enough to act as a cellular messenger. This has the ability to alter research results in and can have severe consequences in many mammalian cells by causing random signals to come and go changing metabolism, stress response, or homeostasis (11).

1.3.4 Oligomerization of GFP

As widely used as GFP is, in certain environments it is not an optimal fluorescent reporter protein. GFP suffers in oxidative environments like the endoplasmic reticulum, an organelle of eukaryotic organisms, where it does not fold properly or fluoresce due to oligomerization and pH sensitivity (19)(20)(25). Oligomerization occurs through disulfide bridges across GFP's two naturally occurring cysteines at positions 48 and 70 (21), causing GFP to be secreted from the cell as a dimer (25), rendering it an ineffective tool. Jain et al. (2006) researched this issue and showed that by substituting the cysteines with serine, a similarly structured amino acid, the folding ability was restored but the fluorescence was reduced (19). Another way this issue was overcome was by creating two new variants of GFP, "fast folder" (FF) and "superfolder" (SF) (26). The fast folder and superfolder GFP were created by starting off with the EGFP mutations, F64L and S65T, then adding the cycle 3 mutations, F99S, M153T, and V163A, and lastly, mutating, Y39N, N105T, Y145F, and I171V to make the fast folder variant and adding S30R and A206V to the fast folder to make the superfolder variant. The addition of all of these mutations improved the folding ability of GFP by 50-fold. Fast folder and superfolder GFP have been shown to be a reliable and useful tool in research for biosensing and monitoring with fusion proteins and in oxidative environments such as the endoplasmic reticulum (26). These GFP variants fold quickly enough that dimers and

oligomers do not form, allowing for it to remain effective and fluorescent (20) and allows for the molecular trafficking through the pH-varied organelles of eukaryotes (25). These fluorescent proteins maintain their fluorescence and are much brighter than only substituting the cysteines out (21).

1.4 Biosensor Capabilities

Green fluorescent protein has many capabilities and has progressively become one of the most used fluorescent proteins for research. One of these discovered functions is as a biosensor. A biosensor is a biological molecule that is used to detect chemicals or changes in the environment. A GFP biosensor changes its fluorescence in the presence of a substance or molecule. GFP has already been used successfully to detect redox reactions (26), calcium ions (27), and reactive oxygen species (1). To determine the redox potential of the environment, Hanson et al. mutated GFP at specific points along the beta barrel around the chromophore. These positions, 147/204 and 149/202, were substituted for cysteine, an amino acid that has a thiol group allowing for a disulfide linkage to form. The paired amino acids are in close proximity to each other to allow formation of a disulfide bridge. The presence of ROS creates an oxidizing environment, which in turn, forms the disulfide bond between them putting strain on the chromophore and changing its excitation spectra. The change in fluorescence can be measured and correlated to the amount of ROS generated in the cell. To keep the biosensor from making mistaken linkages, the two natural cysteines were substituted for serine at 48 and alanine at 70 to hinder any false readings. They concluded that the 147/204 mutant GFP was sensitize to an ROS environment and responded quickly (1).

CHAPTER 2

METHODS

2.1 Creation of yEGFP, royGFP and Fast Folder variants

The two main GFP strains used in this research was yeast-enhanced GFP, yEGFP, and redox-sensitive yeast-enhanced GFP, royGFP. yEGFP and royGFP were both created in Dr. Hindmarsh's lab using Quik-Change Mutagenesis by Claire Jones. Both strains contain the yeast codon optimization because yeast are a part of the CTG clade, where CTG codes for serine instead of leucine and requires optimization of non-yeast proteins to function properly in its cells. These mutations make GFP 75x brighter when transformed into *E. coli* cells (2). The plasmids also contain enhanced mutations, F64L and S65T. These mutations are critical because GFP is from a cold-water jellyfish and they allow for GFP to fold and fluoresce better at higher temperatures like 37°C. royGFP contains two substituted cysteines at positions 147 and 204 that allow it to be a biosensor, yEGFP does not contain these mutations.

yEGFP and royGFP fast folder variants were made in Dr. Hindmarsh's lab by Doug Ferrell. They were created from Ms. Jones' strains and were Quik-Changed with the mutations F99S, M153T, V163A, which are called the cycle-3 mutations. The fast folders also contain Y39N, N105T, Y145F, and I171V. The substitutions of these amino acids allow the proteins to fold much quicker and fluoresce brighter.

2.2 Western Blot of yEGFP

To begin, an overnight culture of yEGFP was performed by adding 5 µL of Ampicillin and a colony from a TSA-Ampicillin plate to a 5 mL TSB tube. The next day, 200 µL of the overnight culture of yEGFP *E. coli* cells were pipetted into a 100 mL TSB flask along with 100 µL of Ampicillin. The culture was incubated for 4-5 hours to reach an optical density of 0.85-0.95. Four separate sterile tubes were filled with 6 mL of culture from the flask. A different concentration of hydrogen peroxide was added to each tube; 0 µL, 40 µL, 160 µL, and 200 µL per milliliter of cells, equally 0, 8.7, 33.8 and 146.7 mM. The cells and hydrogen peroxide mix were then incubated for 4 hours at 37°C in an incubator/shaker. After incubation, 3 mL were removed from each tube and spun down in a 1.5 mL Eppendorf tube for 5 minutes at 14,800 rpm and the supernatant were poured off. This sample was stored at -80°C, if needed.

A lysozyme buffer was created to lysis the cell wall to retrieve the GFPs from the cells. The buffer consisted of 500 µL of 1M Tris 8.0 pH, 3 mL of 5M NaCl, 50 µL of 200x lysozyme, 5 µL of 1M $CaCl_2$, 50 µL of 1 M $MgCl_2$, 10 µL of 0.1% Triton x100, 100 µL of 100x phenylmethylsulphonyl fluoride (PMSF), a protease inhibitor, and 6.375 mL of distilled water. The amount of lysis buffer was added based on the size of the pellet. Most experiments required 400 µL of lysis buffer for the tube with 0 µL hydrogen peroxide due to a larger pellet. Tubes with 8.7 and 33.8 mM of hydrogen peroxide received 200 µL of lysis buffer for a medium sized pellet and the last tube, 146.7 mM of hydrogen peroxide, received 100 µL of lysis buffer due to the smaller pellet. The PMSF and lysozyme was re-added for every repeated experiment attempted to make sure the lysozyme was not degraded for later uses. The samples were incubated for 4 hours at

37°C. The samples were then spun down at 14,800 rotations per minute (RPM) for 5 min creating a pellet and supernatant. The supernatant is transferred to another tube to be tested in a western blot.

The gel electrophoresis was setup in a vertical stand and filled with the running buffer. 15 μL of sample was mixed with 5 μL of dye in a 1.5 mL Eppendorf tube and then heated for 10 min at 95°C. 5 μL of dye was preloaded into the wells to make them more visible and have more dye for the sample. The samples were added in order of increasing hydrogen peroxide concentration, such as 0, 8.7, 33.8, 146.7mM. The gel electrophoresis was run and then soaked in reagent alcohol. The transfer was set up with a thin sheet of paper and a foam pad that was laid on both sides of the gel and soaked in the excess alcohol. The gel was then placed in the transfer mechanism facing the positive side. The transfer was run overnight to get a full transfer to the PVDF membrane. Next, the membrane was blocked with a milk/tween mixture. It consisted of 10 mL of 5x TBS, 40 mL of water, 50 μL of tween and 2.5 g of powdered milk. The membrane was soaked in half the mixture for a one 1-hour segment and then refreshed the mixture for another hour of blocking. Next, the membrane was probed with a tween mixture containing the GFP antibody. It contained 10 mL of 5x TBS, 40 mL of water, 50 μL of tween, and 0.5 μL of antibody. The membrane was washed twice with half of the mixture each time to get full coverage. Lastly, the membrane was washed with the same tween solution without any milk or antibody. This allowed for the wash to remove any unbound antibodies except the antibody that is connected to the protein sample. We then visualized the membrane using enhanced chemiluminescence (ECL).

2.3 Quik-Change Mutagenesis of royGFP Positions 48 and 70

2.3.1 Quik-Change Mutagenesis

Three different mutations were generated to determine if royGFP could maintain fluorescence with substitutions. A polymerase chain reaction (PCR) was completed on royGFP plasmid cells to create the three different mutations. Each PCR tube contained 12.5 μL of water, 5.5 μL of Taq buffer, 5.5 μL of dNTPs, and 1 μL of the forward and reverse primers, and 2 μL of Taq polymerase. The first reaction, N1, had the primers for a cysteine to methionine mutation (C48M) at position 48 on the GFP peptide chain. Reaction two, N2, was cysteine to valine (C48V) at position 48. And lastly, N3, was cysteine to glutamine (C70Q) at position 70. After the PCR reaction, it went through a Dpn1 digestion. DNA extracted from cells have been methylated because Dpn1 digests the methylated template but does not digest the amplicon. This was done by adding 1 μL of Dpn1 and was incubated overnight at 37°C. 120 μL of DH5α *E. coli* cells were put into three 1.5 mL Eppendorf tubes, one for each reaction. 5 μL of the Quik-Change reactions was added to an Eppendorf tube with the cells. These tubes were then incubated for 30 minutes on ice and then switched to 42°C for 90 seconds. 900 μL of TSB was added to each tube and then were placed in an incubator/shaker for one hour at 37°C. After incubation, 185 μL of the sample was plated on a TSA-Ampicillin plate and incubated overnight.

2.3.2 Colony PCR

A colony PCR is performed to determine if the plasmid was transformed into the DH5α *E. coli* cells. Colonies were picked from the plates with a toothpick and added to a 1.5 mL Eppendorf tube with 20 μL of sterile water, which was used as the DNA template

for the colony PCR. The toothpick was twirled and then streaked on a TSA-Ampicillin plate on a grid and then incubated overnight. The plate was checked the next day to determine if the colonies grew and if they had a green tint to them from the protein production. A master mix was made and dispensed to 20 PCR tubes that received 17.5 µL of water, 5.5 µL of Taq buffer, 5.5 µL of dNTPs, 0.5 µL of the forward primer 404, 0.5 µL of the reverse primer 359, and 2 µL of Taq polymerase. Ran at the colony PCR settings and gel electrophoresed to determine the size of the plasmid.

2.3.3 Fluorescence Reading of Quik-Changed royGFP

Cultured 10 positive samples from the Quik-Changed GFPs and added 1 µL of Ampicillin overnight. 300 µL of the samples were added in order of the respective number to the 96 well plate and was read 10 times at 400 nm wavelength in the fluorescence reader against wildtype royGFP as a control.

2.4 Fluorescence Reading Test

yEGFP and royGFP colonies were taken from their respective TSA-Ampicillin plates and placed into a 5 mL TSB tube using a sterile toothpick and were grown overnight without Ampicillin. The next day, the samples were looked at under a microscope to check for density, intensity of fluorescence, and amount of non-fluorescent cells. Samples with higher density, fluorescence, and low amounts of non-fluorescent cells were selected.

Through trial and error, it was found that by adding 10 µL of cells from the overnight culture to a fresh 5 mL of TSB resulted in about a 0.01 OD_{600} starting point. It was then incubated for 2 hours to get to approximately 0.4 OD_{600}, the Log phase of

bacterial growth. As the experiment progressed, the variables were expanded upon, increasing the incubation time to 4-5 hours to acquire an OD_{600} of 0.75-0.85.

150 µL of yEGFP and royGFP cells were placed in a 96 well plate in adjacent rows. Using either hydrogen peroxide or antibiotics, the samples were serial diluted starting at 5% of the well and diluting down to either 10^{-5} or 10^{-9} dilution in TSB. The dilutions were added in order from highest to lowest concentration after adding TSB as the control in the first well. The hydrogen peroxide dilutions were pipetted into the cell samples and mixed thoroughly. Testing the excitation of each well, fluorescence was read five times at one point in time at wavelengths 485 nm, yEGFP's and reduced royGFP's excitation, and 400 nm, oxidized royGFP's excitation, with both having an emission of 525 nm. To put the results into a readable and comparable set of numbers, the 400 nm reads were divided by the 485 nm readings of the same set to give a ratiometric value. As royGFP is oxidized it will have more 400 nm reads increasing its ratiometric value showing ROS production of the stress response. Time of test was increased to allow for more time for the biosensor to work. The fluorescence was read every 15 or 30 minutes for 3 to 12 hours.

2.4.1 Antibiotic Synergistic Fluorescence Test of royGFP

Antibiotic synergistic test was conducted to determine if combining two antibiotics at the same time would result in an increase of ROS production. The same fluorescence test as previously described was set up but with differing dilutions of antibiotics. Kanamycin was tested at 5% of the well's volume, 4.3 mM, as a control and 0.5 % of Kanamycin, .43 mM, was mixed with a similar potency of each antibiotic, 5.3 mM of Chloramphenicol, 14.3 mM of Ampicillin, and 0.23 mM of Norfloxacin.

2.4.2 Fluorescence Test of royGFP with Thiourea and DTT

Fluorescence tests were conducted with the reducing agents thiourea and dithiothreitol (DTT), to support my hypothesis that ROS is actually the molecule oxidizing the cysteines on the royGFP biosensor. The same fluorescence test as previously discussed was conducted with 75, 150, and 200 mM of DTT. DTT was tested against 4.3 mM of Kanamycin and TSB alone.

CHAPTER 3

RESULTS

3.1 Western Blot Analysis of yEGFP

Yeast-enhanced green fluorescent protein, yEGFP, was treated with hydrogen peroxide solutions as an external reactive oxygen species (ROS) source and showed no evidence of oligomerization in western blots as shown in figure 3-1. Initially, the protein was treated for four hours in hydrogen peroxide dilutions and no oligomerization occurred. With further testing, the proteins were treated for fifteen minutes and overnight in differing hydrogen peroxide concentrations. The concentration of hydrogen peroxide and the time of treating, as well as, the amount of protein had no effect on oligomerization. 10 western blots were tested in this manner and in every test the GFP remained as a monomer, a size of 27k Daltons.

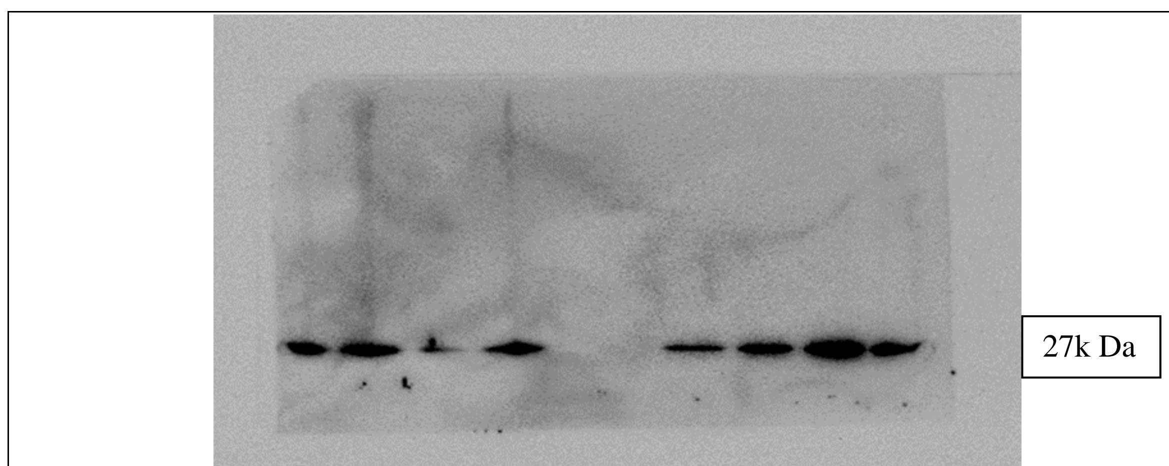

Figure 3-1: Western blot of yEGFP in an SDS-PAGE. From the left, lanes 1-4 are samples treated with 0, 8.7, 33.8, and 146.7 mM of hydrogen peroxide for 15 minutes. Lane 5 is the ladder, not seen due to not using an antibody for visualization. Lane 6 is DH5α, a negative control. Lanes 7-10 are samples treated with 0, 8.7, 33.8, and 146.7 mM of hydrogen peroxide overnight.

3.2 Quik-Change Mutagenesis of royGFP

The Quik-Change mutagenesis to royGFP and yEGFP were successful with royGFP acquiring the cysteine to valine at position 48 (C48V) and yEGFP acquired cysteine to valine at position 70 (C70V). Both strains grew very slowly and inconsistently making it difficult to find an average growth rate for fluorescence test. royGFP formed strands and were not brighter than the wildtype royGFP. The mutant yEGFP could grow as bright as wildtype yEGFP but much less consistently.

3.3 Fluorescence Reading Test

3.3.1 Testing yEGFP vs royGFP in Hydrogen Peroxide

yEGFP and royGFP were grown to 0.90 OD_{600} and tested in a fluorescence reader in differing concentrations of hydrogen peroxide diluted in water. royGFP did not show any sensitivity to the hydrogen peroxide compared to its control and yEGFP as shown in

figure 3-2. yEGFP acted as we predicted, maintaining a ratiometric value similar to its control. These tests hold little value due to the royGFP control being higher than the hydrogen peroxide treatments.

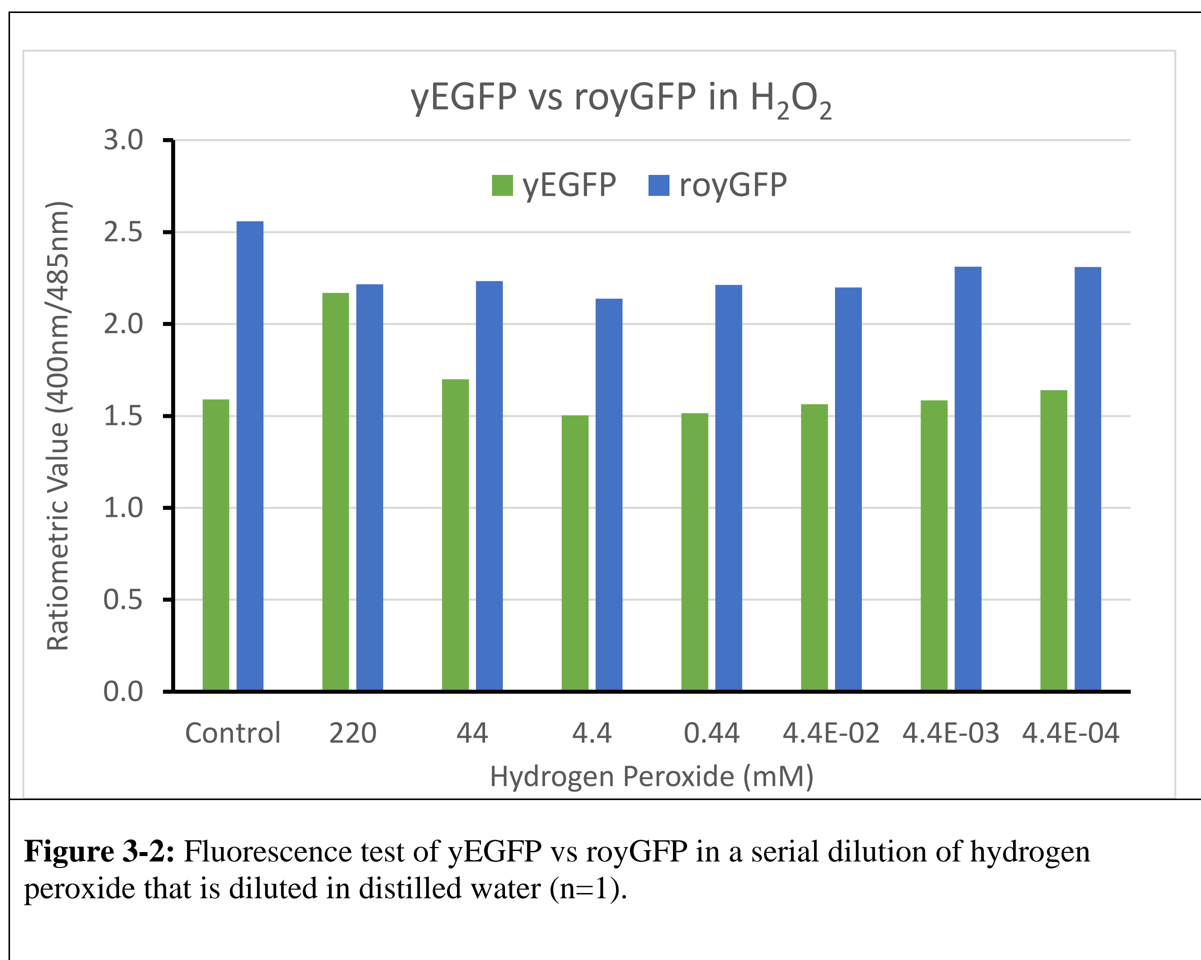

Figure 3-2: Fluorescence test of yEGFP vs royGFP in a serial dilution of hydrogen peroxide that is diluted in distilled water (n=1).

3.3.2 Testing yEGFP vs royGFP with differing medias

In an effort to analyze royGFP's sensitivity to ROS, the fluorescence test was expanded to diluting the hydrogen peroxide in TSB media instead of water. During this time, we also experimented with growing the cells to an OD_{600} of 0.4-0.5. As shown in figure 3-3, the ratiometric values were much more consistent using TSB and made the control values

more similar. royGFP did not show much sensitivity but it was an improvement on the overall test.

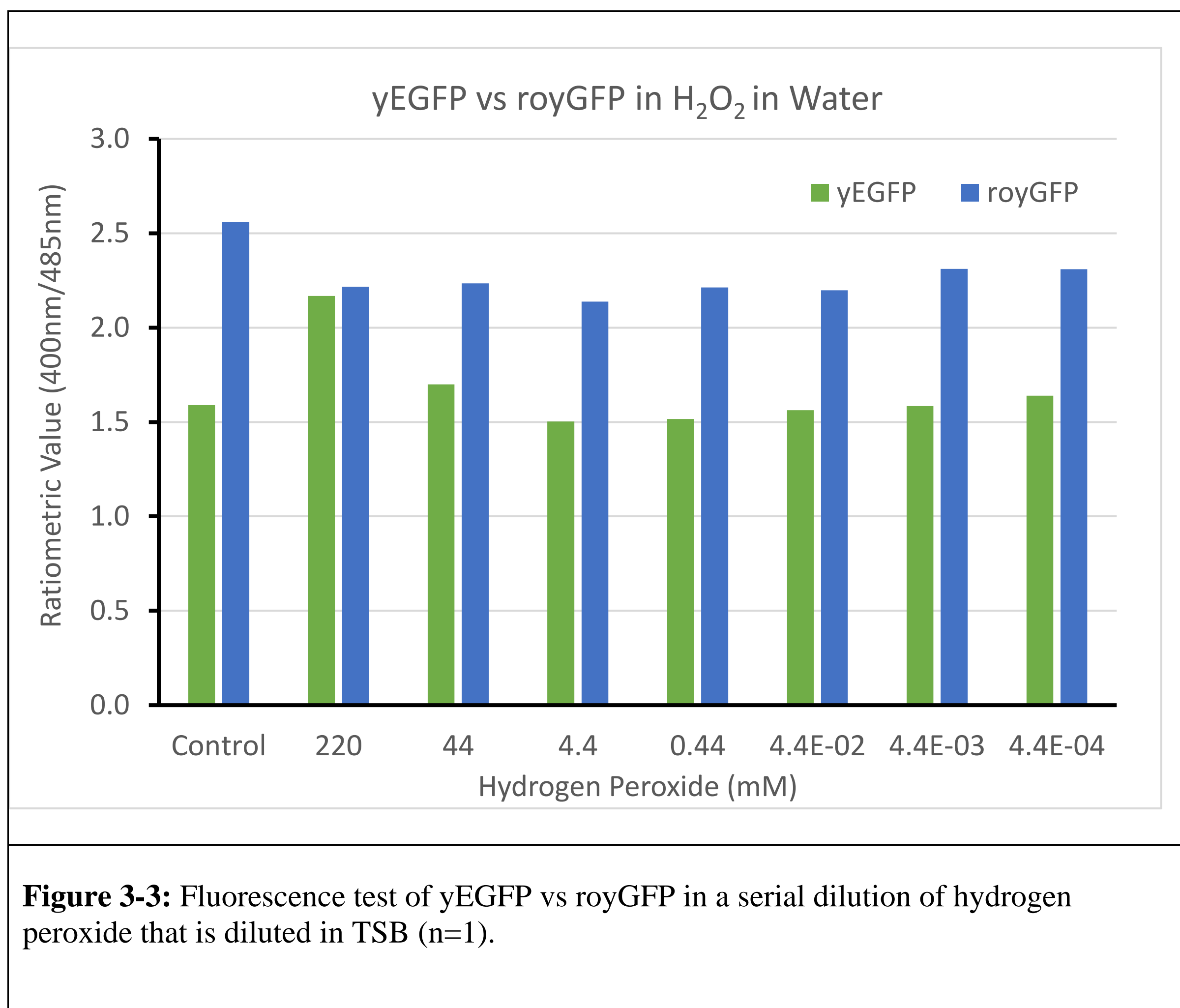

Figure 3-3: Fluorescence test of yEGFP vs royGFP in a serial dilution of hydrogen peroxide that is diluted in TSB (n=1).

3.3.3 Testing yEGFP vs royGFP in the fluorescence reader overtime

Using an external ROS source, I believed the hydrogen peroxide did not have enough time in the few minutes of the test to have an effect on royGFP to show sensitivity to it. The time of test was increased from one to two hours and the fluorescence was read in fifteen-minute intervals. All other variables remained the same to determine if time is a factor. The results of increasing the time showed consistent royGFP sensitivity to its control and to yEGFP. Though, slight at first, but with time, a few dilutions had higher

ratiometric values than the control and yEGFP showing sensitivity. As shown in figure 3-4, the 220 mM of hydrogen peroxide solution created a higher ratiometric value than the other dilutions and control slowly got lower. Another improvement with time, allowed for the control to level out and remain lower than all of the dilutions that did contain ROS.

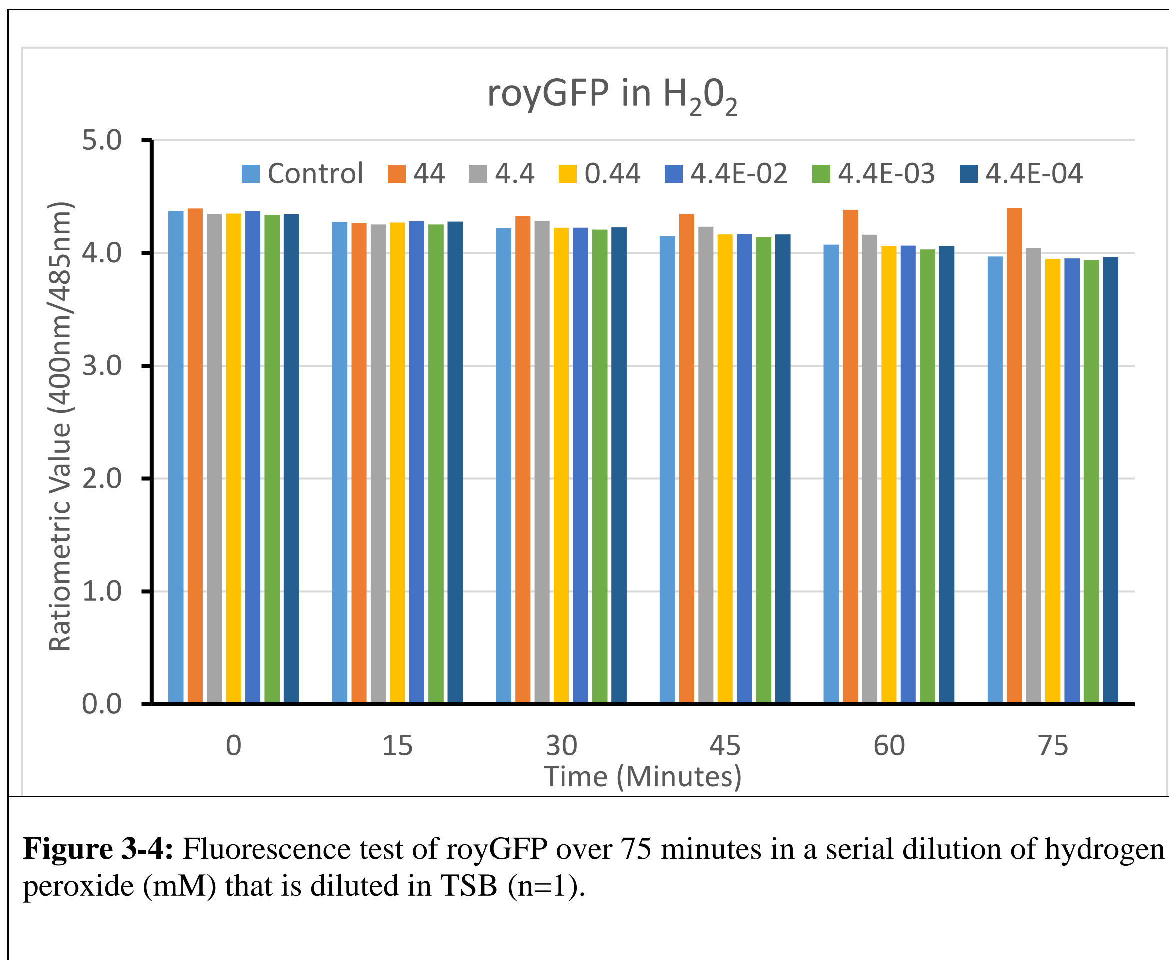

Figure 3-4: Fluorescence test of royGFP over 75 minutes in a serial dilution of hydrogen peroxide (mM) that is diluted in TSB (n=1).

3.3.4 Growing yEGFP and royGFP without Ampicillin

Plasmids usually are maintained in cells using a nutritional or drug marker. Ampicillin is a common drug to maintain plasmids. In the absence of a selectable drug there is no reason for the cell to maintain the plasmid, and plasmids are usually lost over

time. Both yEGFP and royGFP contain an Ampicillin resistance gene that gives it a genetic advantage and allows us to isolate the strains when the cells are cultured. Even though royGFP has started to show slight sensitivity, I believed that growing royGFP with Ampicillin was sensitizing it to other antibiotics. The cells were grown on TSA-Ampicillin plates but subsequently switched to liquid cultures and were grown without Ampicillin. Figure 3-5 shows the cells grow denser and brighter after just one day and maintain this for a week before returning to normal.

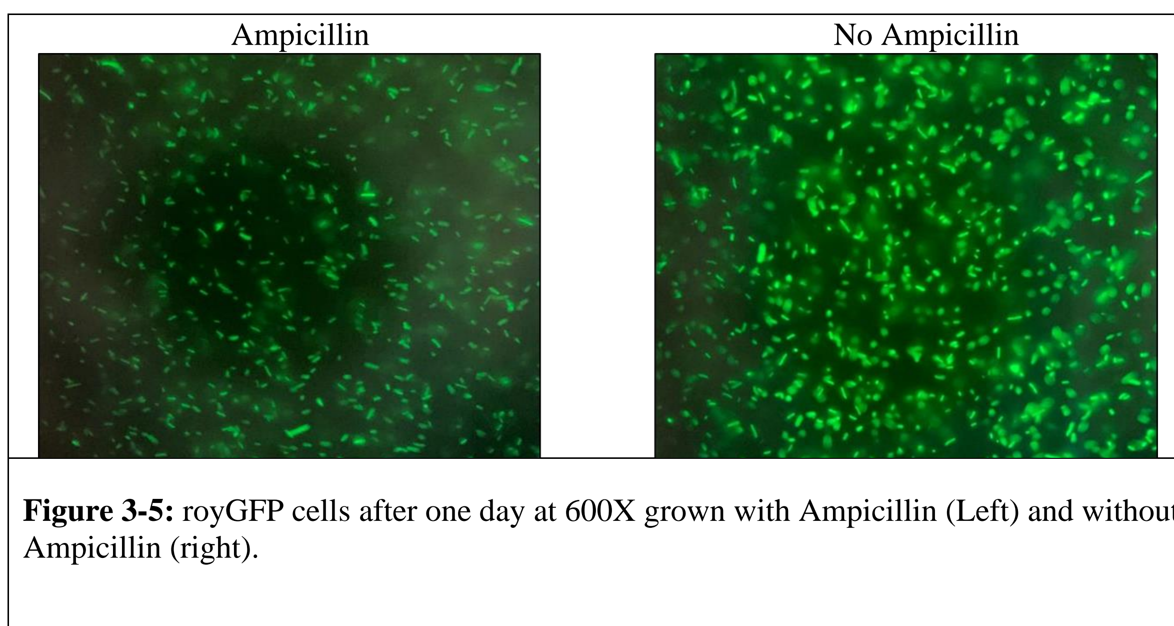

Figure 3-5: royGFP cells after one day at 600X grown with Ampicillin (Left) and without Ampicillin (right).

I repeated the fluorescence test with the cells that were grown without Ampicillin and increased the OD_{600} of the cells to 0.5 and tested it overtime. As shown in figure 3-6, royGFP started showing sensitivity to the ROS from the beginning of the test and increased in ratiometric value over the two-hour test. As well as, more than one dilution of hydrogen peroxide had a higher ratiometric value than the control and yEGFP.

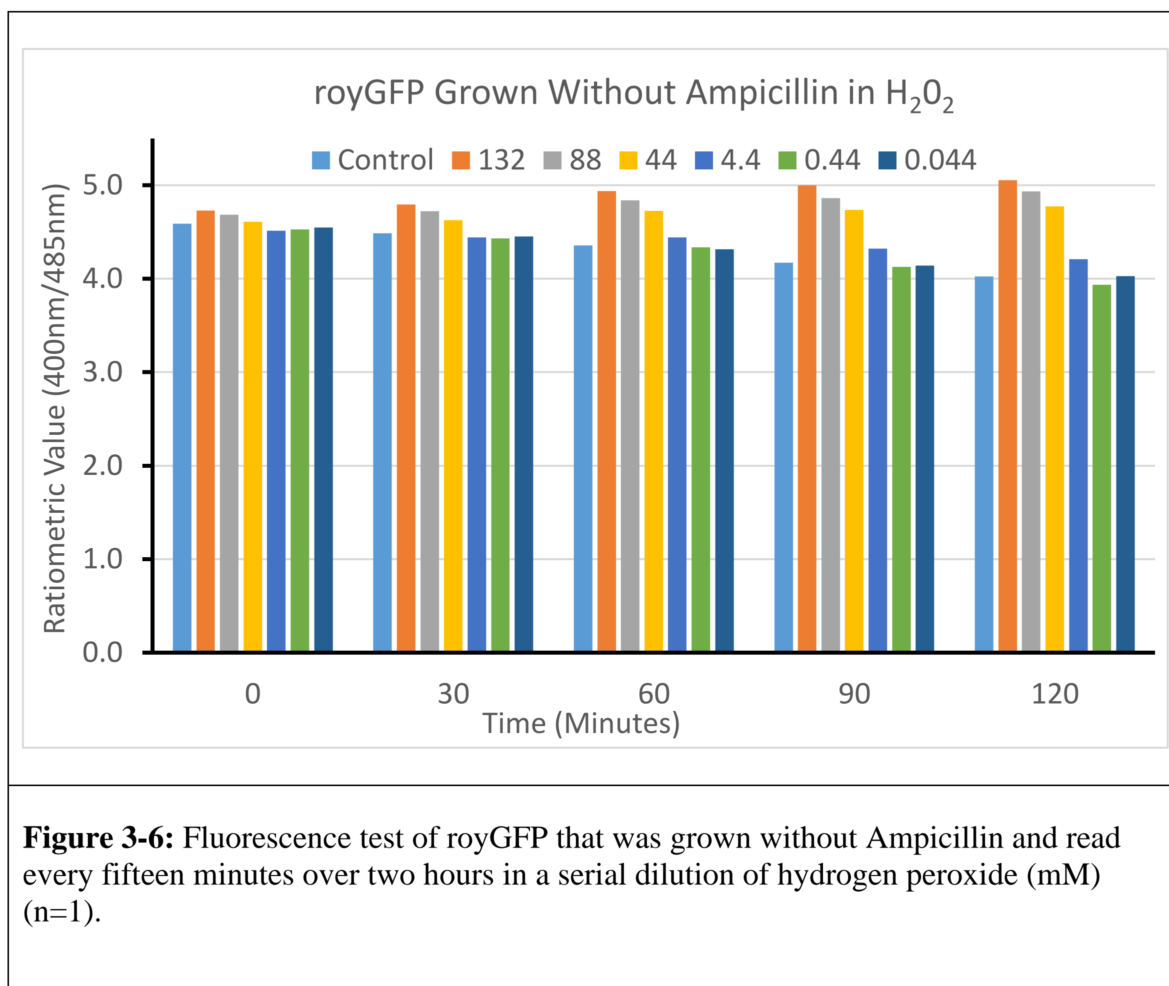

Figure 3-6: Fluorescence test of royGFP that was grown without Ampicillin and read every fifteen minutes over two hours in a serial dilution of hydrogen peroxide (mM) (n=1).

3.3.5 Testing yEGFP vs royGFP in Kanamycin

After stabilizing the fluorescence test and finding the right conditions for growth of the cells and the time of test, I conducted fluorescence test with royGFP and yEGFP in differing concentrations of Kanamycin and/or Ampicillin. Cells were grown to an OD_{600} of 0.75-0.85 and the test was extended to three hours. Increasing the time had a significant effect on royGFP's sensitivity so the test was tested out to twelve hours to find the most optimal time point. In figure 3-7, royGFP's ratiometric value was higher than its own control and higher than the yEGFP ratiometric values in figure 3-8, indicating a sensitivity to ROS production.

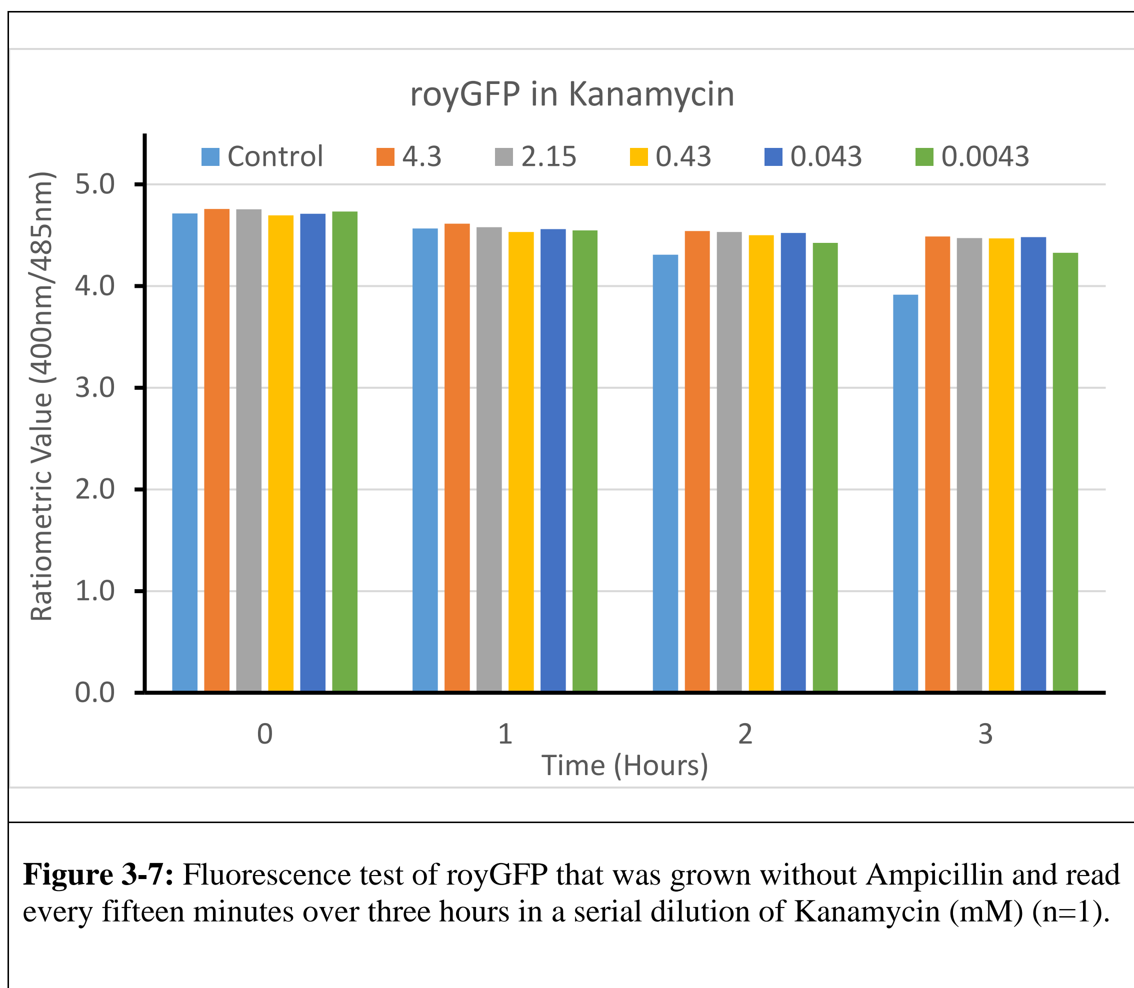

Figure 3-7: Fluorescence test of royGFP that was grown without Ampicillin and read every fifteen minutes over three hours in a serial dilution of Kanamycin (mM) (n=1).

The yEGFP results showed a lower ratiometric value compared to the royGFP ratiometric values but was also increased over its own control. This test was repeated over twelve hours and had its fluorescence read every thirty minutes. The OD_{600} of the test was settled around 0.75-0.80 believing that when they were diluted in half with the antibiotics that they would be beginning their growth phase at 0.37-0.40 OD_{600}.

3.3.6 Twelve-Hour Fluorescence Test

Over twelve hours, figure 3-8 shows that royGFP's sensitivity gets better with time. There is no significant difference between the control and the dilutions for the first two hours but becomes significant at three hours with a p-value of 0.04 and remains

significant with more time with p-values of 0.024 at 6 hours and 0.00044 at 9 hours. Results are not shown after 9 hours due to evaporation of the liquids, concentrating the solution.

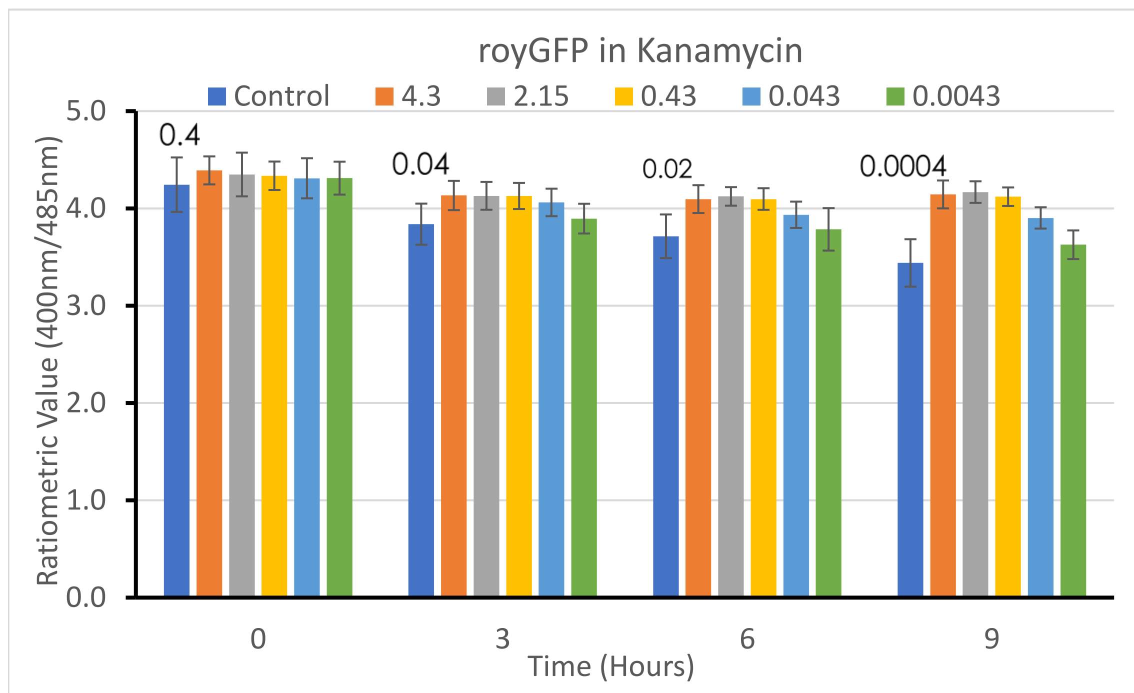

Figure 3-8: Three-day average ratiometric value of royGFP fluorescence read over 12 hours in Kanamycin dilutions (mM) with 95% confidence error bars and p-values between the control and 4.3 mM concentration (n=4).

For yEGFP, the non-redox sensitive strain, a univariate test comparison between the control and the dilutions is not significant at any time point except for the three-hour time point as shown in figure 3-9. yEGFP had a difference between the control and dilutions but not nearly as significant as royGFP. It also had lower ratiometric values for the duration of the test. Comparing the 4.3 mM dilution of Kanamycin for royGFP and

yEGFP on the graphs results in a significant P-value at all time points. This shows that with a lack of ROS, royGFP and yEGFP exhibit the same excitation and no sensitivity.

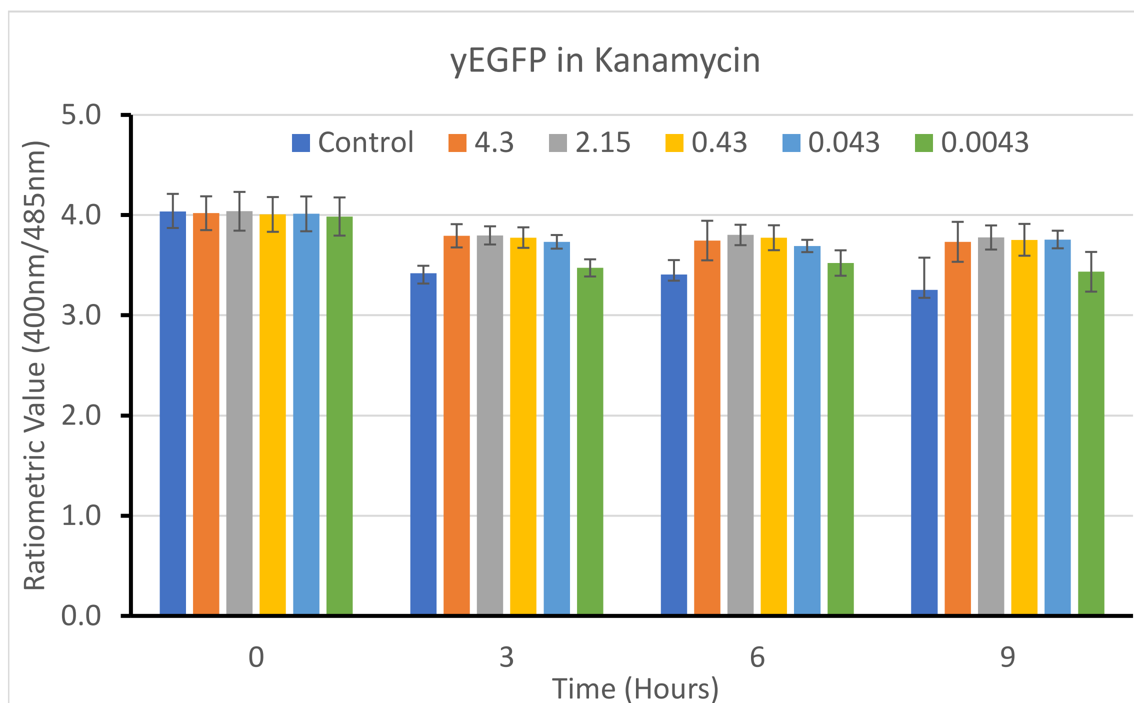

Figure 3-9: Three-day average ratiometric value of yEGFP fluorescence read over 12 hours in Kanamycin dilutions (mM) with 95% confidence error bars and p-values between the control and 4.3 mM concentration (n=4).

Figure 3-10 shows a comparison between yEGFP and royGFP at 9 hours. The control of yEGFP and royGFP act very similar and show no significant difference due to the lack of ROS in the control. This is as we had hypothesized. At the higher Kanamycin concentrations, 8.6 mM, 4.3 mM, and 0.86 mM, royGFP shows a significant difference compared to the same concentrations at yEGFP. The lower concentrations of Kanamycin have overlapping confidence intervals and are not significantly different showing that there is likely not enough ROS production at such low dilutions.

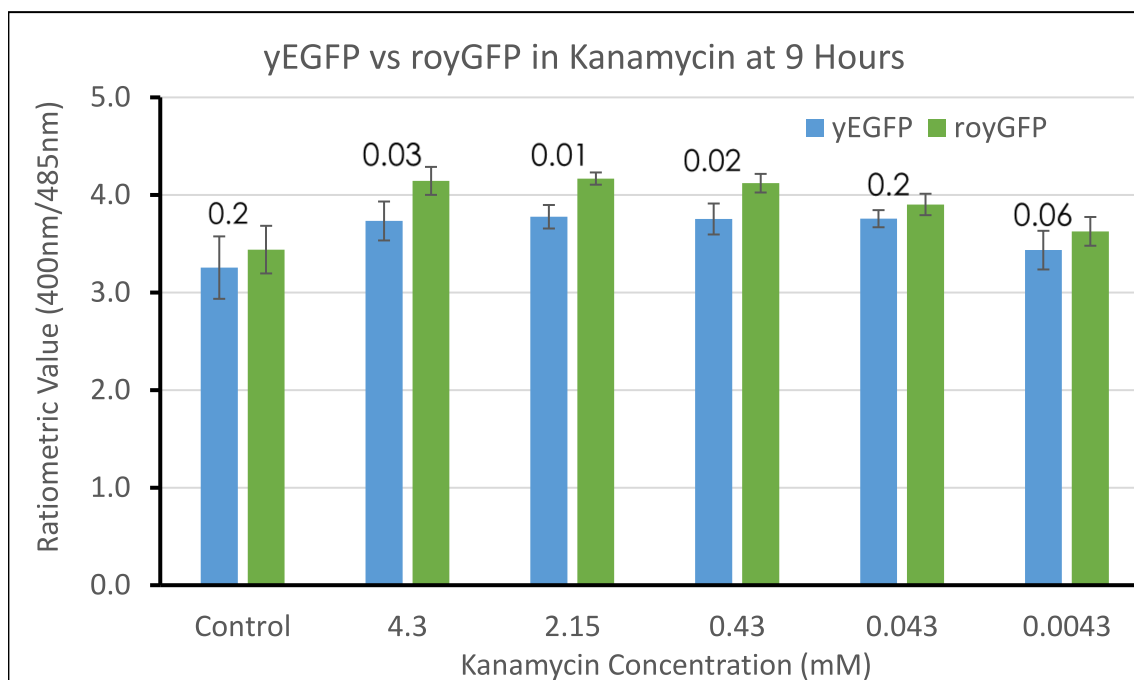

Figure 3-10: Three test average of yEGFP vs royGFP at the nine-hour point of twelve-hour test at the differing Kanamycin dilutions (mM) with 95% confidence error bars and p-values between yEGFP and royGFP at each dilution (n=4).

3.3.7 Testing royGFP in Multiple Antibiotics

After showing royGFP had consistent sensitivity and a significant difference over its own control and yEGFP, it was tested against Chloramphenicol, Ampicillin, Norfloxacin, and streptomycin to determine if it worked with multiple types of antibiotics. Running a twelve-hour fluorescence test with 10%, 5%, and 1% of each antibiotic. As shown in figure 3-11, 5.3 mM of Chloramphenicol triggered some ROS production but not to the same extent as the others. 8.6 mM of Streptomycin and 0.23 mM of Norfloxacin were almost identical to the 4.3 mM of Kanamycin at each time interval, this shows that royGFP works with the same and different types of antibiotics, as well as, shows almost no sensitivity to 14.3 mM of Ampicillin overtime as the cells have

an Ampicillin-resistance gene. With no triggered ROS production, royGFP shows no increase in ratiometric value, supporting our hypothesis that it is ROS triggered by an antibiotic stress response that is causing the change in excitation. At 9 hours, TSB and Ampicillin are not significantly different than each other but they are both significantly lower than Kanamycin, Chloramphenicol, and Norfloxacin.

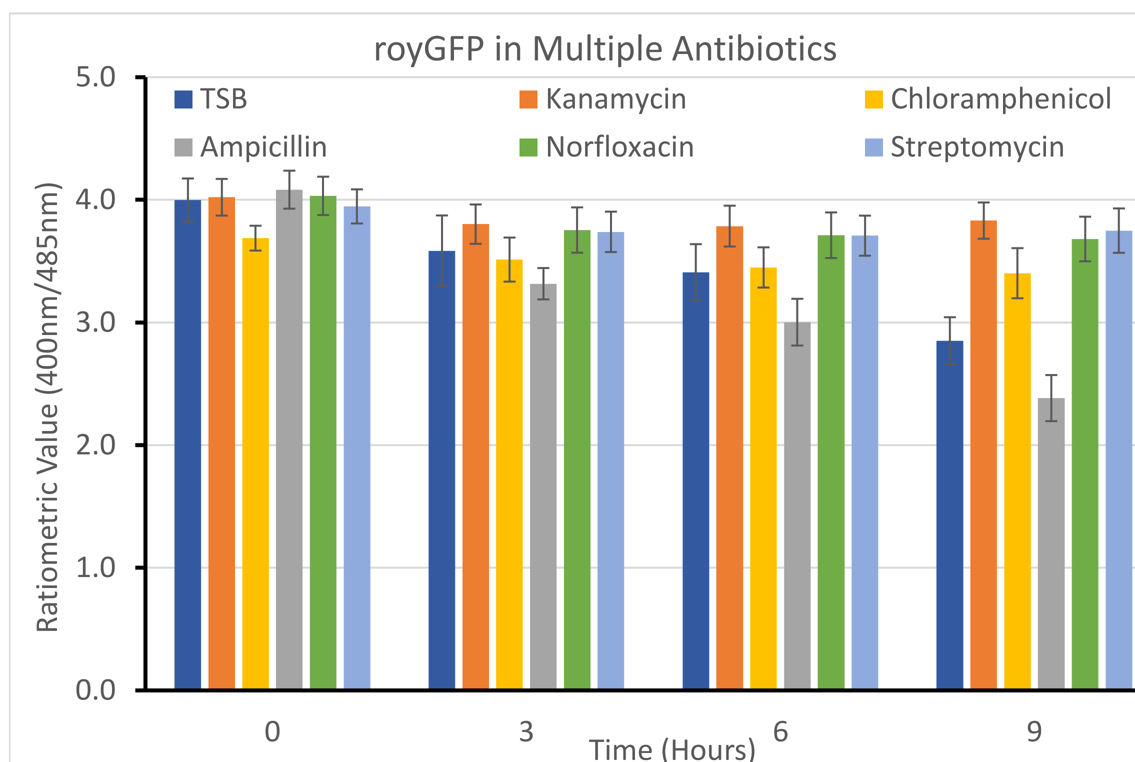

Figure 3-11: Three test average of royGFP in 4.3 mM of Kanamycin, 5.3 mM of Chloramphenicol, 14.3 mM of Ampicillin, 0.23 mM of Norfloxacin, and 8.6 mM of Streptomycin with 95% confidence intervals (n=3).

3.3.8 Testing yEGFP vs royGFP Fast Folders in Kanamycin

Fast folder versions of both yEGFP and royGFP were created in our lab by Doug Ferrell to combat the oligomerization problem of GFP in oxidative environments. The fast folder variants have improved fluorescence and folding ability that hopefully

improve the results of our fluorescence test. As shown in figure 3-12, the fast folder variants acted very similar to the wildtype royGFP and yEGFP. royGFP fast folder gets significantly better with time but due to low control data points, the confidence intervals are much wider than the royGFP wildtypes confidence intervals. royGFP was significant compared to its own control and yEGFP fast folder showing sensitivity to ROS with a p-value of 0.046 at 9 hours which is less significant than the wildtype at 9 hours.

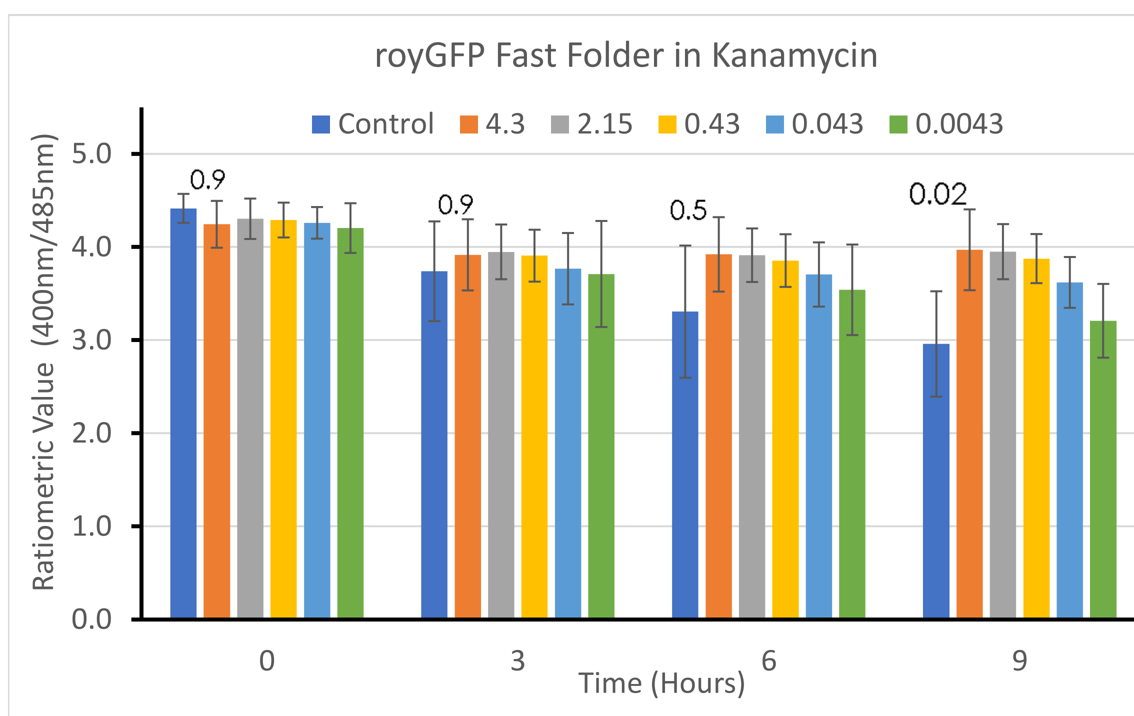

Figure 3-12: Three-day average ratiometric value of royGFP fast folder fluorescence read over 9 hours in Kanamycin dilutions (mM) with 95% confidence error bars with p-values between the control and 4.3 mM dilutions (n=4).

Figure 3-13 shows that yEGFP fast folder was similar to its wildtype variant but had an increased ratiometric value over its own control at nine hours. The values were less than its royGFP fast folder and its brightness seemed to be the brightest of the four fast folder and wild type variants.

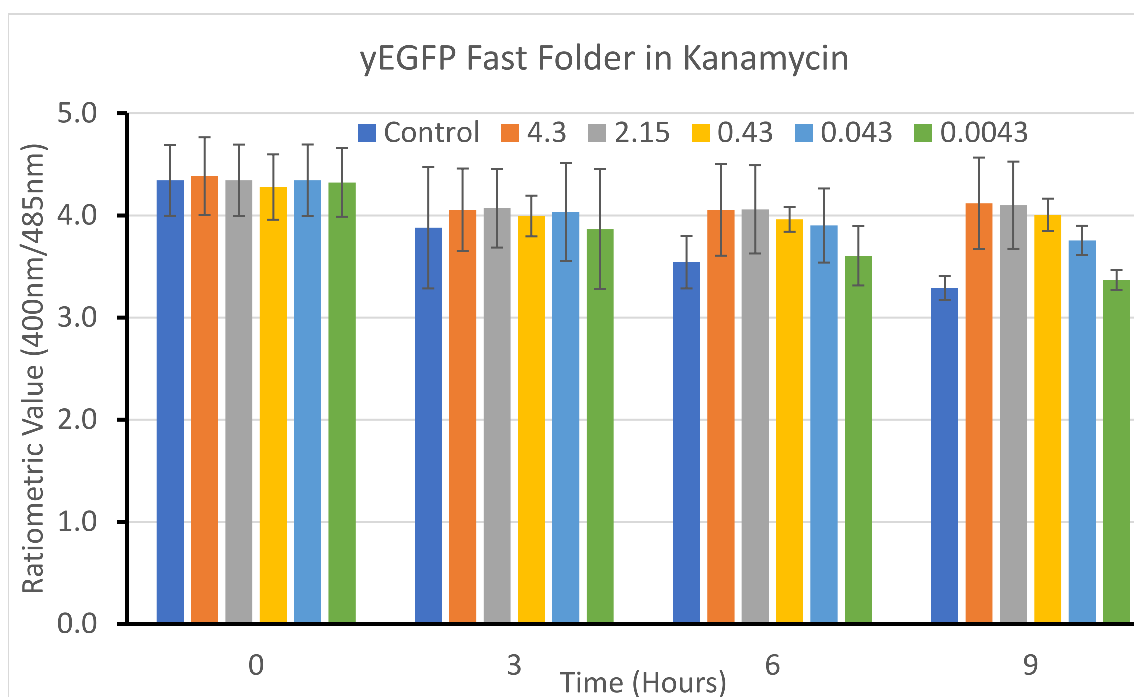

Figure 3-13: Three-day average ratiometric value of yEGFP fast folder fluorescence read over 12 hours in Kanamycin dilutions (mM) with 95% confidence error bars (n=4).

royGFP and royGFP fast folder had no significant difference at any time point or dilution. Figure 3-14 shows that at the 9-hour point, the time of most significance, they are statistically the same. The wild type royGFP on average had a slightly higher ratiometric value and is more significant compare to its own control and yEGFP at 9 hours.

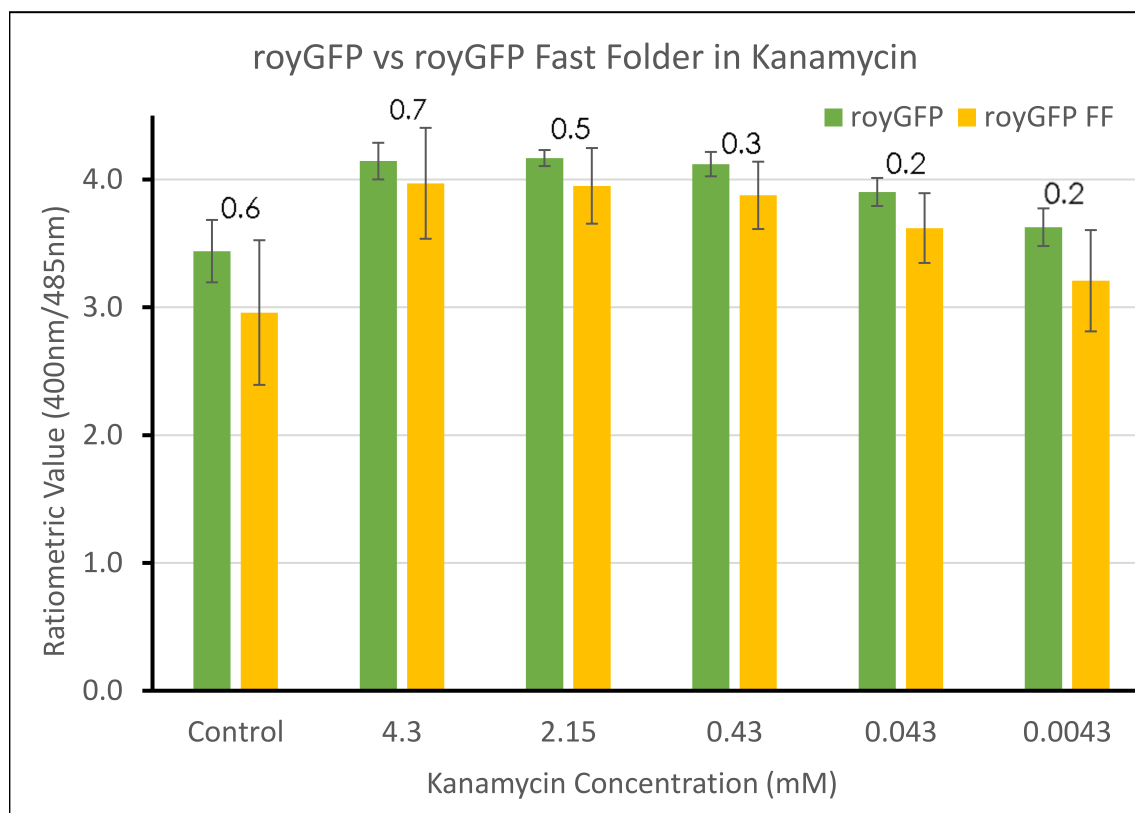

Figure 3-14: Three test average ratiometric value of royGFP vs royGFP fast folder at 9 hours in the differing Kanamycin dilutions (mM) with 95% confidence error bars and p-values between royGFP and royGFP FF at each dilution (n=4).

3.3.9 Testing yEGFP vs royGFP in Synergistic Test

To help combat the rise of antibiotic resistance, differing methods such as using multiple classes of antibiotics to attack the microbe can be a very useful tool at killing them before resistance can be spread. We conducted a fluorescence test with Kanamycin, Ampicillin, Chloramphenicol, and Norfloxacin. Figure 3-15 shows that there is slight increase in sensitivity to a Kanamycin and Ampicillin mix of 0.86 mM and 28.6 mM, respectively. But there was no effect or a worse effect with the other antibiotics. Figure 3-16 shows yEGFP acted as predicted and showed no sensitivity to the ROS production

of the antibiotics and remained consistent throughout the control and dilutions and at a much lower ratiometric value than royGFP.

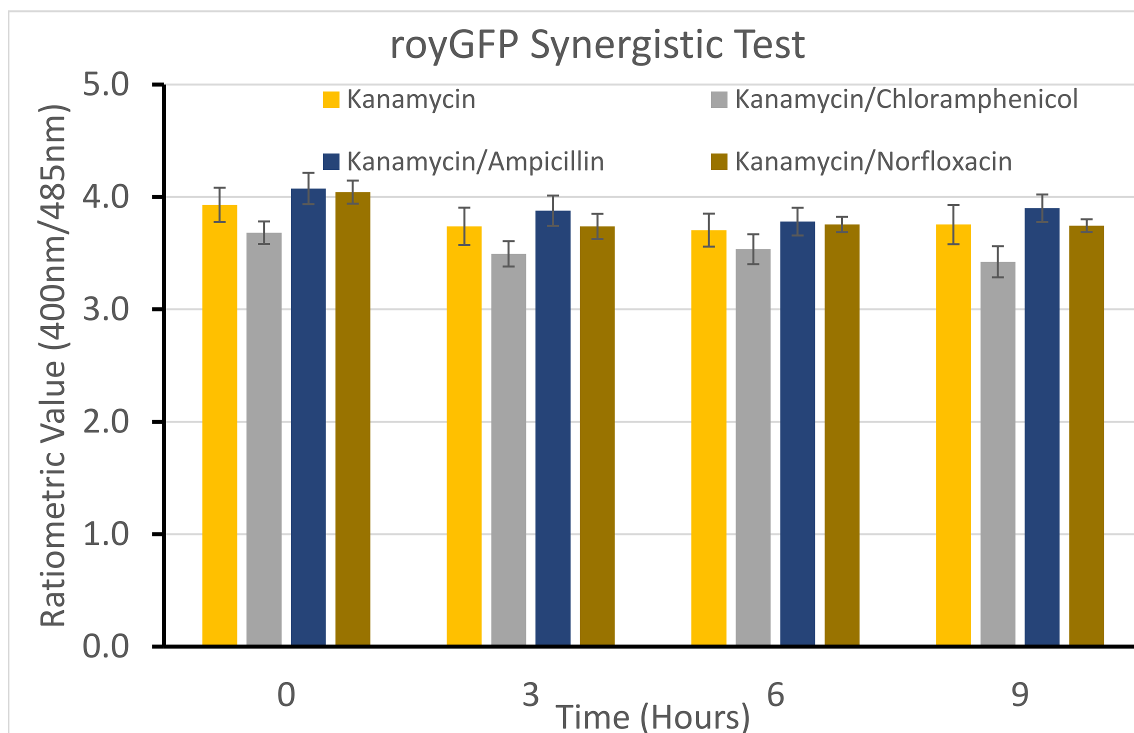

Figure 3-15: Three test average ratiometric value of royGFP fluorescence reads in synergistic dilutions of 8.6 mM of Kanamycin as a control. Then Kanamycin at 0.43 mM with 5.3 mM of Chloramphenicol, 14.3 mM of Ampicillin, and 0.23 mM of Norfloxacin with 95% confidence interval bars (n=3).

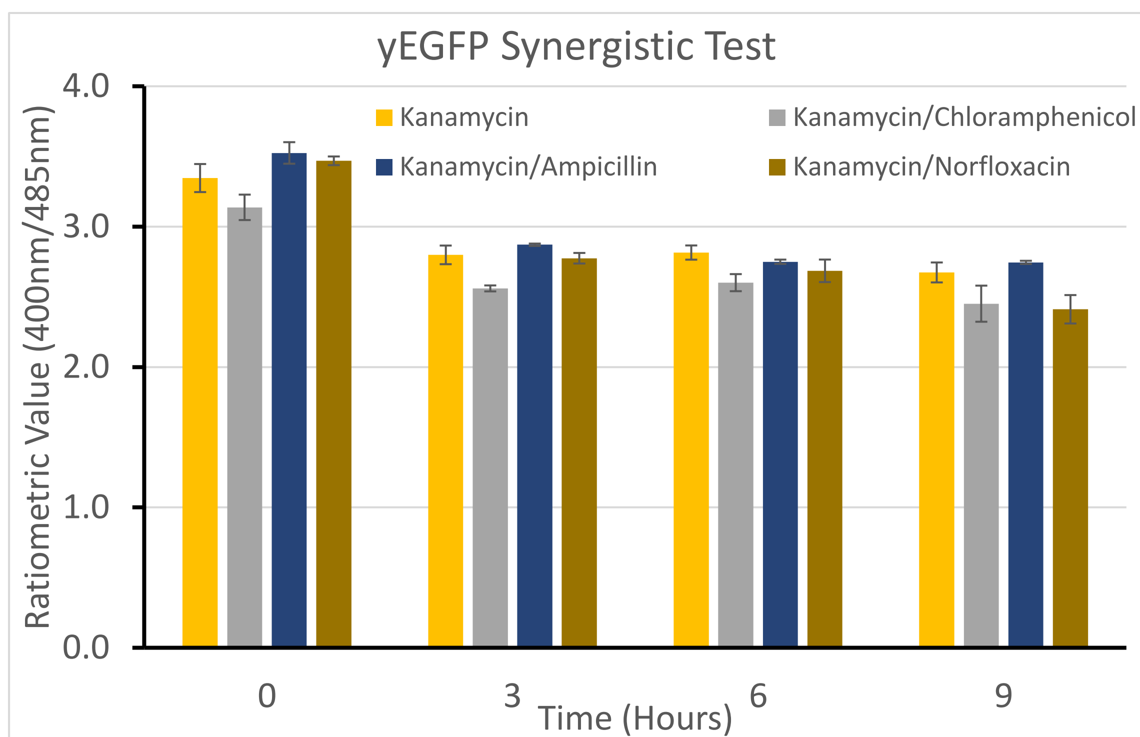

Figure 3-16: Three test average ratiometric value of yEGFP fluorescence reads in synergistic dilutions of 4.3 mM of Kanamycin as a control. Then Kanamycin at 0.43 mM with 5.3 mM of Chloramphenicol, 14.3 mM of Ampicillin, and 0.23 mM of Norfloxacin with 95% confidence interval bars (n=3).

3.3.10 Testing royGFP with DTT and Kanamycin

royGFP was tested with TSB, 4.3 mM of Kanamycin, and 4.3 mM of Kanamycin with 10 mM of dithiothreitol (DTT) over one hour as shown by Figure 3-17. There was no significant difference at time zero but the DTT sample was significantly lower than the Kanamycin alone sample with a p-value of .002 at 10 minutes and .04 at 30 minutes and remained significant for the rest of the test.

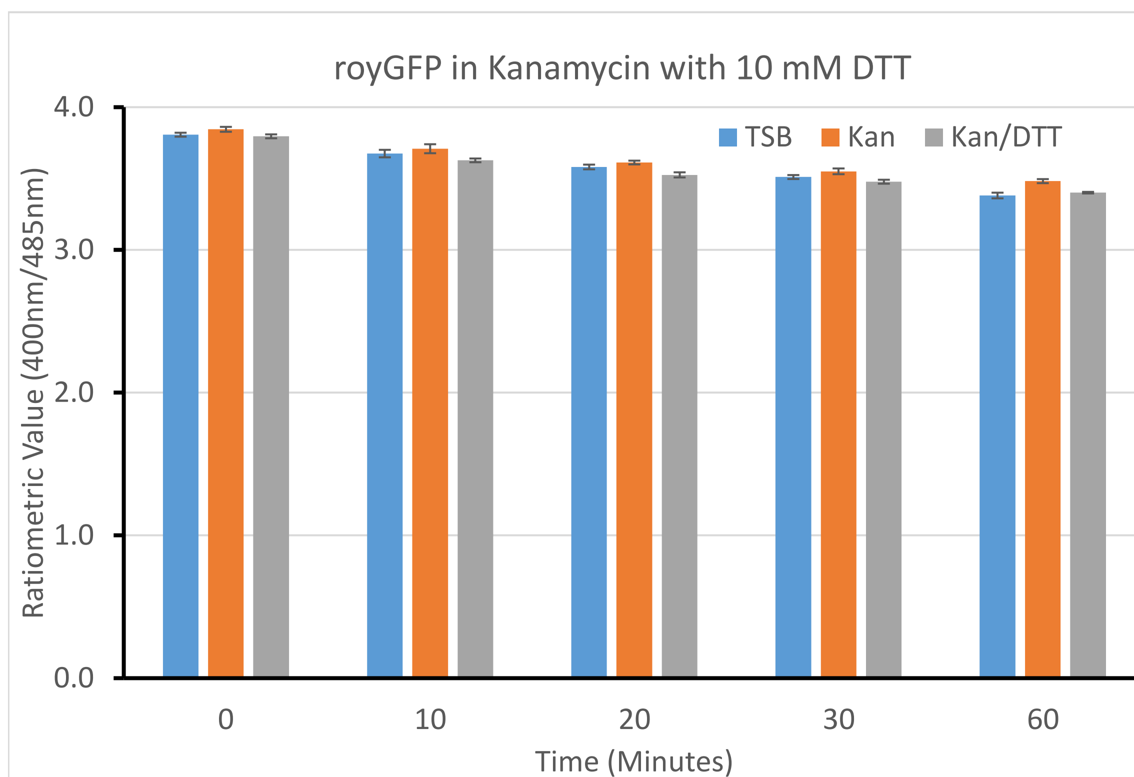

Figure 3-17: Three test average ratiometric value of royGFP fluorescence reads in TSB, 4.3 mM of Kanamycin as a control, and 4.3 mM of Kanamycin plus 10 mM of DTT (n=3).

CHAPTER 4

DISCUSSION

4.1 Western Blotting of yEGFP

GFP has the ability to oligomerize in oxidative environments with its two naturally occurring cysteines causing a significant problem for royGFP, a biosensor designed to change its excitation in the presence of ROS. Determining if GFP oligomerizes in the cytoplasm of bacteria is an objective in this research because this could be a major problem for the ultimate goal, utilizing royGFP to measure the bacterial ROS response to antibiotics. GFP is a resilient protein but has been shown to oligomerize in oxidative environments such as the endoplasmic reticulum (19). Multiple studies have attempted to overcome this problem by substituting the naturally occurring cysteines or increasing folding speed, which worked well at hindering oligomerization but requires many mutations (20). Substituting out the cysteines has been tried and it hindered oligomerization but at the cost of fluorescence intensity (19).

To determine if our GFP will oligomerize in an oxidative environment, we conducted western blots of yEGFP. The results of the western blot showed that yEGFP placed in an oxidative environment had little to no evidence of oligomerization, whether the protein was treated for 15 minutes, 4 hours, or overnight, there was no evidence of oligomerization. This does not support my hypothesis that oligomers would form, but this is a beneficial outcome. This most likely indicates that the oxidative environment

would not affect royGFP due to its similar structure to yEGFP. However, we are not sure the results of the western blots are reliable because there could be other issues such as the protein being in a low concentration to be visible in the western blot or the disulfide bridges being transient. These results do line up with Aronsen et al., stating that no oligomers have been found in gram-negative cells like *E. coli* because they are difficult to observe (20) and due to the bacterial cytoplasm having a reducing environment. Regarding the western blot results, yEGFP and royGFP could still be affected by oligomerization altering our fluorescence test. To resolve this, we are building on Jain et al. research but will take a much deeper dive and attempt to substitute the two cysteines with almost all of the amino acids to determine if oligomerization can be hindered without losing fluorescence. If successful, it will be an important part of future GFP research that involves oxidative environments, allowing for reliable research without the worry of an affected protein due to oligomerization.

4.2 Quik-Change Mutagenesis of royGFP

Oligomerization of GFP in the ROS environment is still a concern due to not being confident in our western blot results. Substituting in amino acids, we hypothesize that the right combination of amino acids in positions 48 and 70 will hinder oligomerization without lowering fluorescence. Jain et al. replaced 48 and 70 for serine due to its similarity to cysteine in its structure. Oligomerization was stopped but its fluorescence suffered significantly (19). Hanson et al. mutated 48 and 70 with serine and alanine, respectively, that stopped oligomerization but alanine was deleterious to their fluorescence of GFP (1). Based off of these studies, we hypothesize that hydrophobic and other similar amino acids such as leucine, isoleucine, methionine, valine, and

glutamine will be good substitutions for these two positions hopefully rendering a brighter biosensor that will not oligomerize.

To begin, three mutations were created separately on royGFP. They were methionine to cysteine at 48 (C48M), valine for cysteine at 48 (C48V), and glutamine for cysteine at 70 (C70Q) using Quik-Change Mutagenesis. Multiple colonies were successfully mutated and cultured with Ampicillin. Unfortunately, after being cultured the cells had a lack of growth and had significant fluorescence loss, going against our hypothesis. There are still many more mutations and combinations to attempt and will be tried as this research continues.

4.3 Fluorescence Reading Test

4.3.1 Testing of yEGFP vs royGFP in Hydrogen Peroxide

With the rise of antibiotic resistance, new and effective antimicrobial molecules are needed to fight bacterial pathogens. Due to the low profit margin of antibiotics, pharmaceutical companies spend little time and effort researching new antibiotics. To help combat this problem, the goal of my research is to create an efficient and cost-effective way to determine the antibiotic activity of molecules with the royGFP biosensor created by Hanson et al. (1). Most antibiotics produce an ROS response in the cell that royGFP is sensitive to, and this increase in ROS changes royGFP's excitation. Correlating the change in excitation to the degree of ROS response can determine its antibiotic activity.

To determine if royGFP is sensitive to ROS, yEGFP and royGFP were tested in the fluorescence reader in dilutions of hydrogen peroxide. Initially, royGFP was not showing much sensitivity, going against our hypothesis. Our control values were very

inconsistent and even higher than the hydrogen peroxide treatment. We began changing variables to find the optimal conditions for this test. Originally, the cells were grown to an OD_{600} of 0.40, diluted the hydrogen peroxide in water, and added the dilutions to the wells by pipetting it on top of the cells. We switched to using TSB as the dilution media and began pipetting the hydrogen peroxide dilutions into the cells, mixing them better. TSB was a better dilatant than water, minimal media, and LB but more research can be done on a wider variety of media to find the most optimal media. Both of these changes leveled out the control and made it more consistent but royGFP still showed little sensitivity. Next, we changed the OD_{600} to 0.45 and began reading the fluorescence every 15 minutes for 2 hours because I predicted that royGFP did not have enough time to become oxidized to an external ROS source in so little time of the test. We found that the longer the test went, the better the sensitivity to ROS there was. After many trial and error runs, royGFP began to show strong and consistent sensitivity to the ROS environment with yEGFP as a non-sensitive baseline. These results confirm Hanson et al.'s study, proving that their roGFP mutations sensitize GFP to ROS through the added cysteines. This is vital to the goal of this research because it shows that royGFP can be expressed and functional in bacterial cells. royGFP showed sensitivity in large amounts of hydrogen peroxide but it has yet to be a proven biosensor to an antibiotic ROS response. The next step is to test royGFP with antibiotics that are known to trigger ROS responses such as Kanamycin and Ampicillin.

4.3.2 Testing of yEGFP vs royGFP in Kanamycin

royGFP showed sensitivity to hydrogen peroxide but has yet to be tested against the ROS triggered from antibiotics. Tests were conducted against a known antibiotic,

Kanamycin that triggers ROS responses in *E. coli*. yEGFP and royGFP were tested in a serial dilution of Kanamycin for 2 hours and read in 15 min increments with the same variables as the hydrogen peroxide test, an OD_{600} of 0.90 and TSB media. During these tests, we wanted to lower the stress on the cells as much as possible. Both yEGFP and royGFP plasmids contain an Ampicillin-resistance gene so they can be isolated in culture and we believed growing them with Ampicillin was sensitizing them to antibiotics. New cultures were taken from a TSA-Ampicillin plate but were cultured without Ampicillin. We hypothesized that the plasmid would be lost if not under selection after two days due to not needing the genetic advantage anymore but to our surprise, the two cultures grew denser and had brighter fluorescence for three to five days after culturing them before falling losing density and fluorescence. Not only were the cells not desensitized to ROS making royGFP a better biosensor, but also the cells grew quicker and had a higher density. This is the most surprising discovered in this research and had a strong positive effect on the sensitivity of royGFP, a big step forward for this research. Using this new technique combined with the variables I used in the hydrogen peroxide test, royGFP showed strong sensitivity to Kanamycin across the two hours and was best at the end of the test. Seeing that royGFP's sensitivity to ROS increased with time, we extended the test to 12 hours and read the fluorescence in 30-minute intervals. Time was shown to be a significant factor for royGFP sensitivity with a p-value of 0.0004 between the control and 4.3 mM dilution at 9 hours compared to a p-value of 0.4 at hour zero, but the dilution of the antibiotic was not a significant factor. We believe time allows for the antibiotic to get into the cell and have an effect on its physiology and ROS production. In addition, test was conducted with the cells at an OD_{600} of 0.375, putting them near the start of their

growth phase where they would be intaking a lot of nutrients, and antibiotics, from its environment. These results show that royGFP can be used as an effective real-time biosensor to measure antibiotic ROS responses. These results are important for the medical field because with an efficient and cost-effective biosensor, new antibiotics can be found quickly to help combat bacterial pathogens.

4.3.3 Testing of yEGFP vs royGFP Fast Folders in Kanamycin

The fast folder mutations to GFP have been shown to hinder oligomerization and increase GFP's folding ability and brightness (20). If these fast folder mutations can be successfully added to our royGFP biosensor, it could allow us to not have to worry about the two naturally occurring cysteines that can oligomerize and can improve the efficiency of the biosensor. The yEGFP and royGFP fast folder mutants were tested in the same fluorescence test as the wildtype variants, a twelve-hour test in dilutions of Kanamycin. The fast folder mutants acted very similar to his wild type variants but was not significantly better. These results could mean that the oligomerization of the proteins is not a problem if the non-oligomerizing fast folder variant was not an improvement on the fluorescence test. This strain may need its own specific environmental conditions to perform better than the wildtype but at the current conditions it performs the same. Due to its increased fluorescence and folding ability it could have a higher potential than the wildtype strain in differing environments and in eukaryotic cells where more proteins would be produced.

4.3.4 Testing royGFP in Antibiotic Synergistic Test

To combat the rise of antibiotic resistance, new ways of attacking the bacteria need to be implemented to successfully kill the cells before a resistance gene can spread. Attacking with two types of antibiotics at has been a strategy to overcome resistance. The stronger effect can wipe out infections due to the chance of having a resistance to both antibiotics.

yEGFP and royGFP were tested in the fluorescence reader in the same conditions as the previous test fluorescence test. The antibiotic combinations consisted of Kanamycin, Chloramphenicol, Streptomycin, Norfloxacin, and Ampicillin. We tested a 1% dilution of Kanamycin mixed individually with the other four antibiotics that were varied from 10, 5 and 1%. royGFP showed higher sensitivity to the combination Kanamycin and Ampicillin but was not significantly more effective. In the first mix, royGFP showed lower sensitivity to the other antibiotics showing that they do not have an additive or synergistic effect. These results show that there is no real synergistic effect with these antibiotics and until further combinations are tested, using multiple antibiotics does not increase ROS production in our *E. coli* strains. The cells could be taking damage much quicker from the two antibiotics, dying before getting the chance to produce more ROS. It is interesting that the combination that had increased sensitivity contained the antibiotic, Ampicillin that the GFP plasmid uses as a resistance gene. It is possible that the threat of Kanamycin lowers its resistance to Ampicillin allowing for it to work on the cells or the energy expenditure to resist Ampicillin can be the increase in ROS production that is showing up on the fluorescence test.

4.3.5 Testing royGFP in DTT

To support our hypothesis that the increase in ratiometric value of royGFP is caused by ROS production, DTT was added to the well to counteract the ROS that was being produced. DTT is dithiothreitol and acts as an ROS scavenger due to its reducing nature. When added to Kanamycin, it has no effect at time zero but with time was significantly lower than the TSB and Kanamycin alone wells.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The goal of this research was to create a cost-effective and real-time ROS biosensor that can measure the stress response of bacteria helping the future discovery of novel antibiotics. To accomplish this, I had two main objectives; one, to determine if GFP oligomerization due to the two cysteines at 48 and 70 would inhibit the biosensor, and two, to find the optimal conditions for this biosensor to work effectively. Through western blot analysis, yEGFP in an oxidative environment showed no evidence of oligomer formation in bacterial cytoplasm, showing it should not inhibit the biosensor. We substituted out the cysteines at 48 and 70 with amino acids valine, methionine, glutamate, and leucine but without a loss of fluorescence. royGFP showed strong sensitivity to a hydrogen peroxide and antibiotically triggered ROS production with time being a significant factor for both royGFP and royGFP fast folder. Time of test and culturing the cells without Ampicillin significantly improved the sensitivity of the biosensor. The biosensor also showed strong sensitivity to multiple antibiotics but was not significantly better when the test were done with multiple antibiotics. royGFP proved to be an effective ROS biosensor with the ability to respond to the stress response of *E. coli* from antibiotics.

5.2 Future Work

Future directions for this research are to further test synergistic effects of antibiotics, as well as switch the GFP plasmid Ampicillin resistance gene with a non-ROS triggering antibiotic so the cells can be isolated better while still maintaining the fluorescence we achieved culturing them without Ampicillin. We would like to find the optimal substitutions for the two naturally occurring cysteines at positions 48 and 70 with amino acids that will improve the fluorescence while maintaining folding ability and growth rate. As well as, test to determine if a royGFP superfast folder variant is sensitive to ROS production.

A ROS biosensor has many capabilities and can be an effective research tool in a wide array of uses. Recent studies conducted have shown that ROS plays an essential physiological role in cells and is not just a destructive by product of the metabolism and aerobic respiration. Functions in pathways in stem cells, cancer, and the human immune system heavily rely on ROS as a messenger and activator to function properly. The ROS biosensor can be an effective tool at determining where and at what levels these systems thrive and can be used to help determine therapeutic answers to problems in these areas. The biosensor will need to be improved upon for it to work in many different types of environments and cells, but it has the potential to make a difference.

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