Optimization of Parameters for the Development of Sensing Systems to Measure Hydrolysis Mechanisms with Enhanced Green Fluorescent Protein

by

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Abstract

The purpose of this research is to develop sensing systems capable of measuring two hydrolysis mechanisms using the pH-dependent fluorescent reporter protein, enhanced green fluorescent protein (EGFP). The enzyme, β-lactamase, catalyzes the hydrolysis of β-lactam antibiotics, notably penicillins, and releases one proton. The hydrolysis of organophosphates, catalyzed by organophosphorus hydrolase (OPH), releases two protons. The release of these protons, in each case, changes the pH of the surrounding environment. The basis for developing sensing systems with these enzymes comes from previous work, in which a fusion protein between EGFP and β-lactamase was developed. This research showed that the pH change from the release of protons through hydrolysis is a local change, and EGFP must be in close proximity to the enzyme domain to detect these changes. To validate this theory, the local pH theory, the goal was to develop separate β-lactamase and EGFP proteins. Additionally, creating a fusion construct with OPH and EGFP to assess the feasibility of other fusion protein systems with EGFP was attempted. The transformation of the separate β-lactamase and EGFP plasmids proved unsuccessful. The two sets of primers designed for the OPH-EGFP experiment were successful in amplifying the EGFP gene, but only one set was sufficient in amplifying the OPH gene. Further optimization of PCR parameters is needed for both experiments to increase the DNA concentration in the samples and ensure successful transformation into DH5α cells. With the threat of the toxic effects of organophosphates, which are found in pesticides and chemical warfare agents, and the growing problem of bacterial resistance to antibiotics, this research has far-reaching implications in the areas of agriculture and medicine. Ultimately, whole cell sensing systems will be developed to assess enzyme kinetics and perform bioavailability and toxicity studies.

Acknowledgements

I would like to start by thanking my family for always supporting me and encouraging me to work toward my goals. I would also like to thank my advisor, Dr. Libby Puckett, for guiding me over the past couple of years both in and outside the lab, and for pushing me to think on a deeper level. Dr. Jennifer Cecile should also be recognized for her contribution to this thesis as my second reader and for taking time out of her busy schedule to help me. Additionally, I would like to acknowledge Holland Howard, Clarissa McCall, and Abi Hawkins for their assistance on this project. Finally, I would like to thank the North Carolina Space Grant, Office of Student Research, and A.R. Smith Department of Chemistry and Fermentation Sciences for making this project possible.

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Introduction

Biosensors and Sensing Systems

A sensor is defined as "a device which detects or measures a physical property and records, indicates, or otherwise responds to it."[1] Sensors are generally made up of a recognition element, which responds to physical changes, and a transducer, which converts the response into a usable signal. Therefore, due to the signal produced, the user is able to monitor system changes.[2]

A type of sensor containing a biological component is called a biosensor. The biological component determines the specific selectivity of the biosensor.[3] The recognition element of a biosensor, or the biological component, is usually one of four materials: enzymes (i.e. glucose oxidase), antibodies (i.e. IgG antibodies), nucleic acids, (i.e. an oligonucleotide with a known base sequence), or receptors (i.e. the acetylcholine receptor), and is coupled to the transducer.[4] Transduction is based on the type of physical change resulting from the recognition element, and is primarily mass based, electrochemical, or optical.[5,6] Biosensors are highly selective, with the ability to discriminate between substrates due to the specificity of biological compounds. Additionally, they are sensitive, sometimes down to the femtomolar range. In practice, however, biosensors generally have longer response times than chemical sensors. This can sometimes last up to thirty seconds, which can be disadvantageous over other sensor types.[4]

Sensing systems, while not true sensors, function similarly. A sensing system differs from a sensor in that it lacks an internal transducer. Therefore, an outside signal detection instrument is required for measurements of system changes.[7] Molecular-based, cellular-based, and tissue-based sensing systems are all types of biosensing systems, categorized based on their sensing component. This research utilizes cellular-based or whole-cell based systems because of

their stability in a range of temperatures and pH values, and the ability to conduct bioavailability studies.[8] For comparison, a molecular-based system requires isolation of specific biological components, which can increase the expense of the assay.[6] A whole-cell based system can be used for a wide array of analytes, including metals, sugars, and viruses, and can employ several different reporter proteins, often luciferase and green fluorescent proteins. [6,8]

Enhanced Green Fluorescent Protein

Many bioluminescent organisms emit light due to various reactions using photoproteins as the primary reactants. About one-third of the two dozen known types of bioluminescent organisms involve these photoproteins. Photoproteins emit light proportional to their amounts. Aequorin, for example, a Ca^{2+} activated photoprotein with a coelenterazine prosthetic group, emits blue lights in aqueous solution, in either the presence or absence of oxygen. Photoproteins, therefore, are useful as reporter proteins and for monitoring biological systems.[9]

The photoprotein, green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria* (Figure 1), is often used in laboratory settings as a reporter protein. Its unique tertiary structure makes it resistant to denaturation under conditions like high pH, high salt concentration, or exposure to organic solvents or detergents. The advantage of GFP over other fluorescent proteins, such as aequorin also from *Aequorea victoria*, is its innate autofluorescence, making additional cofactors or substrates to induce fluorescence unnecessary.[8] GFP contains 238 amino acids (27 kDa) folded into eleven β-sheets arranged in a barrel.[10] The major excitation peaks of GFP occur at 395 nm (λ_{max}) and 470 nm, and the primary emission peak occurs at 508 nm.[11]

Figure 1: The jellyfish *Aequorea victoria*[8]

Several mutant forms of GFP have been developed, which optimize the rate and intensity of GFP fluorescence by the shifting excitation and emission wavelengths. These include enhanced blue fluorescent protein (EBFP), enhanced yellow fluorescent protein (EYFP), and enhanced cyan fluorescent protein, which emit blue, yellow, and cyan wavelengths of light, respectively.[12]

Enhanced green fluorescent protein (EGFP) is the product of over 190 mutations of GFP. EGFP is a pH-dependent, red-shifted variant of the wild type GFP. EGFP is arranged in eleven β-sheets with an axial α-helix surrounding the chromophore (Figure 2).[13] The red shift, or bathochromic shift, of the major excitation peak to 488 nm is due to mutations of several residues near the GFP chromophore region, comprised of the cyclic tripeptide Ser-65-Tyr-66-Gly-67. The chromophore mutation was produced by double amino-acid substitutions (Phe-64 to Leu and Ser-65 to Thr). These mutations, shifting the major excitation peak to longer wavelengths, cause EGFP to fluoresce 35 times brighter than GFP when excited at 488 nm. Additionally, the EGFP mutations make it more amenable for expression in mammalian cells.[12]

GFP variants have been expressed in monkeys, cats, dogs, mice, and pigs (Figure 3).[14] The excitation wavelength of EGFP (488 nm) is commonly used in analytical instruments, including those with argon lasers, which emit light at 488 nm, making EGFP exceptionally useful.[12] Thus, a spectrofluorometer can be used to measure and quantify its fluorescence.[15]

Figure 2: Ribbon structure of EGFP[13]

Figure 3: Mice genetically modified with EGFP[14]

Principle of the Assay

As previously stated, the fluorescence of EGFP is dependent on pH. In an alkaline environment, the hydroxyl group of Tyr-66 is deprotonated, resulting in an increase in the fluorescence intensity. As such, the intensity of the EGFP fluorescence decreases in response to a decrease in pH.[16] Previous work has shown that EGFP can be fused with the enzyme, β-lactamase, to create a fusion protein capable of measuring the hydrolysis mechanism.[13] This fusion protein was created based on the theory that the pH change occurring from the hydrolysis of the β-lactam ring of penicillins catalyzed by β-lactamase, is a local pH change. Thus, in order to detect this change, the EGFP domain must be in close proximity to the β-lactamase domain.[13] This theory, known as the local pH theory, can be validated by using EGFP and β-lactamase to create separate proteins. The fluorescence of EGFP should not change when the two domains are not in close enough proximity. This local pH concept can also be used to monitor other enzymes that alter the surrounding pH and show the functional versatility of EGFP.

β-lactam Antibiotics

Penicillins were first purified from an antibacterial mold in 1928 by Alexander Fleming. He observed a bacteria-free ring around a mold growing on plates containing colonies of *Staphylococcus*. The colonies surrounding the mold were lysed, becoming transparent. Cultures of the mold were purified and the active agent within it, possessing antibacterial properties, became known as penicillin.[17]

Bacterial resistance to antibiotics has dramatically increased over the past few decades, a problem propelled by their overuse.[13,18] The major class of antibiotics, around which this issue revolves, is penicillins, one class of the group of antibiotics known as β-lactam antibiotics.[18] There are four classes of β-lactam antibiotics: penicillins, cephalosporins, monobactams, and

carbapenems (Figure 4). β-lactams are characterized by a four-membered nitrogen-containing ring, known as a β-lactam ring. They target bacterial cell wall synthesis by interacting with D-alanyl-D-alanine carboxypeptidase-transpeptidase, a bacterial protein responsible for crosslinking peptidoglycan in bacterial cell walls.[19] In the absence of this crosslinkage, the rigidity of the cell wall unravels, causing the cell to rupture.[20]

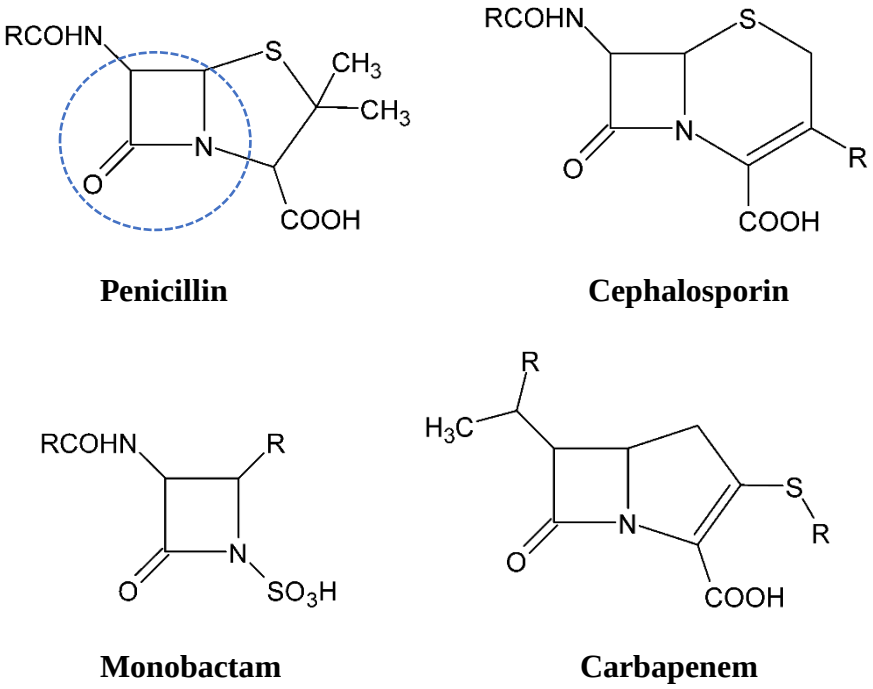


Figure 4: Classes of β-lactam antibiotics with circled β-lactam ring example[21]

As the use of these antibiotics has increased, bacteria have become more resistant through evolution.[22] Some bacteria, such as *Staphylococcus aureus*, produce an enzyme called penicillinase or β-lactamase, which is responsible for their antibiotic resistance. β-lactamase renders the antibiotic inactive by catalyzing the hydrolysis of the β-lactam ring in penicillins and other β-lactam antibiotics.[13,18]

β-lactamase

β-lactamases are a class of enzymes that hydrolyze amides, amines, and other C-N bonds.[23] In many bacteria that produce β-lactamase, the enzyme is either produced on

chromosomal sites or in plasmids within the cell.[24] The structure of β-lactamase shows a homodimer (257 residues, 29 kDa) with two domains. One domain is a β-sheet made up of five antiparallel strands encompassed by three α-helices and the other is several α-helices packed against a face of the sheet (Figure 5). The active site is found in the interface between both domains.[25] Mechanistically, Ser-70 attacks the carbonyl carbon of the β-lactam ring of the substrate to form an acylated intermediate. Lys-73 acts as the catalytic base in the acylation step by deprotonating the hydroxyl group of Ser-70 before Ser-70 attacks the substrate (Figure 6, steps 1 through 3). Glu-166 acts as the activating base of a hydrolytic water molecule in the deacylation step. (Figure 6, steps 3 through 5).[26] The protonation of the nitrogen atom causes the β-lactam ring to open instantaneously, rendering the antibiotic inactive.[27]

Figure 5: Structure of β-lactamase[28]

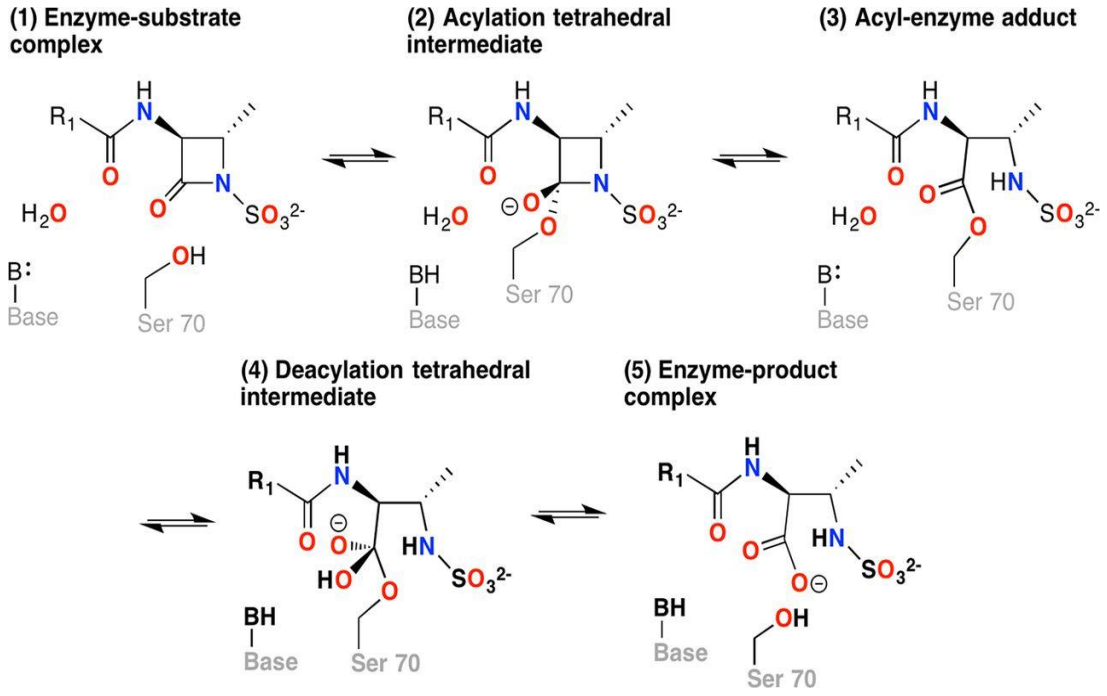


Figure 6: Mechanism of β-lactam ring hydrolysis by β-lactamase active site residues[26]

Validation of the Local pH Theory

The hydrolysis mechanism of β-lactamase results in the release of one proton into the local environment as it catalyzes the hydrolysis of the β-lactam ring of β-lactam antibiotics, indicating the hydrolysis mechanism can be monitored. Since the change in pH is only in the environment surrounding the antibiotic, a pH electrode would not be sensitive enough to detect this change, requiring a different approach. In order for the rate of hydrolysis to be monitored, a reporter protein, like EGFP, must be utilized. Since, previous work has already successfully shown this with a fusion construct between β-lactamase and EGFP, the genes will be purified and expressed in pFLAG-MAC separately to validate the local pH theory.[13] After purifying both proteins from the pEGFP plasmid (Figure 7) and expressing them separately in the pFLAG-MAC expression vector (Figure 8) in an ampicillin solution, the fluorescence of EGFP should

not change. The hydrolysis of ampicillin will be catalyzed by β-lactamase, releasing a proton and causing a decrease in the local pH. However, the fluorescence intensity of EGFP should remain relatively the same since it is not in close enough proximity to the pH change (Figure 9).

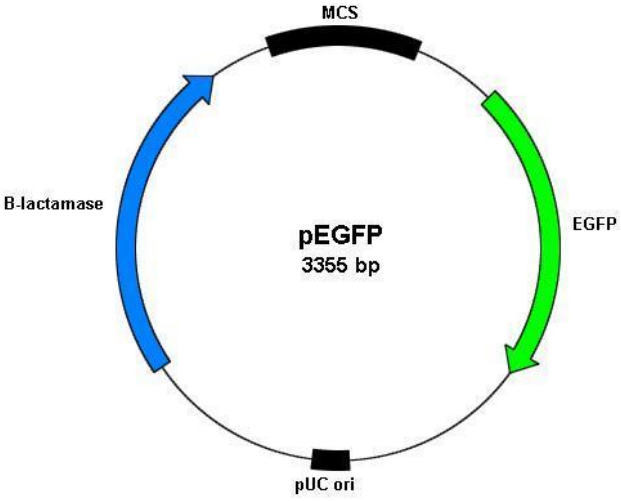


Figure 7: The pEGFP plasmid with the β-lactamase gene

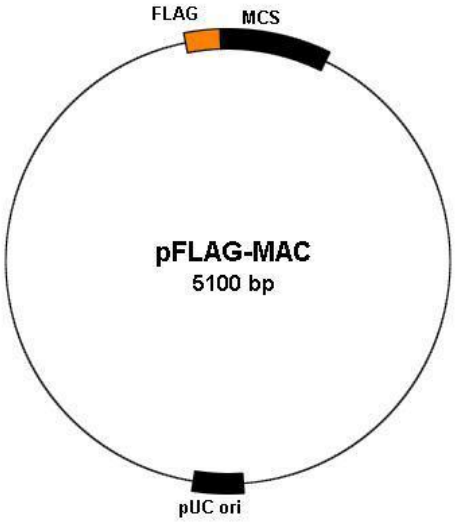


Figure 8: The pFLAG-MAC plasmid

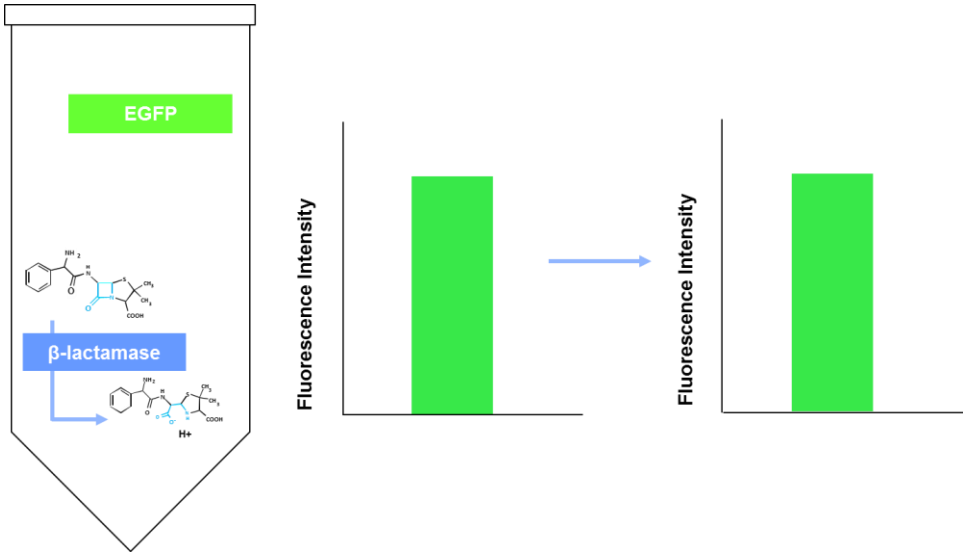
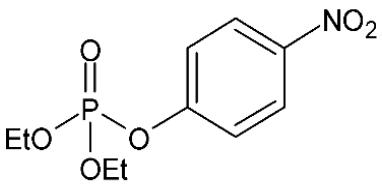


Figure 9: Validation of the local pH theory

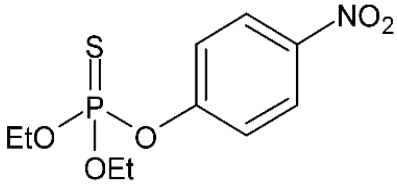
Organophosphates

Organophosphates (OPs) are a group of compounds originally developed in the 1800s for use in insecticides.[29] They were later used to develop chemical warfare agents, especially during World War II.[30] Paraoxon and parathion are the most commonly used organophosphates in insecticides (Figure 10A), and sarin and soman are found in chemical warfare agents (Figure 10B). While the threat of chemical warfare is troubling, the use of OPs in the agriculture industry has a detrimental impact on marine life via run-off from farm land. Not only are shellfish and other commercially-utilized fish negatively affected, but also the microorganisms at the bottom of the food chain in these types of ecosystems. Additionally, OPs have been found to be persistent in the environment for long periods of time, allowing for multiple instances of contamination.[31]

A

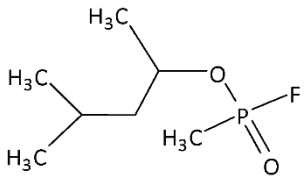


Paraoxon

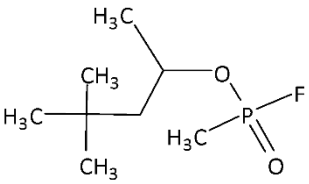


Parathion

B



Sarin



Soman

Figure 10: *A.* Chemical structures of common organophosphates found in insecticides, *B.* Chemical structures of common organophosphates found in chemical warfare agents

OPs are a large class of molecules characterized as esters of phosphoric acid. Some have a high binding affinity to acetylcholinesterase (AChE) and will phosphorylate its active site, making the enzyme inactive. AChE is an enzyme responsible for the metabolism of the neurotransmitter acetylcholine (Figure 11). An inhibited AChE causes the acetylcholine receptor, a Na^+-K^+ ion channel, to stay open for too long. This leads to an excess of acetylcholine within the body, overstimulating nicotinic and muscarinic receptors.[32] Nicotinic receptors function as muscle and neuronal receptors by controlling contractions in the skeletal muscles and contributing to memory formation, learning, and reward.[33] Muscarinic receptors are found in both the sympathetic and parasympathetic nervous systems, but primarily facilitate functions like heart activity, exocrine gland secretions, and contraction of the smooth muscles.[34]

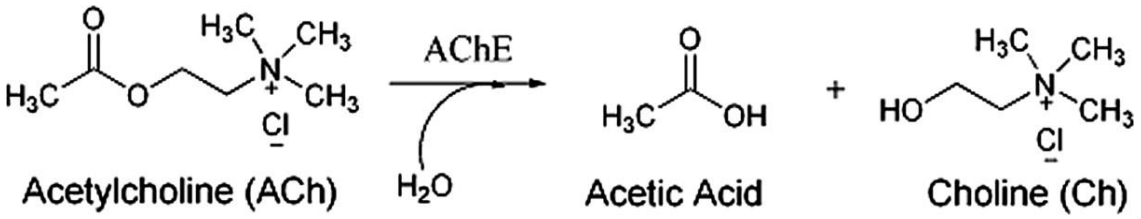


Figure 11: Uninhibited hydrolysis of acetylcholine producing choline and acetic acid

OPs interact with hydroxyl group of the serine in the active site of AChE, creating a Michaelis-Menten intermediate complex and resulting in the phosphorylation and deactivation of the enzyme. The rate of AChE spontaneous reactivation depends on the OP structure inhibiting the enzyme. AChE is able to expel OPs with dimethyl radicals with a 1-2 hour half-life, while the half-life for diethyl radicals is 31-57 hours. OPs with branched radicals, such as sarin and soman, prevent spontaneous reactivation, explaining why they are so deadly.[35]

OP exposure in humans can result in four main neurotoxic disorders: the cholinergic syndrome, the intermediate syndrome (IMS), OP-induced delayed polyneuropathy (OPIDP), and chronic OP-induced neuropsychiatric disorder (COPIND). Symptoms associated with the cholinergic syndrome are directly linked to the level of AChE activity. Acute symptoms can include salivation, respiratory distress, sweating, lacrimation, and nausea, and may be accompanied by symptoms such as dizziness, headache, seizures, coma, and respiratory failure. Psychiatric problems like mood swings, paranoid delusions, and schizoid reactions can occur should an individual survive past the first day of poisoning. IMS generally follows the cholinergic syndrome, with symptoms setting in within 24 to 96 hours after exposure. IMS usually occurs in patients with prolonged and severe AChE inhibition, and is characterized by neck flexion, shoulder abduction, and respiratory insufficiency. OPIDP symptoms present 10 to 20 days after a single exposure. The syndrome does not involve AChE, but instead the phosphorylation of the neuropathy target esterase (NTE), making it mechanistically divergent from the cholinergic syndrome and IMS.[35] Symptoms include ataxia and loss of function of the distal sensory and motor nerves in the peripheral nervous system and in the ascending and descending tracts of the spinal cord.[36] COPINDs are not dependent on AChE inhibition and often occur with a lack of cholinergic symptoms.[37,38] The root of these disorders is still not fully understood, but COPINDs are known to arise in populations where OP exposure is frequent. Symptoms can include chronic fatigue, autonomic dysfunction, peripheral neuropathy, and cognitive deficits (i.e. impairment of memory, concentration, and learning, etc.).[35]

The widespread nature of OPs in agriculture, specifically, requires the development of remediation methods. Enzymatic degradation, by means of phosphotriesterase enzymes, results in the breakdown of phosphoester bonds that make up OPs. Phosphotriesterases are, therefore,

sufficient in the detoxification of a variety of organophosphates due to their broad substrate specificity.[39]

Organophosphorus Hydrolase

Organophosphorus hydrolase (OPH) was discovered in 1976 in the species of bacteria, *Pseudomonas diminuta*, which was found thriving in pesticide-contaminated soil.[39] Later, it was discovered that *opd* (organophosphate degrading), a plasmid-borne gene (pCS1, 66-kb), was responsible for the conferrence of the hydrolytic activity of OPH.[40] While the literature shows the enzyme coded for by the *opd* gene as taking on many names—phosphotriesterase, organophosphate-degrading enzyme, and parathion hydrolase—organophosphorus hydrolase will be used throughout this text. The hydrolysis of OPs catalyzed by OPH occurs through a mechanism similar to an S_N2 mechanism with a nucleophilic attack by a base-activated water molecule. The result is an inversion of the OP substrate at the phosphorus chiral center.[41]

OPH has broad substrate specificity, with the ability to degrade organophosphorus compounds containing P-O, P-CN, P-F, and P-S bonds. It is the only known enzyme with the ability to cleave P-S bonds. OPH is a member of the amidohydrolase family. It is a homodimer (35 kDa per monomer) with 336 amino acids in each subunit (Figure 12).[42] Each monomer is a distorted α/β barrel with eight parallel β-strands and linked by fourteen α-helices on the outer surface.[43] In addition, its activity relies on a binuclear metal center that can bind a wide range of metals aiding in catalysis and contributing to structural function. The native metals at the active site are divalent zincs, but divalent cobalt, nickel, cadmium, and manganese have also shown catalytic activity, with cobalt showing the highest.[42] Several histidine residues are involved in maintaining the geometry of the active site and binding metal ions.[44] The metal center is bridged

by Lys169 and either a water molecule or hydroxide ion, allowing for the nucleophilic attack of an OP phosphorus center (Figure 13).[42]

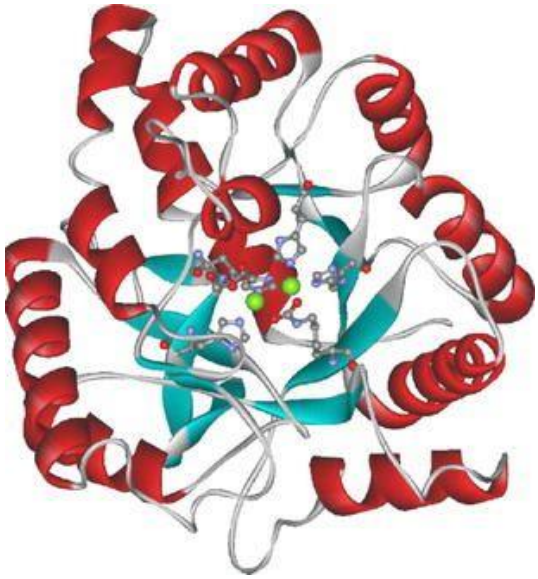


Figure 12: Ribbon structure of OPH[42]

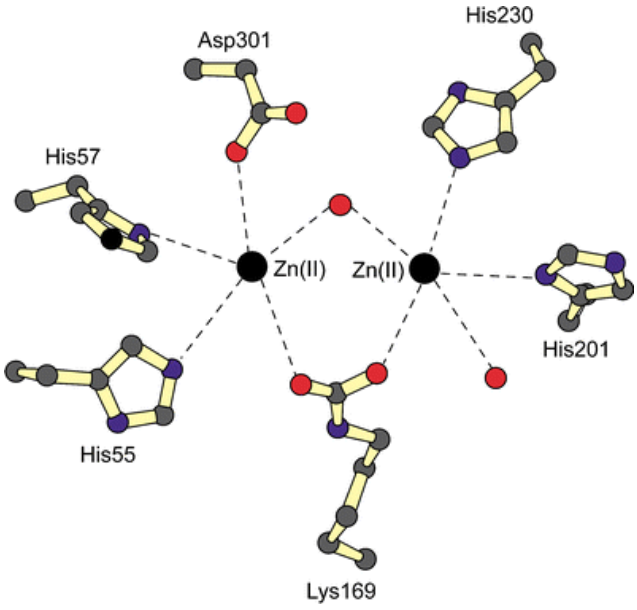


Figure 13: Binuclear metal center of OPH stabilized by His and bridged by Lys[42]

Fusion Protein with EGFP

Upon consideration of the hydrolysis mechanism of OPH, the same logic as the β-lactamase hydrolysis mechanism applies. When OPH catalyzes the hydrolysis of organophosphates, two protons are released into the surrounding environment, indicating EGFP can be used to monitor the hydrolysis reaction. When the pH decreases, the spectral properties of EGFP are changed by reducing the fluorescence. Therefore, a fusion gene containing EGFP and OPH can be created to monitor the hydrolysis reaction (Figure 14). The OPH-EGFP fusion construct will encode for a protein that will be harvested *in vitro*. The fusion protein will be purified and expressed in both pFLAG-MAC and pET-21a expression vectors, and used in an assay to detect the presence of and quantify OPs, acting as a biosensing system monitoring the fluorescence emission over time.[13]

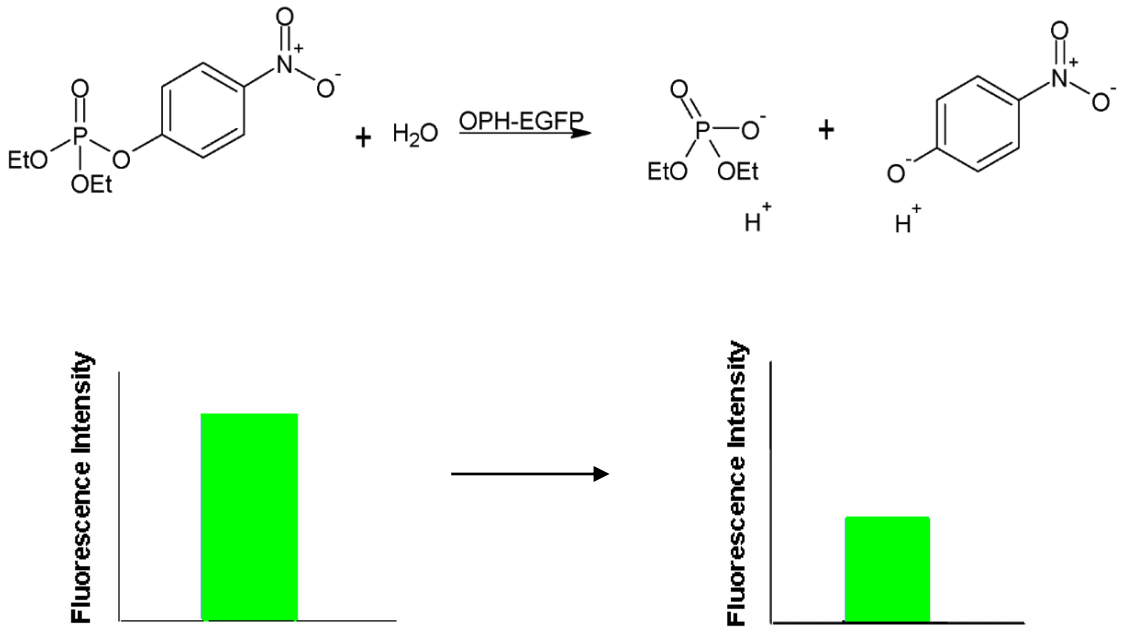


Figure 14: Fusion gene hydrolysis of OP and hypothetical fluorescence intensity

In this research, an OPH-EGFP gene fusion construct and separate β-lactamase and EGFP vectors will be created. Unique vectors containing the β-lactamase and EGFP genes and the fusion construct will be generated using recombinant DNA techniques, including PCR to amplify the genes of interest, gene isolation by gel electrophoresis, overlap extension PCR to fuse the OPH and EGFP genes together, restriction enzyme digestion to create sticky ends, ligation of the constructs into the expression vectors pFLAG-MAC and pET-21a, and transformation into DH5α competent cells. The overall goal is to create cell-based sensing systems that validate the basis of previous work and are capable of detecting and quantifying OPs and monitoring the β-lactamase hydrolysis mechanism.

Experimental Methods

Apparati

Cultures of pEGFP, pJK01, pFLAG-MAC, and pET-21a were incubated overnight in a VWR shaker table (Cornelius, OR). Restriction enzyme digestions were performed in a VWR water bath, and a VWR oven was utilized to incubate transformation plates (Cornelius, OR). Initial gradient PCR was completed in a Veriti 36-well Thermal Cycler obtained from ThermoFisher Scientific (Asheville, NC). Overlap extension PCR was performed in an Eppendorf Mastercycler Personal (Hamburg, Germany). Electrophoresis gels were run on a Shelton Scientific IBI QS-710 Quick Screen (Shelton, CT) connected to a Thermo EC105 power supply (Asheville, NC) and imaged on a UVP UV Transilluminator (Upland, CA). Centrifugation was conducted in an Eppendorf Centrifuge 5424 (Hamburg, Germany). DNA sample concentrations were determined using a Thermo Scientific NanoDrop (Asheville, NC). Thermo Scientific Finnpipettes (Asheville, NC) were employed to measure volumes of reagents ranging from 0.5 µL to 1000 µL. The steam sterilizer autoclave was purchased from Tuttnauer (Hauppauge, NY).

Reagents

Luria Bertani Broth (LB Broth) and Luria Bertani Agar (LB Agar) were purchased from Difco (Lawrence, KS). Ethidium bromide and ampicillin sodium was purchased from Sigma Aldrich (St. Louis, MO). Agarose powder was obtained from Phenix (Candler, NC). DNA ladder (1 Kb Plus) and 10x BlueJuice loading buffer were obtained from Invitrogen (Vilnius, LT). RedSafe nucleic acid staining solution was purchased from VWR (Cornelius, OR). Restriction enzymes *Bam*HI, *Xho*I, *Eco*RI, *Kpn*I, *Hin*dIII, and 10x FastDigest Buffer were purchased from Thermo Fisher Scientific (Vilnius, LT). S.O.C. media, DH5α maximum efficiency cells, and

nuclease-free water were purchased from Invitrogen (Carlsbad, CA). PCR primers were custom-created by Eurofins MWG Operon (Huntsville, AL). T4 DNA ligase and T4 DNA ligase buffer were purchased from Promega (Madison, WI). The pEGFP vector was obtained from Clontech Laboratories (Palo Alto, CA). The pFLAG-MAC vector was purchased from IBI Kodak (New Haven, CT). The pJK01 plasmid was provided by F.M. Raushel at Texas A&M University. The pET-21a vector was donated by Megan Culpepper at Appalachian State University. The QIAprep Spin Miniprep kit, the QIAquick PCR Purification kit, and the QIAquick Gel Extraction kit were purchased from Qiagen (Germantown, MD).

Preparation of Luria-Bertani Broth (LB Broth) Growth Media

Luria-Bertani broth powder (8 g) was dissolved in 400 mL of DI water in a 500 mL glass media bottle. The solution was sterilized by the autoclave and allowed to cool to 50°C. Ampicillin (0.040 g) was added to the solution to obtain a concentration of 100 µg/mL.

Preparation of Luria-Bertani Agar (LB Agar) Plates

In a 500 mL glass storage bottle, 14g of LB agar powder was dissolved in 400 mL of DI water. The solution was sterilized in the autoclave. The mixture was cooled to 50°C prior to the addition of 0.040 g (100 µg/mL) of ampicillin. The solution was poured into petri dishes, adding approximately 20 mL to each plate, and allowed to solidify. The LB-ampicillin plates were stored in the refrigerator.

Bacterial Cultures and Miniprep

Bacterial cultures of pEGFP, pJK01, pFLAG-MAC, and pET-21a were grown from colonies obtained from agar plates. Source DNA was transferred from the plates to clean, labeled Falcon tubes containing 3 mL of LB-ampicillin broth via an autoclaved toothpick. The cultures were placed on a shaker table with proper ventilation and were incubated overnight at 37°C,

shaking at 200 rpm. DNA was isolated from bacterial cells following the procedures provided in the Qiagen miniprep kit. Aliquots of the bacterial cultures were transferred to 1.5 mL Eppendorf tubes and centrifuged (60 seconds at 13,000 rpm). The supernatant was decanted, leaving only the pellet. This process was repeated to transfer the remaining contents of the Falcon tubes to maximize the amount of isolated DNA. The pellets were resuspended via a pipette in 250 µL of Buffer P1 containing RNAse. To wash, 250 µL of Buffer P2 was added to the Eppendorf tubes, which were inverted 4-6 times to mix the contents. Immediately after the addition of Buffer P2, the tubes were centrifuged for 10 minutes at 13,000 rpm. The contents of the Eppendorf tubes were then transferred to QIAquick columns (800 µL at a time), centrifuged (60 seconds, 13,000 rpm), and the flow-through liquid was discarded. This step was repeated until the Eppendorf tubes were emptied. A volume of 750 µL of Buffer PE was added to the QIAquick columns, which were then centrifuged, and the flow-through liquid was discarded. The columns were centrifuged an additional 60 seconds to ensure that any residual wash buffer was removed before the columns were transferred to clean 1.5 mL Eppendorf tubes. To elute the DNA, 30 µL of Buffer EB was added to the center of each column. After allowing the columns to stand for 60 seconds, they were centrifuged again (60 seconds, 13,000rpm). The columns were discarded, and the Eppendorf tubes containing isolated DNA were placed in the refrigerator.

Isolation and Amplification of OPH, β-lactamase, and EGFP via Polymerase Chain Reaction

Following preparation of the DNA samples, the OPH gene was isolated and amplified from pJK01, and the β-lactamase (β-lac) and EGFP genes were isolated and amplified from pEGFP by PCR. The 50 µL PCR reaction mixtures for the β-lactamase and EGFP experiment contained 21 µL sterile water, 2 µL DNA sample (EGFP or β-lac), 1 µL respective forward primer, 1 µL respective reverse primer, and 25 µL PCR MasterMix. Primers were designed with

respect to the multiple cloning sites (MCS) of the pFLAG-MAC and pET-21a vectors (Figures 15 and 16). The 50 μL PCR reaction mixtures for the OPH and EGFP experiment contained 17 μL sterile water, 4 μL DNA sample (EGFP or OPH), 2 μL respective forward primer, 2 μL respective reverse primer, and 25 μL PCR MasterMix. Restriction enzymes were chosen with consideration of the restriction sites within the multiple cloning site of each vector, the number of base pairs between cut sites, and the gene sequences of β-lactamase, EGFP, and OPH. The specially designed primers for the isolation of the β-lactamase and EGFP genes are shown in Table 1, and the primers designed for the amplification of the OPH and EGFP gene sequences for insertion into each of the expression vectors are shown in Tables 2 and 3.

The set of β-lactamase and EGFP primers for individual gene isolation contained two different EGFP forward primers, with one incorporating the start codon (ATG) and the other leaving it out. The underlined sequences in each primer signal a restriction site. The β-lactamase primers did not contain the signal peptide in the gene sequence. Each primer was designed with respect to the multiple cloning site of pFLAG-MAC. The reaction mixtures were placed in the thermocycler using the following the parameters under the program: 94ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 2 minutes with a total of 30 cycles performed.

The forward primer for OPH contains a start codon followed by a complementary sequence to select for the beginning of the OPH gene. The underlined sequence is a restriction site. The EGFP reverse primer selects for the end of the EGFP gene and contains a restriction site. The overlap OPH primer contains the spacer sequence (in bold) 5’-TCCTCCTCCTCC-3’ followed by a sequence complementary to the 3’ end of the OPH gene. The EGFP overlap primer includes a sequence that selects for the 3’ end of the OPH gene followed by the spacer (in bold) 5’-GGAGGAGGAGGA-3’ which will base pair with the spacer included in the overlap

OPH primer. The final sequence of the EGFP overlap primer selects for the beginning of the EGFP gene. These primers were designed with respect to the multiple cloning sites of the two expression vectors. The reaction mixtures were placed in the gradient PCR thermocycler starting at 94ºC for 1 minute, then using varying annealing temperatures for 1 minute, and ending at 72ºC for 2 minutes with a total of 50 cycles performed.

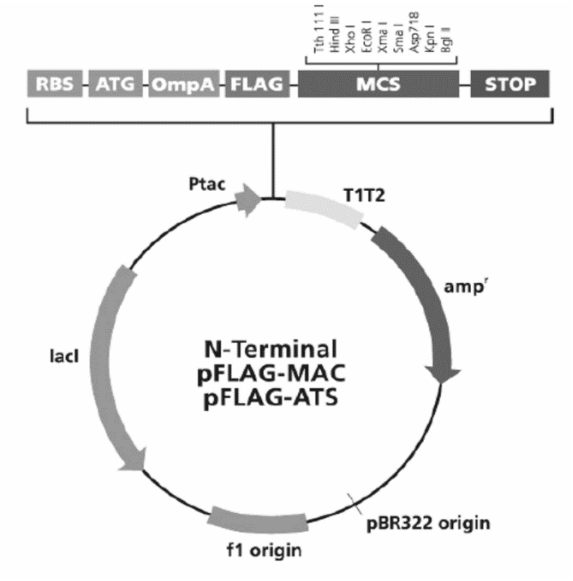


Figure 15: The pFLAG-MAC plasmid with detailed MCS[45]

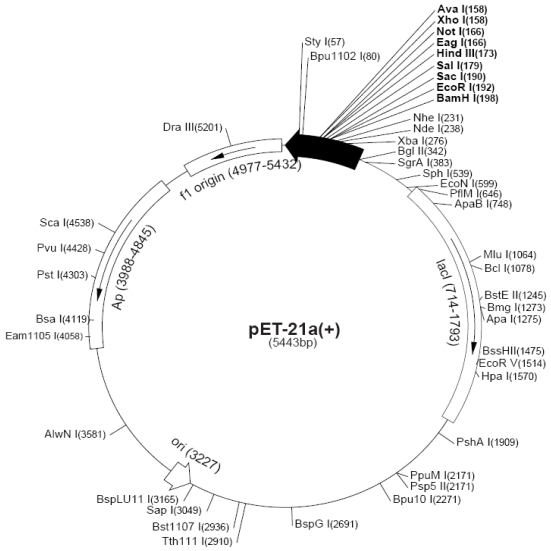


Figure 16: The pET-21a plasmid with detailed MCS[46]

Table 1: **Primers for separate expression of β-lac and EGFP in pFLAG-MAC vector**

β-lac	Forward Primer	5'ACCATCGCAGTAAAGCTTCACCCAGAAACGCTGG TGAAAGTA 3' HindIII
	Reverse Primer	5'ACCGCCGCAGTGAATTCATTACCAATGCTTAATC AGTGAGGC 3' EcoRI
EGFP	Overlap Primer	5' ACCTACGCAGTTAAGCTTGTGAGCAAGGGCGAG GAGCTG 3' HindIII
	Forward Primer #2	5' ACCTACGCAGTTAAGCTTATGGTGAGCAAGGGCG AGGAGCTG 3' HindIII
	Reverse Primer	5' TACACCGCAGTGAATTCATTACTTGTACAGCTCG TCCAT 3' EcoRI

Table 2: Primers for gene fusion of OPH and EGFP for pET-21a vector

OPH	Forward Primer	5'ACCACCTACGGATCCATGCCGGGTATCGATCGGCACAGG 3' (BamHI)
	Overlap Primer	5' **TCCTCCTCCTCC**TGACGCCCGCAAGGTCGGTG 3'
EGFP	Overlap Primer	5' TTGCGGGCGTCA**GGAGGAGGAGGA**GTGAGCAAGGGCGAGGAG 3'
	Reverse Primer	5' ACCACCTACGCTCGAGTTTTACTTGTACAGCTCGTCCATGCC 3' (XhoI)

Table 3: Primers for gene fusion of OPH and EGFP for pFLAG-MAC vector

OPH	Forward Primer	5'ACCACCTACGTGAATTCAATGCCGGGTATCGATCGGCACAGG 3' (EcoRI)
	Overlap Primer	5' **TCCTCCTCCTCC**TGACGCCCGCAAGGTCGGTGA 3'
EGFP	Overlap Primer	5' TTGCGGGCGTCA**GGAGGAGGAGGA**GTGAGCAAGGGCGAGGAG 3'
	Reverse Primer	5' ACCACCTACGTGGTACCATTACTTGTACAGCTCGTCCATGCC 3' (KpnI)

Gel Electrophoresis and Gel Extraction

Agarose gels were prepared by using a microwave to dissolve approximately 0.5 grams of agarose powder in 50 mL of 1X TAE buffer in a 125 mL Erlenmeyer flask. TAE buffer (25X) was diluted to 1X (121.0 g tris base, 28.6 mL glacial acetic acid, 18.6 g $Na_2EDTA \cdot 2H_2O$, and DI water to 1.0 L). Once the agarose powder was completely dissolved in solution, 2 µL of RedSafe was added to the mixture. The gel was then poured into a casting tray equipped with a well comb and allowed to solidify. The gel was transferred to the Quick Screen and covered with 1X TAE buffer. PCR products were prepared with 5.4 µL of BlueJuice loading buffer, and 25 µL of each sample was placed in the gel wells. In addition, 5 µL of 1 Kb DNA ladder was added to one well for reference. The gel was run at 100 volts for 35 minutes. The gel was imaged on the UV

transilluminator, and a DNA ladder was used to identify desired DNA fragments. EGFP and β-lactamase bands are approximately 800 base pairs and OPH bands are close to 1000 base pairs. The bands were excised using a clean razor blade and placed in labeled 1.5 mL Eppendorf tubes. Gel extractions were performed following procedures in the QIAquick gel extraction kit. To each Eppendorf tube, 3 volumes of buffer QC to 1 volume of gel was added. The tubes were warmed by hand and intermittently vortexed until the gel was completely dissolved in solution. The mixtures were transferred to QIAquick spin columns and centrifuged for 1 minute, discarding the flow-through. The samples were washed with 750 µL of Buffer PE and columns were centrifuged for 1 minute (13,000 rpm). The flow-through was discarded, and the columns were centrifuged for an additional minute to remove any residual wash buffer. The columns were transferred to new 1.5 mL microcentrifuge tubes, and 30 µL of Buffer EB was added to the center of each column. They were then allowed to stand for one minute before eluting the DNA. The columns were centrifuged for one minute, and the extracted samples were stored in the refrigerator.

Preparation and Isolation of EGFP and OPH Fusion Gene Construct

Samples were prepared from gel extracted DNA. The 50 µL overlap extension PCR mixture contained 13 µL sterile water, 4 µL EGFP DNA, 4 µL OPH DNA, 2 µL forward primer, 2 µL reverse primer, and 25 µL PCR MasterMix. Only the forward OPH primer and reverse EGFP primer were used. Overlap cycling parameters were as follows: 94°C for 1 minute, 45°C for 1 minute, and 72°C for 3.5 minutes for 10 cycles, 94°C for 1 minute, 50°C for 1 minute, and 72°C for 3.5 minutes for 20 cycles. The PCR products were isolated by gel electrophoresis and extracted following the QIAquick gel extraction procedure.

Enzyme Digestion and Purification

The EGFP and β-lactamase gene sequences along with the pFLAG-MAC vector were concurrently digested using *Hin*dIII and *Eco*RI. Each reaction mixture received 24.0 µL of DNA, 3.0 µL of REact 2 buffer, 1.5 µL of *Eco*RI, and 1.5 µL of *Hin*dIII. The mixtures were placed in a 37ºC water bath for 3 hours. The digestion was stopped by running an agarose gel. The new DNA products were excised around 800 bp for EGFP and β-lactamase and 5000 bp for pFLAG-MAC and extracted using the gel extraction kit.

The OPH-EGFP fusion construct sample and vector samples were digested sequentially, with a PCR clean up in between digestions. Each digestion mixture contained 25.5 µL of extracted DNA sample, 1.5 µL of restriction enzyme, and 3 µL of corresponding buffer. For the first digestion, the restriction enzymes were determined based on star activity. *KpnI* and 10X FastDigest buffer were employed for the fusion construct amplified with the primers designed for pFLAG-MAC, and *XhoI* and 10X FastDigest buffer were added to the pET-21a primer samples. The vectors themselves were digested in the same way as their respective inserts. The samples were incubated in a 37°C water bath for 15 minutes. Digestions were halted by PCR purification following the procedure of the Qiagen PCR purification kit. Buffer PBI (5 volumes of buffer to 1 volume of PCR product) was added to the digestion mixture and vortexed. The samples were transferred to a QIAquick column and centrifuged for one minute, discarding the flow-through. Buffer PE (750 µL) was added to the column, which was centrifuged again for one minute. The flow-through was removed, and the columns were centrifuged again to remove excess wash buffer. The columns were transferred to sterile 1.5 mL microcentrifuge tubes, and 30 µL of Buffer EB was added to the center of each column to elute the DNA. The columns stood for one minute before being centrifuged for one minute. The second digestions were

performed using the remaining restriction enzymes. *EcoRI* and 10X FastDigest Buffer were added to samples intended for the pFLAG-MAC vector. *BamHI* and 10X FastDigest Buffer were added to the pET-21a samples. Digestion preparation was identical to the previous protocol, and the digestion was stopped by running the samples on a gel. The products were excised around 1800 bp for the OPH-EGFP construct and about 5000 bp for pFLAG-MAC and pET-21a. The DNA was extracted using the gel extraction kit.

NanoDrop

The NanoDrop was utilized to determine nucleic acid concentrations of the purified PCR products. The platform was cleaned with 3 µL of DI water and allowed to sit for two minutes. To blank the NanoDrop, 2 µL of Buffer EB was added to the platform. DNA sample concentrations of OPH, EGFP, β-lactamase, pFLAG-MAC, and pET-21a were measured by adding 2 µL of each sample to the platform, blanking with elution buffer between samples.

Ligation

Ligations were performed based on concentrations determined from the NanoDrop. Volumes of DNA were added to the reaction mixture following a gene insert to vector ratio of 3:1. The designated volumes of the separate EGFP and β-lactamase gene inserts and the corresponding volume of the pFLAG-MAC vector were added to ligation reaction mixtures. Each mixture contained 4.0 µL T4 DNA ligase buffer, 2.0 µL T4 DNA ligase, the corresponding volume of either EGFP or β-lactamase, the appropriate volume of the pFLAG-MAC vector, and DI water to equate to 20 µL. The ligation mixtures were placed in the thermocycler at 16ºC overnight. The same procedure was followed for OPH-EGFP fusion construct and the pFLAG-MAC and pET-21a vectors.

Transformation

Transformation mixtures were prepared in Falcon tubes with 1 µL of each ligation mixture and 20 µL of DH5α competent cells. The mixtures were incubated on ice (10 minutes), heat shocked in a water bath (42ºC, 45 seconds), and placed back on ice (2 minutes). SOC media (350 µL) was added to each tube. The mixtures were then incubated on the shaker table (37ºC, 15 minutes). The transformation cultures were plated on LB-ampicillin plates, spreading 10 µL of sample on one half and 20 µL on the other. The cultures were incubated overnight at 37ºC.

Results and Discussion

Validation of the local pH Theory with β-lactamase and EGFP

Isolation of the gene sequences with PCR

To determine that the primers intended for the isolation of β-lactamase and EGFP were designed properly, a gel was run using PCR samples of both genes (Figure 17). Several trials were completed yielding similar results so a representative gel is shown. Both β-lactamase and EGFP are around 800 bp, and bands were present for both samples, indicating the primers were designed correctly. The 800 bp bands were excised and the DNA was extracted.

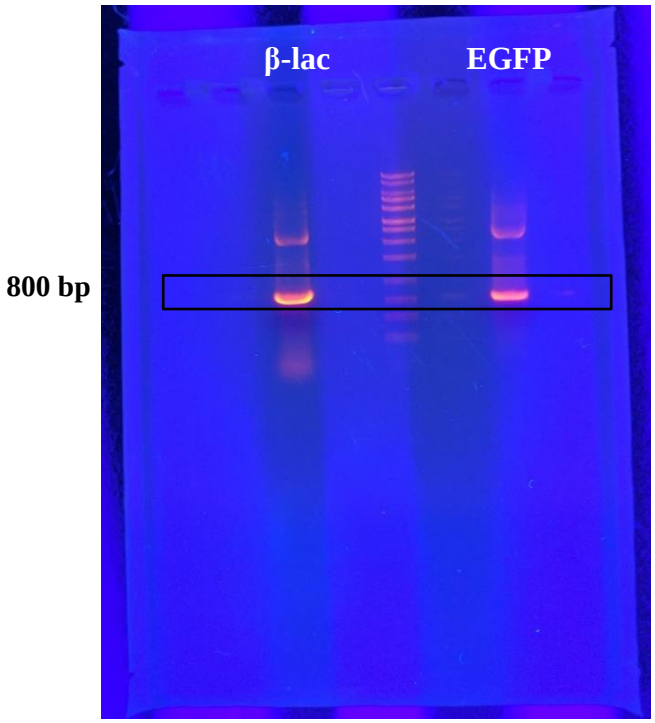


Figure 17: Representative gel electrophoresis results of PCR with β-lac and EGFP

Restriction Digestion Reactions

Several trials of β-lactamase and EGFP samples isolated from the gel concurrently digested with *Hin*dIII and *Eco*RI were completed. A 30 µL digestion mixture was made for both EGFP and β-lactamase as well as the pFLAG-MAC vector using the reagents and volumes discussed in the Methods section. To halt the digestion, a gel was run with each sample (Figure 18). As before, a representative gel is shown because each trial gave similar results. A band around 5000 bp was seen for the pFLAG-MAC sample, and bands around 800 bp were seen for both β-lactamase and EGFP. This suggests the digestions successfully cut the vector and each gene, leaving the entirety of the sequences of interest. The bands were excised, and the DNA was extracted.

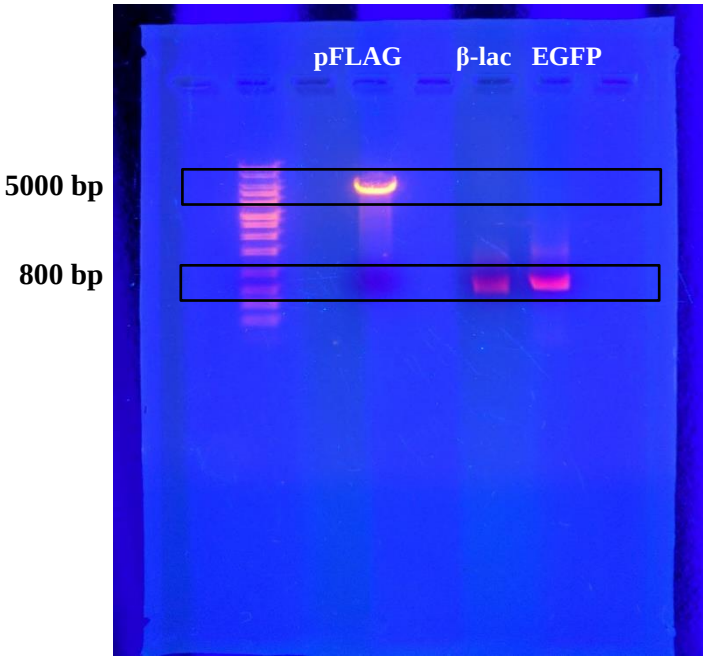


Figure 18: Representative gel electrophoresis results of digestion reaction of pFLAG-MAC, β-lac, and EGFP with restriction enzymes

NanoDrop Data

A NanoDrop was used after the digestion and subsequent gel extractions to determine the nucleic acid concentration in each sample. The resulting data, summarized in Table 4, showed moderate amounts of DNA in each sample. While the concentrations were not exceptionally high, they were within a range that suggested there was enough DNA for each of the samples to be used for further work.

Table 4: NanoDrop data following restriction digestion of each sample

DNA Sample	Concentration (ng/μL)
pFLAG-MAC	59.5
β-lactamase	52.0
EGFP	27.1

Ligation Reactions

Ligation mixtures (20 μL) using a 3:1 ratio of insert to vector were prepared using the concentrations from the NanoDrop data. The amounts of each reagent used are summarized in Tables 5 and 6 for the ligation of EGFP into the pFLAG-MAC vector and β-lactamase into the pFLAG-MAC vector, respectively. The ligation mixtures were subsequently transformed into DH5α high efficiency cells and spread on LB-ampicillin plates, however, no colonies were observed for each transformation trial. This suggests that either the digestion was incomplete where some gene sequences did not have sticky ends, the ligation procedure was unsuccessful and the genes of interest were not ligated into the vectors, or that the transformation procedure failed, such that antibiotic resistance was not conferred to the DH5α cells.

Table 5: EGFP ligation mixture resulting from NanoDrop DNA concentrations

Component	Volume
EGFP insert	8 μL
pFLAG-MAC vector	1 μL
Nuclease-free water	5 μL
T4 ligase buffer	4 μL
T4 DNA ligase	2 μL
Total	20 μL

Table 6: β-lactamase ligation mixture resulting from NanoDrop DNA concentrations

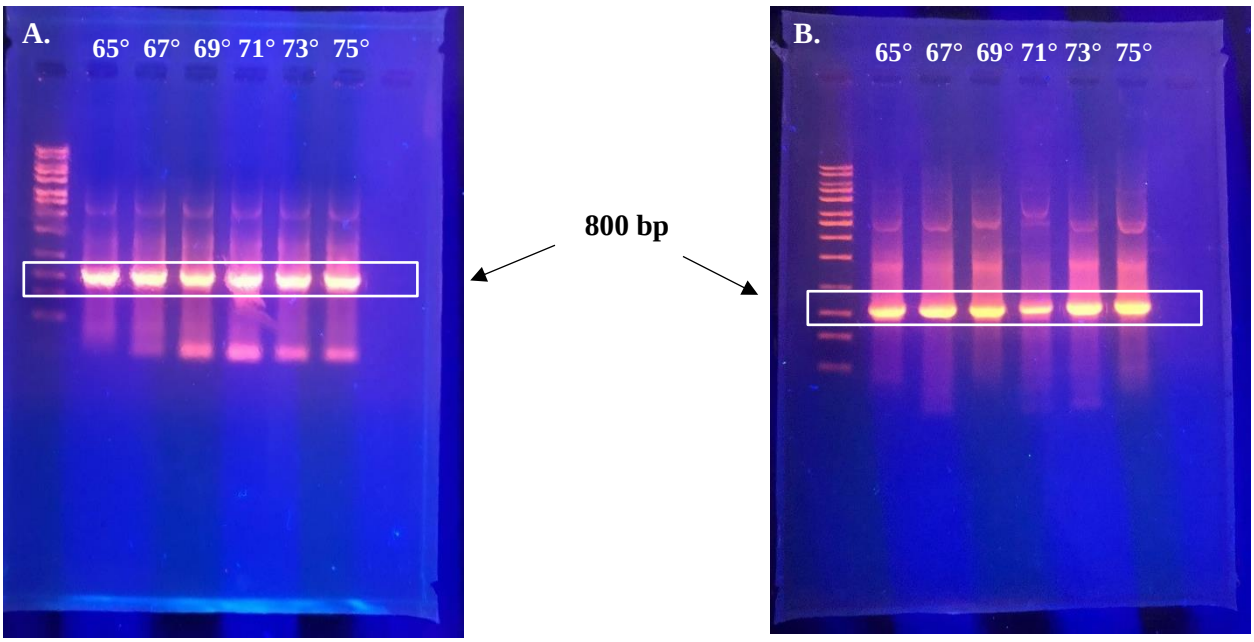
Component	Volume
β-lactamase insert	7 μL
pFLAG-MAC vector	2 μL
Nuclease-free water	5 μL
T4 ligase buffer	4 μL
T4 DNA ligase	2 μL
Total	20 μL

OPH-EGFP Fusion Protein

Primer Optimization

Once the DNA in the OPH and EGFP was eluted, primer annealing temperatures of the newly designed primers were optimized using gradient PCR. In the first trial, annealing temperatures increased by increments of 2ºC from 65ºC to 75ºC. One aliquot of each PCR sample of the separate OPH and EGFP genes was set at each temperature and the products were isolated on a gel (ethidium bromide, 3 μL, was used before RedSafe was purchased). The EGFP

primers for pFLAG-MAC showed bands at each of the six temperatures (Figure 19A), indicating all temperatures were sufficient for this set of primers. Due to the less concentrated amount of primer dimers in the 67ºC lane, that temperature was chosen as the optimum temperature. The EGFP primers for pET-21a showed similar results (Figure 19B). Each temperature resulted in a band on the gel, with low amounts of primer dimers present. The 69ºC lane showed the least amount of primer dimers while still maintaining a bright EGFP band, therefore it was chosen as the optimum temperature. The OPH primers for pFLAG-MAC only showed two faint bands in the 67ºC and 73ºC lanes, and large amounts of primer dimers (Figure 19C). These two bands were excised, but the annealing temperatures were lowered in subsequent trials. The OPH primers for pET-21a showed no bands and high amounts of primer dimers (Figure 19D), again, suggesting the annealing temperatures for that set of primers should be lowered.



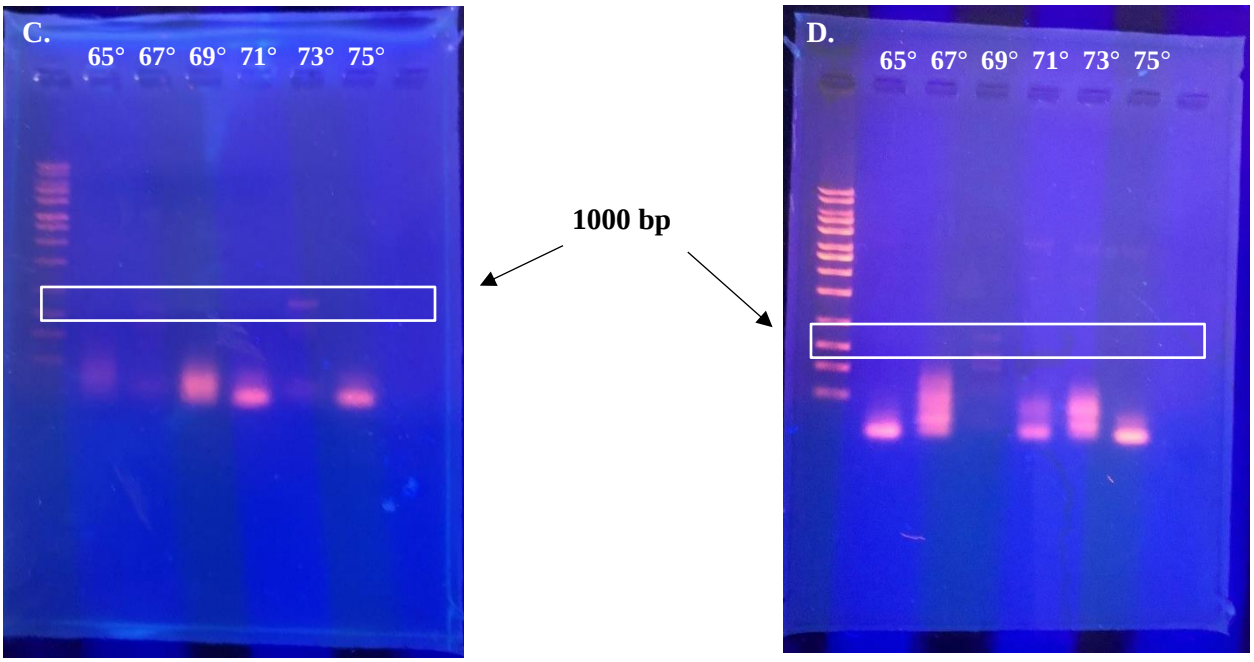


Figure 19: Gel electrophoresis results of gradient PCR of *A.* EGFP with pFLAG-MAC primers, *B.* EGFP with pET-21a primers, *C.* OPH with pFLAG-MAC primers, *D.* OPH with pET-21a primers

In the next trial, only the OPH primers were tested. The annealing temperatures were lowered based on the results of the previous trial. Here, the temperatures increased in increments of 2ºC from 60ºC to 70ºC. The OPH primers for pFLAG-MAC showed high amounts of primer dimers and no bands at 1000 bp (Figure 20A), suggesting the annealing temperature must be lowered even more. The OPH primers for pET-21a, again, showed large amounts of primer dimers, but also exhibited a band in the 60ºC lane (Figure 20B). This band was excised, and PCR parameters continued to be optimized.

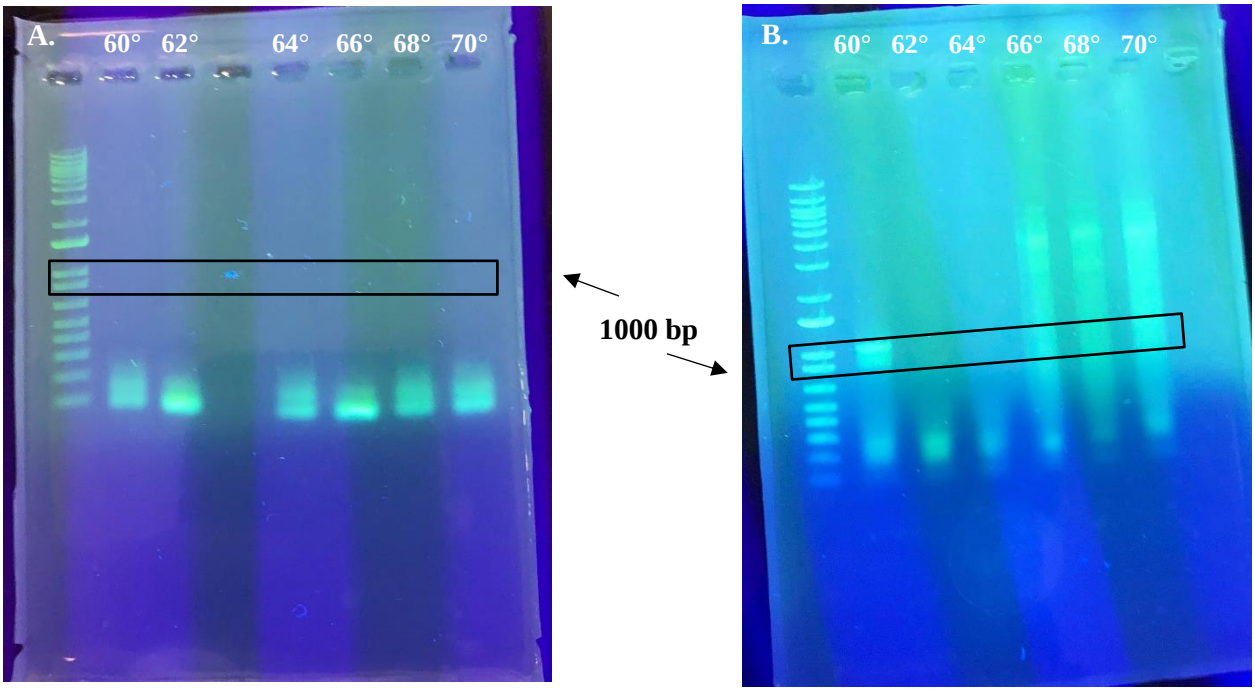


Figure 20: Gel electrophoresis results of gradient PCR of *A.* OPH with pFLAG-MAC primers, *B.* OPH with pET-21a primers

The final optimization trial, again, used only the OPH primers. Annealing temperatures were lowered to 54ºC-58ºC, increasing in increments of 2ºC. The OPH primers for pFLAG-MAC were loaded into wells 2 through 4, while the samples using the pET-21a primers were loaded in wells 5 through 7 (Figure 21). No bands were observed using the pFLAG-MAC primers, but bands were observed and excised at each temperature with the pET-21a primers. For the pET-21a primers, 54ºC was chosen as the optimum temperature. Due to each trial using the OPH primers for pFLAG-MAC resulting in no usable bands, this set of primers suggests re-evaluation is necessary to determine if any sequences in the primers interfere with either the pFLAG-MAC sequence or OPH gene sequence. Therefore, only the OPH primers for pET-21a were used for the remainder of the research.

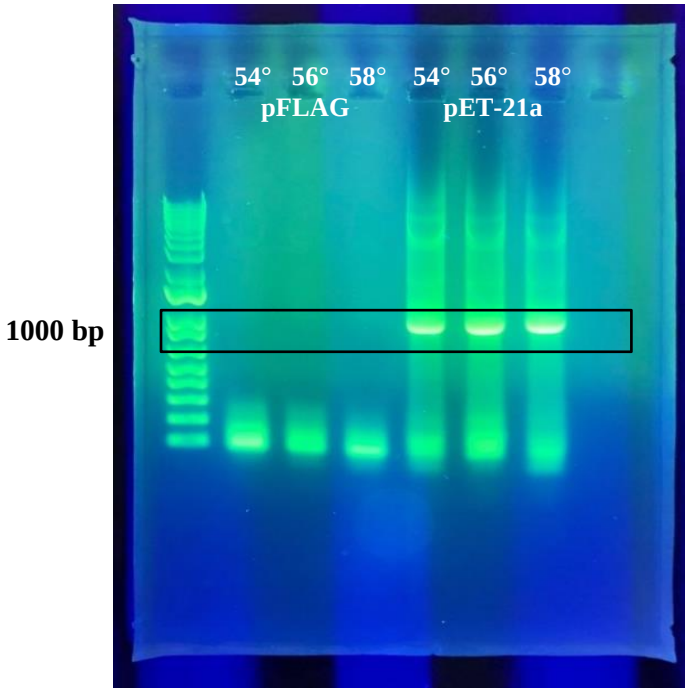


Figure 21: Gel electrophoresis results of gradient PCR of OPH with pFLAG-MAC primers and pET-21a primers

Overlap Extension PCR

An overlap extension PCR program was created using the parameters outlined in the Methods section. The samples used were those excised from the pET-21a EGFP and OPH gels, and only the OPH forward and EGFP reverse primers for pET-21a (Table 2) were used to amplify the fusion gene construct. The resulting fusion construct was expected to be about 1800 bp due to EGFP being about 800 bp and OPH being about 1000 bp. Two samples were loaded onto an agarose gel, but no bands were seen around 1800 bp (Figure 22). Bands were seen around 800 bp, suggesting the primers were insufficient in fusing the genes and/or there was likely no OPH gene present. Only one trial of overlap extension PCR was performed because no usable samples of the OPH gene were isolated from subsequent PCR reactions.

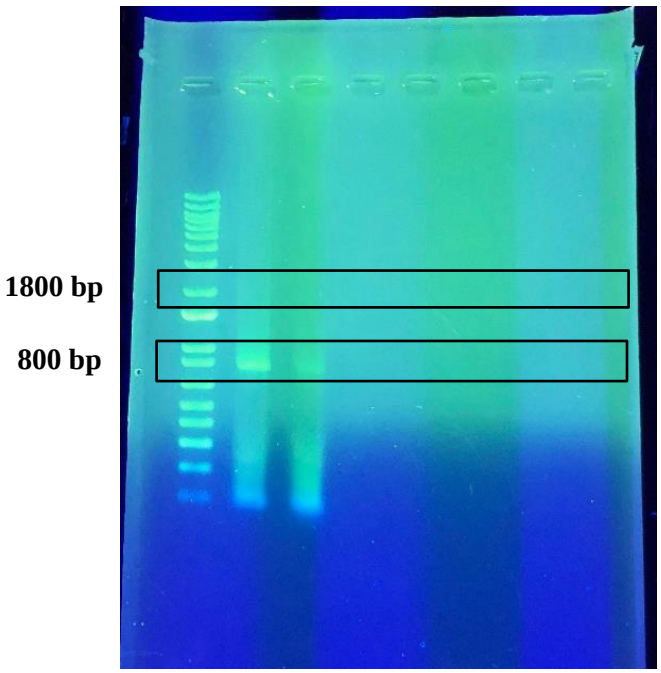


Figure 22: Gel electrophoresis results of overlap extension PCR with pET-21a primers

Conclusions and Future Work

The motivation for creating separate EGFP and β-lactamase proteins came from validating the theory of previous work in which a fusion protein between the two proteins was created. The newly designed primers for this experiment successfully isolated both gene sequences from the pEGFP plasmid. Transformations following restriction digests with *Hin*dIII and *Eco*RI and ligation into the pFLAG-MAC vector were unsuccessful for both the β-lactamase gene and EGFP gene. Further work optimizing PCR parameters may be necessary, as well as sequencing, purifying, and expressing the individual proteins.

The purpose of the OPH experiment was to create an OPH-EGFP fusion gene, ligate the construct into both pET-21a and pFLAG-MAC vectors, and transform the plasmids into DH5α maximum efficiency cells. The primers that were newly designed for this experiment were successful in amplifying the EGFP gene, but the pFLAG-MAC primers were not sufficient for amplification of the OPH gene, suggesting the pET-21a vector will be better suited for *in vitro* protein purification assays. The future course of this project will include the continuation of optimization of PCR parameters and the redesign of the pFLAG-MAC primers to ensure OPH amplification.

Overall, the project was intended to develop proteins that could be used in whole-cell sensing systems. A cellular host would provide both protein stability and allow for bioavailability studies of OPs and β-lactam antibiotics. Additionally, the *in vivo* assays could be used to assess enzyme kinetics and the toxicity of OPs as well as find new inhibitors of both enzymes.

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