Spring 4-27-2017

CLONING AND EXPRESSION OF PSEUDOMONAS AERUGINOSA ELASTASE FOR ELUCIDATING THE PATHOPHYSIOLOGICAL EFFECT ON LUNG TISSUE

Derek T. Draper
University of Texas at Tyler

CLONING AND EXPRESSION OF PSEUDOMONAS AERUGINOSA ELASTASE FOR ELUCIDATING THE PATHOPHYSIOLOGICAL EFFECT ON LUNG TISSUE

by

DEREK DRAPER

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology
Department of Biology

Ali O. Azghani, Ph.D., Committee Chair

College of Arts and Sciences

The University of Texas at Tyler
May 2017

The University of Texas at Tyler
Tyler, Texas

This is to certify that the Master's Thesis of

DEREK DRAPER

has been approved for the thesis requirement on
March 29th, 2017
for the Master of Science in Biology Degree

Approvals:

Thesis Chair: Ali O. Azghani, Ph.D.

Member: Brent Bill, Ph.D.

Member: Dustin Patterson, Ph.D.

Chair, Department of Biology

Dean, College of Arts and Sciences

Acknowledgments

First and foremost, I would like to thank my family for their support in pursuing an advanced degree in biology. Without their support, I wouldn't be the person I am today. I would like to express my immense gratitude towards Dr. Ali Azghani for supervising my project entirely and coordinating my efforts towards the wholesome fulfillment of my thesis. I would like to take this opportunity to express my profound and deep regards to my committee members, Dr. Dustin Patterson and Dr. Brent Bill, who have been instrumental in guiding me during the development of my research project and through the perfection of the final product. I want to extend my thanks towards Dr. Srini Kambhampati who gave me the opportunity to learn and grow as a researcher at this esteemed establishment. Also, I want to acknowledge the undergraduate researchers Christy Hjorth, Andrea Hernandez, and Jessica Bird that were invaluable in helping me complete the cloning and expression process. And lastly, I want to thank all the members of faculty and staff not mentioned that have been integral in my thesis accomplishment.

Table of Contents

List of Tables

List of Figures

Abstract

CLONING AND EXPRESSION OF PSEUDOMONAS AERUGINOSA ELASTASE FOR ELUCIDATING THE PATHOPHYSIOLOGICAL EFFECT ON LUNG TISSUE

Derek Draper

Thesis Chair: Ali O. Azghani, Ph.D.

The University of Texas at Tyler
May 2017

Pseudomonas aeruginosa causes acute nosocomial as well as community acquired pneumonia. In cystic fibrosis patients, this organism causes recurrent infections and inflammation with a high rate of morbidity and mortality. To support research in elucidating the molecular mechanisms of *Pseudomonas* elastase (PE) on disease progression, PE was expressed in *E. coli* and the protein purified for further application. This study represents a valid experimental protocol to produce. Genomic DNA was isolated from *P. aeruginosa* and selected regions were amplified using PCR with lasB gene specification. Amplified DNA was ligated into plasmids and *E. coli* were transformed to support the recombinant vector and produce a viable elastase product. The recombinant DNA was sequenced and accurately matched the pro-Elastase sequence (1497 bp in length). Purification of enzyme produced via heterologous expression in ClearColi LPS free *E. coli* was aided via Ni-NTA Liquid Chromatography system by the incorporation of a 6x Histidine affinity tag engineered on the C-terminus of elastase. Analysis by SDS-PAGE revealed a product of ~34 kDa in molecular weight consistent

with the molecular weight of the naturally produced mature Elastase. Proteolytic activity was determined via Skim Milk Agar diffusion. Elastolytic activity was determined by Elastin-Congo Red substrate to be two times the activity of the commercial enzyme. Cellular anatomy and physiology experiments confirmed the conserved pathologic effects of the Recombinant Elastase. Immunofluorescent study demonstrated preliminary data that Recombinant Elastase had the ability to disrupts tight junction structure as evidenced by fragmentation of ZO-1 proteins in Calu-3 cell monolayers.

Chapter One

Introduction

Significance and Historical Relevance of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic Gram-negative bacterium that typically affects hospitalized patients, presents life-threatening responses in hosts with compromised immune systems, and recurrent infections in patients with cystic fibrosis (CF) (1, 2). Among other species, *P. aeruginosa* is normally present in small numbers in the human intestines and freely living in water and soil. This organism is termed "opportunistic" because it is normally dealt with by the defenses of the body, however, when the opportunity arises, as in immunocompromised individuals, it can cause local or systemic infection (1).

This bacterium was discovered in 1882 by Carle Gessard (2), a chemist and bacteriologist from France. Dr. Gessard set out to determine why he kept observing blue and green coloration on the bandages of soldiers. He identified water-soluble pigments that were derived from the microbe in the pus of infections. These pigments turned blue-green after exposure to ultra-violet light. He termed this invader *Bacillus pyocaneus* and the cause of "blue pus" that was widely observed throughout many infections, especially in children. This bacterium, now known as *Pseudomonas aeruginosa* (*P. aeruginosa*), possesses the ability to build resistance to most antibiotics making it particularly dangerous (3, 4, 5, 6, 7). There are numerous cases of infection by *P. aeruginosa* since its

discovery in 1882, including an epidemic of diarrhea in the 1940s of 24 new-born infants. The initial infection was caused by improper sterilization of the milk supply and cleaning techniques utilized by the hospital staff (7, 8).

In 1928, the first true antibiotic, penicillin, was discovered by Alexander Fleming. Through his work, he incidentally observed a clearing in petri dishes containing *Staphylococcus* where the mold, *Penicillium notatum* resided. The clearing zones were caused by the production of an antibiotic by the mold which killed any bacteria nearby. The United States utilized penicillin in World War II to fight off infection of bacteria in battle wounds. However, due to its overuse, severely burned patients frequently displayed *P. aeruginosa* infections that were typically fatal because of the intrinsic ability of *P. aeruginosa* to develop resistance to antibiotics (9). *Pseudomonas* septicemia had been the major cause of death after the initial two-day post-burn period. Statistically, this bacterium is the cause of most fatalities from infection post severe burns (10, 11, 12, 13, 14). In 1954, a study was conducted to determine the epidemiology of this infection through the hospital and how to potentially contain the pathogen. Infected burns appeared to be the most important reservoirs. About 3 % of the stools of normal subjects and of patients with intestinal symptoms carried the organism, which was also isolated from the nose, throat, nasopharynx, skin and ear of a small proportion of patients and staff in the burns wards. The hands of the nurses were often contaminated with the bacterium which would then be spread to various sources through contact (15).

As the knowledge of this bacterium spread and the concurrent use of antibiotics increased, the relevance of *P. aeruginosa* amplified. Among the list of infections, corneal destruction is one of the most commonly observed. After cataract extraction, a ring

similar in pigment to intra-capsular cataracts is present in many patients. This was determined to be a pyocyaneous infection (*P. aeruginosa*) and was treated with streptomycin therapy because this strain was resistant to penicillin (16). In the cornea, it's been observed that *P. aeruginosa* causes acute ulceration, hyperacute conjunctivitis, infection and scarring (17, 18, 19, 20).

Because of *P. aeruginosa's* prominence in the hospital environment and post-surgical procedures, studies were directed to the cause of infection and initial treatment of conditions. A major cause for concern has been in pulmonary medicine. In this realm, the bacterium contaminates inhalant equipment and becomes a chronic inducer for sustained infections with ulcer of the lung epithelium and hemorrhage sequel (21).

Today's impact of *P. aeruginosa* pathogenesis is centered toward patients suffering from acute and chronic infections accompanied with a life-threatening injury, compromised immune system, or illness, such as Cystic Fibrosis (CF). Patients with CF develop chronic infections because the lung epithelium produce thick and sticky mucus instead of the typical thin and slippery product. This is due to defective chloride channels that prevent chloride from exiting the cell, in turn water is abnormally retained and the mucus becomes thick and immotile. The stagnant mucus becomes a breeding ground for bacteria and fungi. The most prevalent bacterium in patients with CF is *P. aeruginosa* which replaces other causative bacteria. There is no cure for CF, but different treatment options reduce symptoms and extend life expectancy. Many options include: medications such as antibiotics, chest physical therapy, pulmonary rehabilitation, kalydeco (improves function of defective channels), and lung transplant. Due to recurrent *Pseudomonas* infections and increase in antibiotic usage in CF patients, *P. aeruginosa* has become more

pathogenic and resistant to drug treatment and options become limited for patients in later stages of this disorder (22). Remedies used today to alleviate infection by *P. aeruginosa* include antimicrobial combination drug therapy (beta-lactam antibiotic with an aminoglycoside). The family of beta-lactam antibiotics are broad-spectrum and consist of penicillin derivatives, cephalosporins, monobactams, and carbapenems. Further, the aminoglycoside reduces protein synthesis in Gram-negative bacteria. However, the choice of specific antibiotic depends on the site and extent of the infection and on the resistance of the present strain (23, 24). Good examples of these therapy interventions include the use of the Dutch regimen, an intermittent/combination therapy option in which patients are treated with a parenterally administered combination of a β-lactam antibiotic with a β-lactamase inhibitor called Piperacillin-tazobactam or the use of TOBI podhaler that releases a dry powder directly into the respiratory epithelium (25). Furthermore, Azghani and colleagues published work on the use of liposomal antibodies, antibodies surrounded by lipids, to increase the effectiveness of antibodies and decrease the inhibitory effect in the sputum (26).

Cellular and Molecular Pathogenesis of *P. aeruginosa* Infection

Normally, the bacterium doesn't take up a large amount of the whole microbiome in individuals. However, hospitalized patients are at much greater risk for *P. aeruginosa* infection and spreading (1, 2, 27). Thus, when the opportunity arises, this bacterium makes use of the susceptible hosts (1, 28).

I. Bacterial Adhesion

A common laboratory reference strain is the *P. aeruginosa* PAO1, a spontaneous chloramphenicol-resistant mutant of the original PAO strain (earlier called "*P. aeruginosa* strain 1"). PAO1 was isolated in 1954 from a wound in Melbourne, Australia and provides a genome of reference for one of the most virulent strains isolated from a patient and is the most commonly studied strain of *P. aeruginosa* available (29, 30).

The first step in *P. aeruginosa* adhesion is colonization of altered epithelium. Once initially adhered, the bacteria begin to expand and divide into mature, stable colonies. Many studies describe the use of various pili that allow for complete adherence to host cells. Further, it has been shown the flagella and other bacterial products act in adhesion as well (31, 32, 33). It was discovered that the flagella and pili are not necessary for the initial attachment and formation but do increase the probability of resistance development (34).

P. aeruginosa is known to produce a biofilm as part of the infection process due to chronic infections. The process of biofilm formation begins with the initial infection to the surface of tissue, followed by attachment and formation of microcolonies, and lastly the biofilm maturation. Once a biofilm is matured in formation, it retains the ability of antimicrobial resistance caused by many chronic and persistent infections (35, 36, 37, 38). The PAO1 strain was utilized in experiments to study the formation of biofilms. A Green Fluorescent Protein (GFP) tagged PAO1 wild type elucidated the ability of *P. aeruginosa* to infect hosts by studying the composition and adherence of the bacterium in the formation process of mature biofilms (34).

II. Virulence Factors of *P. aeruginosa*

Both cell-associated and extracellular products of *P. aeruginosa* contribute to its virulence. Surface structures, including pili and the polysaccharide capsule or glycocalyx, appear to mediate the initial attachment of *P. aeruginosa* to its prospective host, thus permitting colonization (31, 39, 40, 41, 42). Extracellular enzymes such as alkaline protease, elastase, phospholipase C, and exotoxin A interrupt cellular metabolism, degrade infected tissues and promote bacterial invasion. When spreading occurs, systemic disease results, often with fatal consequences (39, 40, 43).

After years of study by pioneers including Morihara et al., Barbara Iglewski and colleagues, it became apparent that the bacterium's virulence depends on many cell-associated and extracellular factors. An initial break in the skin and mechanical boundaries can cause an infection by this bacterium because of its repertoire of virulence factors and host compromised immune systems (44, 45, 46). For instance, *in vivo* studies described the use of mutant strains that did not produce exotoxin A, exoenzyme S, elastase, or alkaline proteases (47, 48). It was discovered that these elements are necessary for *P. aeruginosa* infection; however, the anatomical location determines the ability of any given virulence factor in the infection establishment (47, 48, 49). Various strains have adapted to antibiotics and therefore, there is an increased demand for studying antimicrobial resistance, mechanisms of virulence including secreted toxins, and ability to form biofilms (50, 51, 52).

III. *P. aeruginosa* Extracellular Toxins

The most well-known research on extracellular toxins was performed by Justinus Kerner, a German poet, practicing physician, and medical writer was the first to

completely and accurately describe the symptoms of botulism and the botulism toxin that caused the side-effects such as cramps, vomiting, breathing problems, difficulty swallowing, double vision, and weakness or paralysis. Botulism is caused by the neurotoxin produced by various strains of the bacterium *Clostridium botulinum* (53, 54, 55). Kerner went on to describe a therapeutic use of the botulism toxin based on a set of experiments he performed using the toxin in clinical and animal experiments. Interestingly, modern developments made by Alan Scott in 1980 have allowed for the medicinal purpose of these toxins and are some of the most widely used pharmaceuticals in treating patients with strabismus (crossing eyes), cosmetic applications, abnormal disorders associated with movement, and even pain (53, 56).

This observation by Kerner was a huge shift in the understanding of disease and infection by bacteria. No longer were bacteria the exclusive cause, but substances released by the bacteria were acting in on tissues to allow them to infect, propagate, survive and thrive. There are two types of toxins that bacteria can express: exotoxins or endotoxins. Exotoxins are directly secreted by bacteria (54, 55). They cause damage to tissue of the host by destroying key proteins that maintain tissue structure or cellular metabolism. Further, these exotoxins can also be released during lysis of the cell. On the other hand, an endotoxin located in the outer membrane of Gram-negative bacteria, also known as lipopolysaccharides (LPS), illicit strong responses of inflammation and other immune responses when exposed to a host (53, 57).

Early observations of *P. aeruginosa* by Morihara's lab pointed to the production of toxins but it wasn't until the 1970s that these toxins were properly characterized. *P. aeruginosa* produces an expanse of extracellular toxins (exotoxins), which include

phytotoxic factor, pigments, hydrocyanic acid, proteolytic enzymes, and phospholipase (58, 59). The most important factor in the pathogenicity of *P. aeruginosa* is the elaboration of a group of protein exotoxins including Exotoxin A, the first studied exotoxin by this bacterium. This enzyme enters host cells through receptor-mediated endocytosis and lands in the cytoplasm where it catalyzes ADP-ribosylation inactivating Eukaryotic Translation Elongation Factor 2 (eEF-2), a protein necessary for ribosomal function, leading to a standstill in protein biosynthesis and the host cell's eventual death (60, 61, 62). This and the many other exotoxins altogether can produce a variety of disease symptoms including leukopenia, acidosis, circulatory collapse, necrosis of the liver, pulmonary edema, hemorrhage, and necrosis of kidneys. The proteolytic enzyme, *P. aeruginosa* Elastase, that breaks down peptide bonds of proteins produced by *P. aeruginosa* are responsible for the hemorrhagic and necrotic changes in the skin, as well as devastation of the cornea in eye infections. The hemolytic phospholipase C enzyme may be responsible for the damage and overall destruction of pulmonary surfactant. When this occurs, the result is typically atelectasis or the collapse/closing of the lungs (63). This effect, together with inflammatory response and the necrosis of lung parenchyma are important in the pathogenesis of the damage to lungs due to *Pseudomonas*-induced pneumonia. Further, it has been implicated that various strains of *P. aeruginosa* produce an enterotoxin responsible for gastrointestinal diseases (54, 55, 56).

In 1985, Dr. Iglewski's team employed a genetic approach to determine the contribution of Exotoxin A, exoenzyme S, Elastase and alkaline protease to the pathogenesis of *P. aeruginosa*. They introduced mutations into various strains with

chemicals or transposons (48). Pathogenesis of the mutants were then identified using immunological, chemical, or toxicity assays to observe their changes and/or increased resistance or pathogenesis. Mutants were extensively characterized *in vitro* to ascertain that they were identical to their parent strain except for the production of the desired product that could be utilized for further studies as knockouts, knockdowns, or dysfunctional for the appropriate exotoxins or enzymes produced. One such model, PAO-E64, was a mutant that produced an antigenically identical elastase except for its deficiency in elastase activity (48, 64). The idea was to better understand how these various toxins produced by *P. aeruginosa* acted alone, together, or in synergy. This comparison of the overall function of this bacterium signified infection is not due to a single secreted factor alone. As expected, the data indicated that virulence of *P. aeruginosa* is multifactorial and the relative contribution of a given strain (mutation) of *P. aeruginosa* product may vary with the type of infection and the location of tissue because of the different cell types and immune responses (44, 64, 65).

Until the 1960s and 70s, the least-characterized, yet most likely major virulence factor, was the trypsin-sensitive, heat-labile exotoxin, *P. aeruginosa* Elastase. This toxin had been shown to be lethal for mice and can elicit hypotensive shock in monkeys and in dogs after intravenous administration. Previous investigations in the Liu laboratory showed that Elastase inhibits uridine and amino acid uptake by cultured cells and inhibits the respiration of mitochondria derived from mouse liver cells (65, 66). The ability of this metalloprotease to degrade several extracellular proteins including elastin and collagen and functionally inactivate human immunoglobulin G, serum alpha1-proteinase inhibitor, and several complement components accentuates its potential importance as a virulence

factor (67). Because of the emphasis of my thesis research, the remainder of this introduction focuses on the characterization of Elastase, disruption of tight junctions and increased epithelial permeability, and the interplay of signal transduction pathways involving *P. aeruginosa* Elastase (PE).

IV. Characterization of *P. aeruginosa* Elastase

Morihara and his coworkers demonstrated in 1965 that most strains of *P. aeruginosa* produce three distinct proteinases and one, a semialkaline proteinase, possesses elastolytic activity as a single homogenous enzyme (68, 69). *In vivo* studies revealed the pathologic effects of purified PE in animal models. Intracutaneous injection of PE in microgram amounts had the ability to cause hemorrhage, cellular infiltration of the subcutaneous tissue and muscular layer, and degeneration of endothelial cells (70). Similar results occurred via intraperitoneal injections in mice causing pulmonary and subcutaneous hemorrhaging (71). The production of PE is strain specific and accompanied to taxonomic origin (72). Inhibition of elastase was achieved temporarily with phosphoramidon, a powerful inhibitor for many proteases and elastases. The elastolytic effect was inhibited for a period of twelve hours following the addition of the compound to an elastase solution injected into the eyes of rabbits and subcutaneously into mice (73, 74).

Further biochemical and molecular studies characterized the specific gene coding for PE. A DNA fragment from *P. aeruginosa* was cloned into *E. coli* via plasmid transformation. Deletion mutant analysis confirmed the full-length gene coding for elastase must be approximately between 1.0-1.3 Kb in length (75). The DNA segment encoding Elastase was cloned and the complete nucleotide sequence was determined in

1989. The primary structure of Elastase was determined to be like thermolysin produced by *Bacillus thermoproteolyticus* because the functionally important amino acid sequences and residues are identical and both are Zn-metallo proteinases. These proteinases are found in many microorganisms but also in snake venom and within the organs and tissues of mammals. Mature elastase is an enzyme containing 301 amino acids with a molecular mass of 32,926 daltons (33kDa). Further, a "pro" sequence was determined containing 197 amino acids in length. Initially, Elastase is expressed as an inactive pro-peptide similar to other proteases, and this "pro" domain is cleaved in the periplasmic space to produce the active Elastase (76, 77, 78). A more in depth molecular characterization of the Elastase structural gene (lasB) was performed by Iglewski et al. in 1988. The nucleotide sequence of the lasB gene containing 1,491 base pairs encoded a protein of 498 amino acids with a molecular mass of 53,600 daltons. Using PAO mutants with inactive elastase, two structural genes (lasA and lasR) were quantified and verified necessary for the activation of the mature elastase (67, 79, 80, 81). LasR was identified as a requirement for the transcription of lasB and lasA protein had the ability to enhance the elastolytic activity of mature PE (81, 82, 83).

Pathophysiological Effect of PE

I. Electrophysiology and Paracellular Permeability

Normal respiratory epithelium plays a key role in the immune response to pathogens and as a protective barrier against foreign microorganisms and macromolecules. During embryogenesis and cell differentiation, layers of epithelium are specially developed to create a barrier for the body's defense and integrity. Along with a barrier function, polarized epithelial cells are key to the exchange of ions and water

across membranes within the lungs and other tissues (84, 85). However, the normal release of minute amounts of exotoxins by a bacterium, such as *P. aeruginosa*, might cause a breach in this barrier increasing the susceptibility to bacterial invasion and infection. One *in vivo* study sought to elucidate this idea and observed the effect of PE on the lungs of guinea pigs. This demonstrated that aerosols of elastase increased the clearing rate of labeled albumin. Further, PE was traced to interruptions specifically in the tight junction structures that help in the creation of a tight monolayer of epithelial cells (86, 87). Therefore, an increased clearing rate of a substance in the lungs coincides with a decreased barrier function caused by PE addition.

To help form regulatory barriers, the epithelial cells create intercellular junctions called tight junctions. Tight junctions are considered the ultimate gateway between the cells and the underlying tissues. The complexes that form tight junctions are composed of various proteins including occludin, claudin, and the cytoplasmic scaffolding proteins zonula occludens (ZO-1, -2, -3) (88, 89, 90). Disruption of the proteins that form the complexes for tight junctions can cause an adverse effect in the transport and filtering functionality of the epithelium (91, 92, 93). Therefore, as demonstrated previously, any alteration by bacterial toxins can interfere with normal barrier functions (94, 95). Madin-Darby canine kidney (MDCK) epithelial cells demonstrated the ability of PE to perturb the function of the tight junctions by altering the concentration of ZO-1 proteins without membrane disruption. This indicated that PE has a toxic effect on the barrier function of epithelial monolayers and alters at least one protein in the tight junction complex (96). Further, virulence factors released from *P. aeruginosa* including PE and exotoxin A exhibited an ability to increase epithelial permeability by the measurement of Trans-

epithelial Electrical Resistance (TER), however, during experiments with acute pneumonia, data suggested that PE breaks down tight junctions increasing its virulence. And in synergy with lipopolysaccharide (LPS), PE demonstrated an increase in epithelial permeability and facilitated the influx of neutrophils causing an inflammatory response (97, 98, 99, 100).

II. Molecular Mechanism of the Disruption in Epithelia Integrity

Since PE disrupts the function of tight junctions without resulting in cell injury or death, investigations to explain this effect were explored. A plausible theory pointed in the direction of host signaling pathways involved in the formation of tight junctions because previous research determined the ability of *Pseudomonal* proteases in stimulating the extracellular regulated kinase (ERK) signaling pathway, enhancing IL-8 production *in vitro*, and activating other inflammatory responses through certain protease-activated receptors (101, 102, 103). Therefore, an exploration in the role of PE on the pathway of tight junction formation was studied. It is known that tight junction assembly and sealing, cellular localization of occludin and ZO-1, along with the previous pathways described, are in part regulated by protein kinase C (PKC) signaling (104, 105, 106). Our lab incorporated a study that discovered the impact of PE-induced PKC signaling and the redistribution of the multifunctional complex that defines tight junctions. The results demonstrated PE pathogenesis targets occludin, ZO-1 and F-actin by elevating PKC levels and causing tight junction translocation and cytoskeletal reorganization (107). These experiments all elucidated the mechanistic action of PE on paracellular permeability and tight junction disruption. The next discoveries allowed for an enhanced explanation of this distinct effect on cell signaling.

III. Signal Transduction Pathways Involved in Pathogenesis of PE-induced injury

The epidermal growth factor receptor (EGFR) is a complex signaling pathway involving a number of signaling cascades. This receptor is common to many cell types including lung epithelium. Receptor tyrosine kinases, like EGFR, are transmembrane receptors composed of an intracellular domain, extracellular ligand-binding domain, and a transmembrane portion (108). The EGF family of receptors are initially activated via ligand-receptor complex formation. It is known that members of this family of tyrosine receptors are activated by a multitude of ligands including Epidermal growth factor (EGF), Transforming growth factor (TGF – α), heparin binding (HB)-EGF, amphiregulin, β-cellulin and epiregulin (109, 110). Once ligand bound, a conformational change occurs within EGFR, dimerization of the receptors, and then autophosphorylation of the tyrosine residues results. Transient phosphorylation of tyrosine residues on internal cellular substrates activates downstream signaling including phospholipase C-y (PLC-y), PKC, mitogen-activated protein kinases (MAPK), and ras GTPase-activating protein (GAP) (111). This ultimately leads to increased activity in angiogenesis, cell differentiation, proliferation, survival, progression of many cancers, and more recently reported regulation of tight junctions via PKC signaling (104, 105, 106, 112, 113, 114, 115).

A consequence of autophosphorylation of EGFR, as mentioned previously, is the activation of downstream elements like MAPK. Mitogen-activated protein kinases include ERK 1/2, ERK 3/4, ERK 5, ERK 7/8, c-Jun N-terminal kinase (JNK) 1/2/3 and p38 isoforms. It is known that ERK signaling is involved in cell proliferation and through extracellular transient activation by EGFR, can regulate tight junction protein assembly

and cytoskeletal rearrangement (116). Our lab demonstrated the ability of PE to activate the epidermal growth factor receptor and further elicit downstream activation of the ERK 1/2 arm of the MAPK pathway within 5 minutes of apical exposure of Calu-3 cell monolayers. This activation preceded the disruption in tight junction structure by the loss of localization of occludin, ZO-1, and cytoskeletal reorganization (Castillo, Azghani, unpublished). Further, this ability of PE to activate this signaling cascade was revealed through inflammatory pathways that displayed an enhanced IL-8 production via NF-κB activation (117).

Another family of downstream signaling affected by EGFR activation is the Rho family of small guanosine triphosphate (GTP) – binding proteins. Rho proteins are identified to be important in the regulation of tight junction structure, function and assembly (115). This is important in the context of mediating Rho GTPases because previous studies of bacterial toxins on tight junction and actin cytoskeleton documented their ability to reorganize actin cytoskeleton, disrupt tight junction structure and function, and causing the formation of gaps in cell monolayers (100). This was further illustrated by additional studies displaying Rho protein importance in tight junction structure and function regulation (118, 119). Experiments ran in our lab verified a similar effect caused by PE on cell monolayers. RhoA GTPase was activated through EGFR and MAPK signaling by PE treatment on cell monolayers and confirmed by morphological analysis with fluorescent microscopy (Pal, Azhgani, unpublished). However, even with all the knowledge accrued, there are still questions that need to be answered. Therefore, new methodology in our lab seeks to make the studies of elucidating the mechanisms more efficient.

Goals of this Thesis

- Synthesize a Recombinant Elastase
- Measure and compare activity of Recombinant Elastase to commercial product
- Define pathophysiologic effects of Recombinant Elastase in comparison to published data on *P. aeruginosa* Elastase

Synthesizing a Recombinant Elastase

I. History of Cloning and Recombinant Technology

The process of molecular cloning dates to the late 1960s with the discovery of restriction endonucleases, better known as restriction enzymes, that possess the ability to cleave specific regions of DNA. With this molecular machinery, along with reverse transcriptase and polymerase chain reaction (PCR), recombinant technology expanded exponentially as techniques improved manipulating the genetic material. The basic concept in cloning involves (1) the isolation of coding DNA fragments through PCR amplification, (2) ligation of isolated DNA, termed inserts, into an appropriate plasmid genetic carrier, (3) transformation of plasmid into a suitable host for propagation, (4) screening and selecting for hosts containing the intended plasmid, and lastly (5) expression of recombinant protein in new host organism (120). The process of manipulating DNA and creating suitable recombinant hosts for expression is straight forward, however, the selection of primers, vectors, extraction techniques, percent yield, and activity of recombinant product defines the actual efficiency of any select protocol.

Bever and Iglewski performed the first cloning and expression study of PE in 1988. They utilized a pUC18 plasmid to transform *E. coli.* The specific *E. coli* employed

in this cloning process was the TB1 strain that has an *hsdR* mutation which allows for the efficient transformation of unmethylated DNA from PCR amplification. Further, they implemented an *E. coli* cell-free transcription-translation system that resulted in small amounts of gene products. Elastolytic activity was measured by spectrophotometry readings using Elastin-congo red (ERC) of total cell lysates (80). The next group of researchers that attempted to clone and express PE utilized an HB101 *E. coli* strain with pUC18. This strain displays high stability of recombinant vector, high efficiency of transformation, and doesn't require a heat shock step. However, their representation of recombinant products does not account for purification technique or possible interaction by lipopolysaccharide (76, 77). More recent developments attempted the expression in a methylotrophic yeast, *Pichia pastoria*, which allowed the researchers to produce high yields of protein with an inexpensive induction promotor, AOX1, by methanol. Although yeasts produce a high yield of recombinants and are economically friendly, in comparison to *E. coli,* yeast are slow growers, protein production may take days versus hours, they must be produced immediately before use, and usually many clones need to be screened for protein production before a good producer is found (121).

An improved cloning technique was presented in 2013 by utilizing a plasmid containing a 6-consecutive histidine residue tag and transforming *E. coli* BL21 for expression (122). The BL21 strain is deficient in Lon protease and OmpT protease and is therefore generally preferred for recombinant protein expression. Furthermore, this strain of *E. coli* contains an RNA polymerase that recognizes the lac promoter necessary for Isopropyl β-D-1-thiogalactopyranoside (IPTG) induced expression. However, Raftari

et al. (2013) used a 6x histidine tag for verification via western blot analysis. However, they state their recombinant product to be 53 kDa in size rather than the 33 kDa product that should be expected (122).

The improvement of recombinant technology over time has dramatically increased our potential to create a more economically friendly enzyme, however, more work is necessary to produce an experimentally useful recombinant product. There are several concerns not accounted for with the previous research including lipopolysaccharide (LPS), efficient selection and ability to freeze stocks, easy purification techniques, correct size and activity of recombinant enzyme, and the ability to represent identical pathophysiological function to the naturally produced enzyme. Therefore, our overall goal is to improve the technology to account for these concerns and prove the existence of an identical recombinant Elastase product.

II. Purpose of Developing a Recombinant *P. aeruginosa* Elastase

P. aeruginosa elastase has demonstrated an invalidating ability to cause detrimental effects to the human body through *in vivo* signs and symptoms, such as: edema, fibrosis of the lungs, tight junction disruption, increased paracellular permeability, and induced inflammatory responses. However, much research remains to elucidate the molecular mechanisms for the activation of the pathways involved. To further our laboratory's research efforts, it was decided to clone and purify PE instead of purchasing the commercially produced and costly product. The purpose of this thesis is to offer an alternative process that is cost effective, efficient, and corrects for concerns from previous cloning techniques. This thesis elaborates on the ability to produce a recombinant PE that can be utilized for research purposes that further characterize the

precise nature of the previously studied effects *in vitro* and in an animal model of pulmonary infection and inflammation. This provides a laboratory setting that indubitably becomes self-efficient, an opportunity to reduce funding expenses for further project goals, and more knowledge in the process of PE production and characterization.

Hypothesis

In this study, evidence is provided in support of the hypothesis that *P. aeruginosa* Elastase can efficiently and effectively be cloned and expressed in *E. coli.* The significance of this study proves the identity of the recombinant enzyme (RecE) produced in terms of enzymatic activity and physiologic effects *in vitro*. The hypothesis was addressed by DNA isolation from strains of *P. aeruginosa* that produce PE and cloning the gene responsible for PE, lasB, with specifically designed primers. Follow up biochemical analysis and *in vitro* studies confirmed RecE identity as well as its pathophysiological activity.

Chapter Two

Materials and Methods

1) *Bacterial strains:*

   a) *Pseudomonas aeruginosa:* The strain of *Pseudomonas aeruginosa* (*P. aeruginosa*) is a clinical strain isolated from a patient positive for cystic fibrosis disorder (UT Health Northeast, Tyler, TX). For our experiments, this isolate was sub-cultured into 3 mL of Luria-Bertani (LB) broth at 37°C for 24 hours before DNA isolation.

   b) *Escherichia coli:* Two separate strains of *Escherichia coli* (*E. coli)* were used in this experiment. The first strain, 10G electrocompetent; Lucigen, Radnor, PA, was used for plasmid amplification and second strain, ClearColi BL21; Lucigen, Radnor, PA, was utilized for protein expression. ClearColi is a genetically modified *E. coli* that contains an altered form of lipopolysaccharide that does not trigger an endotoxic response in human host.

2) *Amplification of lasB gene:*

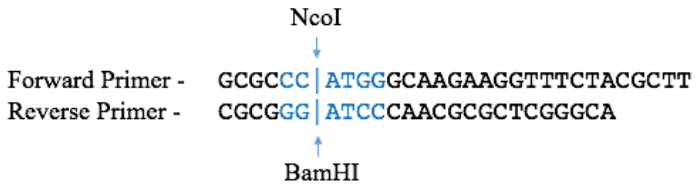
a) *Bacterial genomic DNA isolation:* Ultraclean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA) was used to isolate bacterial DNA according to the manufacturer's protocol. DNA was analyzed for purity using a Thermoscientific NanoDrop 2000 Spectrophotometer (ThermoFisher, Waltham, MA) to assess the quality of the isolated DNA. *P. aeruginosa* DNA was evaluated by absorbance at 260 nm.

b) *Primer Design:* Primers used for amplification of the LasB gene (*P. aeruginosa* Elastase coding gene) were the Forward LasB and Reverse LasB primers as follows:

5'>GCGCCCATGGGCAAGAAGGTTTCTACGCTT<3' (sense primer containing ATG start codon), containing a NcoI restriction site and

5'>CGCGGGATCCCAACGCGCTCGGGCA<3' (anti-sense primer), containing a BamHI restriction site (Eurofins Genomics, Louisville, KY). The highlighted regions contain the restriction enzyme recognition sites. These primers were developed by analyzing the beginning and end of the publicly available gene sequences for LasB on GenBank. The gene was amplified by Polymerase Chain Reaction (PCR) utilizing the *PfuUltra* II Fusion HS DNA Polymerase (Agilent Technology, Santa Clara, CA) in a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA).

PCR specifications were: Heating at 95°C for 5 minutes, Initial Denaturation at 95°C for 30 seconds, Annealing at 52°C for 30 seconds, Elongation at 72°C for 5 minutes, and 40 rounds. After amplification, the product was analyzed using agarose gel electrophoresis and sent for sequencing to Eurofins Genomics (Louisville, KY) to verify the amplified gene. The recombinant lasB gene sequence was deposited in the NCBI GenBank database (accession number KY861927).



3) *Transformation of E. coli:*

a) *Digestion and Ligation with bacterial plasmid:* The pET28b plasmid (Novigen Sciences, Inc, Washington, DC) was utilized for LasB gene incorporation, which installed the sequence tag containing 6 histidine residues (6xHis tag) to the C-terminus of Recombinant Elastase (recE) allowing single column purification by nickel affinity chromatography. Amplified LasB gene and pET28b vector were digested with NcoI and BamHI restriction enzymes (New England Biolabs, Ipswich, MA). The digested LasB gene and pET28b vector were purified using the Qiagen MinElute Reaction Cleanup Kit (Qiagen, Hilden, Germany). Purified LasB and pET28b were mixed and ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's directions. Subsequently, the ligation mixture was transformed into *E. coli*

10G electrocompetent cells (Lucigen, Radnor, PA) by electroporation using an Eppendorf Eporator (ThermoFisher, Waltham, MA). Cells were grown on an LB agar plate containing kanamycin (50 μg/mL) for selection of the pET28b plasmid. Colonies were screened for the presence of LasB via colony PCR and positive hits were subsequently grown up in LB broth. The DNA was extracted from selected colonies and sent for sequencing (Eurofins, Louisville, KY). Plasmids with a confirmed sequence for LasB were transferred into ClearColi (Lucigen, Radnor, PA) and successive expression experiments were carried out.

b) *Inductive Expression of Recombinant Elastase:* For expression, 5 mL LB broth cultures containing kanamycin were inoculated and grown overnight at 37°C. These cultures were later transferred to 1 L shaker flasks that were sterilized and made lipopolysaccharide (LPS) free by baking at 350°C for 3-4 hours. We incubated the flasks with shaking at 250 rpm at 37°C. Upon reaching an OD_{600} between 0.6 – 0.8, cells were induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Carbosynth, San Diego, CA) and allowed to incubate overnight at a reduced temperature of 30°C. Cells were harvested by centrifugation (Rotor TA-14-50, Beckman Coulter, Allegra 25R Centrifuge) and stored at -20°C until purification.

4) *Protein Harvest and Purification:*

a) *Cell Lysing:* Harvested cells were thawed at 4°C and resuspended in Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4). Lysis was carried out by sonication in a 50 mL conical tube on ice using a Qsonica Sonicator (Qsonica, LLC, Newtown, CT) with 70% amplitude, a process time of 5 minutes, pulse-on for 1 second, and pulse-off for 2 seconds. After sonication, the whole-cell lysates were centrifuged (Rotor TA-14-50, Beckman Coulter, Allegra 25R Centrifuge) at 14,000 rpm for 40 minutes at 4°C to separate the insoluble cell matter from soluble supernatant containing our recombinant Elastase (recE).

b) *Fast Protein Liquid Chromatography (FPLC):* Purification of recE was performed on a Bio-Rad FPLC NGC Chromatography System (Bio-Rad, Hercules, CA) the supernatant was applied to a nickel-nitrilotriacetic acid (Ni-NTA) affinity column pre-equilibrated with Wash Buffer (50 mM Tris, 300 mM Sodium Chloride, pH 7.5) using a DynaLoop 90 (Bio-Rad, Hercules, CA). Subsequently, the column was washed with 10 column volumes of Wash Buffer, then 10 column volumes of low concentration imidazole buffer (50 MM Tris, 300 mM Sodium Chloride, 25 mM Imidazole, pH 7.5), to remove non-specifically bound proteins, before final elution with Elution Buffer (50 MM Tris, 300 mM Sodium Chloride, 250 mM Imidazole, pH 7.5). During the elution process, absorption of eluent was monitored with Bio-Rad FPLC NGC Chromatography System

(Bio-Rad, Hercules, CA) and fractions collected with a BioFrac Fraction Collector (BioRad, Hercules, CA). Fractions showing strong absorption at 280 nm were kept for analysis on a 12% Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a Mini-Protean electrophoresis apparatus (BioRad, Hercules, CA) to verify the molecular weight of the purified product expected to contain recE. The recE containing fractions (~33kDa) were combined and dialyzed 4x with Tris Buffer (5 mM Tris-HCl, 0.5 mM Calcium Chloride, pH 7.5) for four hours each time to reduce the imidazole concentration. The dialysis tubing was 45 mm in width, 29 mm in diameter, and contained a molecular weight cutoff (MWCO) of 3,500 (Sigma-Aldrich, St. Louis, MO). Once dialysis was complete the recombinant solutions were centrifuged at 13.3 rpm for 10 minutes to remove any precipitated matter (inactive enzyme). Then, the final concentration of recE per solution (mg/ml) was determined by UV-Vis absorption with Thermoscientific NanoDrop 2000 Spectrophotometer (ThermoFisher, Waltham, MA). The Extinction Coefficient (ε) was calculated by translating the gene sequence to amino acids using an online DNA Translator (fr33.net) and subsequently using the software program Protein Calculator v3.4 (C. Putnam, The Scripps Research Institute, U.S.A.) to determine ε at A_{280} Molar Extinction with all disulfides.

5) *Recombinant Elastase Activity Assessment:*

a) *Proteolytic Activity:* Proteolytic activity was assessed by Skim Milk Agar Diffusion Plate analysis using varying concentrations of recE solution (7.36U/ml, 3.68U/ml, 1.84U/ml, and 0.74U/ml). Petri-dishes containing 1.5% skim milk powder and 1% agar were used to place round filter papers loaded with 10 μL of recE solution and observed for casein diffusion every 30 minutes for 4 hours, then overnight.

b) *Elastolytic Activity:* Elastolytic activity was assessed by Elastin-Congo Red Assay (ThermoFisher, Waltham, MA) by two separate methods. In the first method and step, a standard Optical Density (OD) per mg of elastin using Porcine Pancreatic Elastase (Worthington Biochemical Corporation, Lakewood, NJ) was developed to compare our recE for activity. Varying concentrations of elastin substrate (0, 2, 4, 6, 8, and 10 mg/ml) were added to 1 mL eppendorf tubes with constant volumes of Porcine Pancreatic Elastase (50 μL/ml) and left on a shaker for 1 hour at 37°C. OD was determined by spectrophotometry at A_{485} using the Bio-Rad SmartSpec Plus spectrophotometer (BioRad, Hercules, CA) and the average OD per mg substrate was calculated (+/- 5%). In step two, we ran an activity assay for our recE unknown by keeping the concentration of enzyme constant (50 μL/ml mixture) and increasing amounts of the elastin substrate (0, 2, 4, 6, 8, 10 mg). This elastolytic assay ran on a shaker for 1 hour at 37°C before reading OD. An OD per mg RecE was calculated to

determine if enzyme displayed a linear increase in solubilization with increasing substrate. To determine the mg of substrate solubilized, the OD readout was subsequently divided by the standard OD per mg substrate previously determined in the first step. Then, the units of activity per mg of recE was calculated by dividing the mg of substrate solubilized by the mg of recE used. One unit of activity equals the mg of elastin solubilized in 1 hour at 37°C. Equation 1 below demonstrates the previously mentioned calculations.

Equation 1:

$$\text{Units/mg} = \text{mg Substrate Solubilized/ mg Elastase} = (\text{OD Observed})/(\text{OD/mg Substrate})$$

The second method to analyze elastolytic activity utilized the same methodology listed above except for being read at 495 nm for absorbance. Following OD readouts, specific activity was determined by dividing the activity of the recombinant elastases by the activity of the commercial elastase and subsequently multiplying by 100 to represent a percentage.

6) *Virulence activity of RecE in vitro:*

a) *Cell Culture:* A human bronchial adenocarcinoma epithelial cell-line (Calu-3, ATCC; Manassas, Virginia) was used as an *in vitro* model to study the physiological effects of RecE. The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (Gibco, Life Technologies, Grand Island, New York) at 37°C in a humidified

atmosphere of 5% CO_2 / air. The medium was supplemented with 10% Fetal Bovine Serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 1% L-glutamine (Cellgro, Herndon, VA) and an antibiotic mixture of 10% Penicillin (100μg /ml). Cells were maintained in tissue culture flasks (Corning, Corning, NY) and were discarded after being passaged 20 times. The medium for the flasks was changed every other day to avoid nutrient depletion. Trypsin/EDTA (0.25%, Gibco, Life Technologies, Grand Island, NY) was used to transfer the cells from flasks to the appropriate cell culture ware according to the experimental design.

b) *Impact on Cell Morphology:* To identify morphological changes in response to concentration and time of treatment with RecE, 24-well cell culture cluster plates (Costar, Corning, NY) were seeded at $1x10^5$ cells with Calu-3 cells and grown to confluency before experimentation. Cells were serum-starved overnight (12 hours) before subsequent treatment with varying concentrations of RecE (1-8 U/ml) and observed using a Nikon TMS Inverted Microscope (Nikon, Tokyo, Japan) every 15 minutes for 2 hours. Images were captured using 8 MP, f/2.2, 29mm, phase detection autofocus Apple camera at 200x total magnification (Cupertino, CA).

c) *Immunofluorescence microscopy:* Fluorescence microscopy was employed to investigate the subcellular distribution of target tight junction proteins in Calu-3 cells during Recombinant Elastase exposure. Calu-3 cells were

seeded into Lab-Tek Chamber Slides an 8 Well Permanox system (ThermoFisher, Waltham, MA) at $1x10^5$ cells per chamber. Cells were treated with 1-2 U/ml of RecE for up to 90 minutes and a control (Carrier MEM alone) for 90 minutes. Following treatment, cells were washed with Phosphate Buffered Saline (PBS) 2x and fixed with 4% fresh paraformaldehyde solution in PBS for 10 minutes at room temperature. After an additional 2x wash with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. The cells were washed 2x with PBS before being blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature. After blocking, the monolayers were incubated with FITC conjugated Mouse Monoclonal Antibody against ZO-1 (10μg/ml; Invitrogen, Carlsbad, CA) at room temperature for 60 minutes in the dark. The monolayers were washed 2x with PBS before the addition of secondary antibody (Goat anti-Mouse IgG) conjugated with Alexa Fluor 488 (10μg/ml; ThermoFisher, Waltham, MA) at room temperature for 60 minutes in the dark. The monolayers were then washed 2x with PBS and then mounted using ProLong Gold antifade (ThermoFisher, Waltham, MA) before microscopic observation. The monolayers were examined under Zeiss LSM 5 Pascal Laser scanning Confocal Microscope equipped with an Axiocam HRc Color Digital Camera (One Zeiss Drive, Thornwood, NY). Photomicrography was performed under 500x total magnification with oil and images were captured for visualization in LSM software by Zeiss. Differences in the

localization of tight junction protein, Z0-1, were visually observed and documented.

Chapter Three

Results

Cloning and Sequence Analysis of Recombinant Elastase Gene from P. aeruginosa

The coding gene for *P. aeruginosa* Elastase (lasB) was amplified from the genomic DNA of two separate strains of *P. aeruginosa*, a clinical strain (from our clinical isolates collection from CF patients) and PAO1 from ATCC. It is reported that some strains of *P. aeruginosa* do not produce elastase, therefore, two strains were utilized to confirm for the elastase gene contained in the genome. An Ultraclean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc, Carlsbad, CA) was used to isolate the genomic DNA. Purified DNA was verified by NanoDrop for quality and respective absorbance was read at 260 nm. Forward and reverse primers were designed based on the nucleotide sequence coding elastase from *P. aeruginosa* reported in GenBank Accession no. M194721 followed by amplification. Figure 1 demonstrates the amplification

products of the two strains of *P. aeruginosa*. The amplified product following PCR was

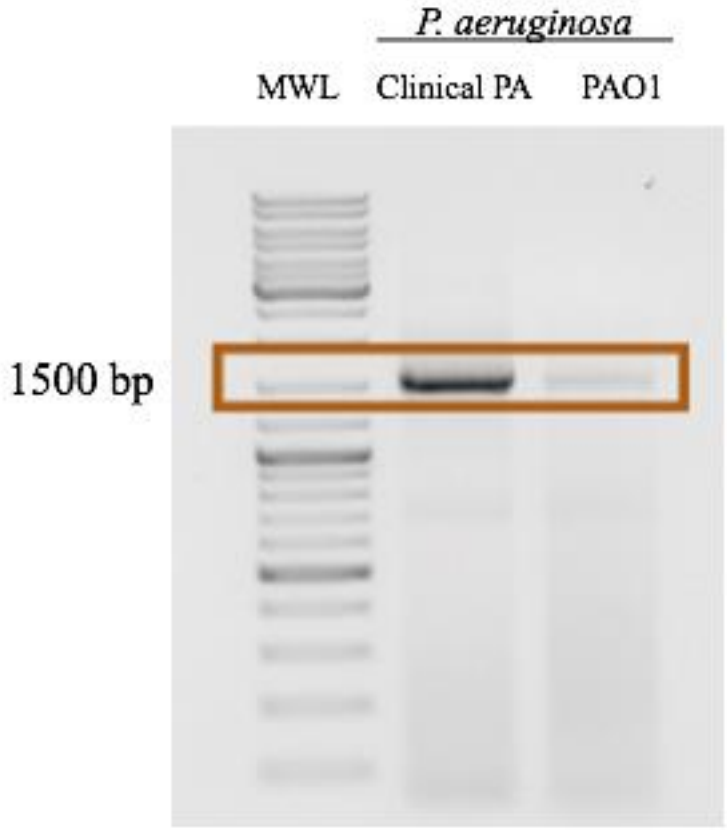


Figure 1: Agarose gel electrophoresis displaying PCR amplification of the lasB gene*:* The amplified DNA sequences from Clinical and Laboratory strains PAO1 of *Pseudomonas aeruginosa* displayed a PCR product approximately the size of native elastase gene (1,491 bp).

comparable to the reported size of the lasB gene of 1,491 base pair.

The Clinical strain displayed a higher amplification product and was thus the only strain used in the rest of the experimentation. After amplification of the lasB gene, a process of digestion and ligation was performed to insert the newly synthesized PCR product into the pET28b plasmid. Initially the amplification product from Fig. 1 would not properly ligate into the pET28b plasmid vector until the required conditions of ligation and digestion were optimized. Ligation reaction was used to transform an initial

electrocompetent cell line of *E. coli* known as 10G Electrocompetent cells. Integration of plasmid provided kanamycin resistance that allow for screening for incorporation within cells. Figure 2 demonstrates the correct band size (~1,500 bp) for the lasB gene, from colony 28, in lane 5 following colony PCR of randomly selected 10G Electrocompetent cells expressing kanamycin resistance.

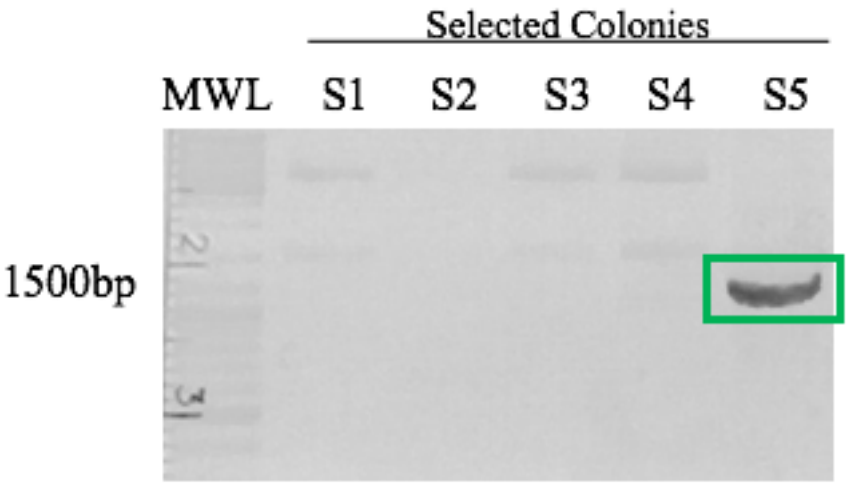


Figure 2: Recombinant lasB gene expression in Electrocompetent cells: Agarose gel electrophoresis after ligation and transformation into 10G Electrocompetent cells and colony PCR. Lane 1 is the Molecular Weight Ladder, Lanes 2-4 were not clones, and Lane 5 shows the proper length of the lasB recombinant gene at approximately 1491 bp.

Each well was loaded with 10 µl of sample (S1 – S5) or molecular weight markers and gel ran for 45 minutes before visualization. Several of the colonies present on the plates were resistant to kanamycin but did not contain the recombinant gene as visible in the gel electrophoresis in lanes 1-4 of Fig. 2. After gel electrophoresis of our recombinant gene confirmed the size, colonies were grown up, DNA extracted, and sent for sequence confirmation through Eurofins in the forward and reverse direction. Figure 3

displays the result we received after splicing the gene sequences together from the forward and reverse runs.

```
1     ATG GGC|AAG AAG GTT TCT ACG CTT GAC CTG TTG TTC GTT GCG ATC ATG
49    GGT GTT TCG CCG GCC GCT TTT GCC GCC GAC CTG ATC GAC GTG TCC AAA
97    CTC CCC AGC AAG GCT GCC CAG GGC GCG CCC GGC CCG GTC ACC TTG CAA
145   GCC GCG GTC GGC GCT GGC GGT GCC GAC GAA CTG AAA GCG ATC CGC AGC
193   ACG ACC CTG CCC AAC GGC AAG CAG GTC ACC CGC TAC GAG CAA TTC CAC
241   AAC GGC GTA CGG GTG GTC GGC GAA GCC ATC ACC GAA GTC AAG GGT CCC
289   GGC AAG AGC GTG GCG GCG CAG CGC AGC GGC CAT TTC GTC GCC AAC ATC
337   GCT GCC GAC CTG CCG GGC AGC ACC ACC GCG GCG GTA TCC GCC GAG CAG
385   GTG CTG GCC CAG GCC AAG AGC CTG AAG GCC CAG GGC CGC AAG ACC GAG
433   AAT GAC AAA GTG GAA CTG GTG ATC CGC CTG GGC GAG AAC AAC ATC GCC
481   CAA CTG GTC TAC AAC GTC TCC TAC CTG ATT CCC GGC GAG GGA CTG TCG
529   CGG CCG CAT TTC GTC ATC GAC GCC AAG ACC GGC GAA GTG CTC GAT CAG
577   TGG GAA GGC CTG GCC CAC GCC GAG GCG GGC GGC CCC GGC GGC AAC CAG
625   AAG ATC GGC AAG TAC ACC TAC GGT AGC GAC TAC GGT CCG CTG ATC GTC
673   AAC GAC CGC TGC GAG ATG GAC GAC GGC AAC GTC ATC ACC GTC GAC ATG
721   AAC AGC AGC ACC GAC GAC AGC AAG ACC ACG CCG TTC CGC TTC GCC TGC
769   CCG ACC AAC ACC TAC AAG CAG GTC AAC GGC GCC TAT TCG CCG CTG AAC
817   GAC GCG CAT TTC TTC GGC GGC GTG GTG TTC AAA CTG TAC CGG GAC TGG
865   TTC GGC ACC AGC CCG CTG ACC CAC AAG CTG TAC ATG AAG GTG CAC TAC
913   GGG CGC AGC GTG GAG AAC GCC TAC TGG GAC GGC ACG GCG ATG CTC TTC
961   GGC GAC GGC GCC ACC ATG TTC TAT CCG CTG GTG TCG CTG GAC GTG GCG
1009  GCC CAC GAG GTC AGC CAC GGC TTC ACC GAG CAG AAC TCC GGG CTG ATC
1057  TAC CGC GGG CAA TCA GGC GGA ATG AAC GAA GCG TTC TCC GAC ATG GCC
1105  GGC GAG GCT GCC GAG TTC TAT ATG CGC GGC AAG AAC GAC TTC CTG ATC
1153  GGC TAC GAC ATC AAG AAG GGC AGC GGT GCG CTG CGC TAC ATG GAC CAG
1201  CCC AGC CGC GAC GGG CGA TCC ATC GAC AAC GCG TCG CAG TAC TAC AAC
1249  GGC ATC GAC GTG CAC CAC TCC AGC GGC GTG TAC AAC CGT GCG TTC TAC
1297  CTG TTG GCC AAT TCG CCG GGC TGG GAT ACC CGC AAG GCC TTC GAG GTG
1345  TTC GTC GAC GCC AAC CGC TAC TAC TGG ACC GCC ACC AGC AAC TAC AAC
1393  AGC GGC GCC TGC GGG GTG ATT CGC TCG GCG CAG AAC CGC AAC TAC TCG
1441  GCG GCT GAC GTC ACC CGG GCG TTC AGC ACC GTC GGC GTG ACC TGC CCG
1489  AGC GCG TTG|GGA TCC GGC GGA CTG GTG CCG CGC GGC AGC GGA CAT CAC
1537  CAT CAT CAC CAC TAA GAG CTC
```

Figure 3: Sequence results of the recombinant lasB gene match lasB in GenBank: The above sequence is the resulting sequence obtained from Eurofins. Vertical line: beginning and end of gene; Green font: start codon; Purple font: base changes. The obtained 1557 bp recombinant nucleotide sequence contained homology with lasB from *P. aeruginosa* PAO1 (GenBank Accession no. M194721).

The entire sequence return was 1,557 nucleotides in length and a 1,497 base-pair product was obtained after removing the histidine residue sequences and the bases coding for thrombin (for removal of tag) located immediately in between the end of the sequence and the histidine tag. This product corresponds directly to the product size on a previous

study attempting to clone lasB into *Picha pastoris* GenBank Accession no. EU265777. The start codon is highlighted in green and the vertical lines mark the beginning and end of the lasB gene displayed in Fig 3. Compared with the other elastase gene sequences reported in GenBank, the nucleotide sequence of the cloned DNA shares 99% homology with lasB from *P. aeruginosa* PAO1 (GenBank Accession no. M19472.1) (Figure 3). Only four bases changed (336 C→T, 1029 T→C, 1110 A→T, 1122 C→T) were observed as highlighted in purple of Fig 3. Regardless, when translated, there was no difference between deduced amino acid sequence and native elastase from *P. aeruginosa*.

Figure 4 displays the resulting amino acid sequence from our gene sequencing data.

```
1    MGKKVSTLDL LFVAIMGVSP AAFAADLIDV SKLPSKAAQG APGPVTLQAA VGAGGADELK
61   AIRSTTLPNG KQVTRYEQFH NGVRVVGEAI TEVKGPGKSV AAQRSGHFVA NIAADLPGST
121  TAAVSAEQVL AQAKSLKAQG RKTENDKVEL VIRLGENNIA QLVYNVSYLI PGEGLSRPHF
181  VIDAKTGEVL DQWEGLAHAE AGGPGGNQKI GKYTYGSDYG PLIVNDRCEM DDGNVITVDM
241  NSSTDDSKTT PFRFACPTNT YKQVNGAYSP LNDAHFFGGV VFKLYRDWFG TSPLTHKLYM
301  KVHYGRSVEN AYWDGTAMLF GDGATMFYPL VSLDVAAHEV SHGFTEQNSG LIYRGQSGGM
361  NEAFSDMAGE AAEFYMRGKN DFLIGYDIKK GSGALRYMDQ PSRDGRSIDN ASQYYNGIDV
421  HHSSGVYNRA FYLLANSPGW DTRKAFEVFV DANRYYWTAT SNYNSGACGV IRSAQNRNYS
481  AADVTRAFST VGVTCPSALG SGGLVPRGSG HHHHHH
```

Figure 4: Complete amino acid sequence of recombinant lasB gene containing the 6x His-tag: The amino acid sequence was translated from the coding strand of the RNA template of the recombinant lasB gene. The sequence contains identity comparable to the naturally produced elastase and contains 6 histidine residues at the end for our purification tag (blue font).

The importance of translating the code to amino acids is to present the presence of 6 concurring histidine residues that served as a tag when we purified through the Ni-NTA

liquid chromatography system. Our recombinant gene displays the histidine residues, highlighted in blue, necessary for this step.

Expression of Recombinant Elastase in ClearColi

DNA was extracted from the 10G Electrocompetent cells and subsequently introduced into our expression vector ClearColi. Post induction with IPTG, ClearColi were incubated overnight at a reduced temperature of 30°C before cell harvest. We utilized this induction condition to optimize the production efficiency. Under these conditions, ClearColi expressed about 1.083 mg recE/L of cells.

After the expression incubation on the shaker, the cells were subsequently harvested by sonication and recombinant products separated from the cell debris by centrifugation. The supernatant was harvested and through a process of Ni-NTA liquid chromatography, our recombinant elastase was collected and purified as detailed in methods. The different methods were utilized for protein purification. The initial batch of recombinant elastase used a standard chromatography column with Ni-NTA liquid

chromatography and fractions were collected manually as depicted in Figure 5.

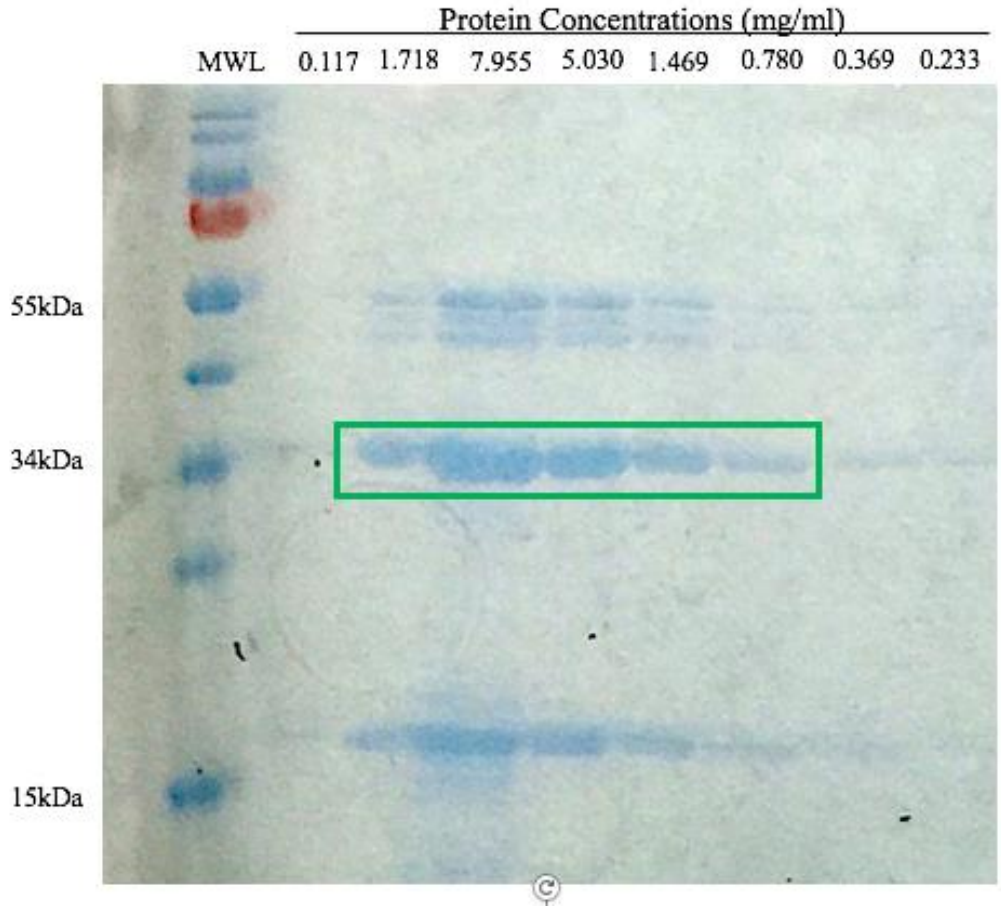


Figure 5: SDS-PAGE analysis of fractions from Ni-NTA chromatography of recombinant elastase: The gel displays the correct size of elastase (34 kDa) and pro-elastase (55 kDa) with various smaller fragments (~17 kDa).

Ten µl of each collected fraction was added to the SDS-PAGE wells in Fig. 5 and are in order from first collected eluent to last. As expected, lanes 3 through 6 contained the highest concentration (mg/ml) of recombinant proteins displaying correct size (~34 kDa).

The second (RecE2) and third (RecE3) batch of recombinant elastase were purified using a fast protein liquid chromatography system (FPLC). The fractions were automatically analyzed and collected during the elution process. Only fractions containing the recombinant elastase displaying proper absorbance (280 nm) were

combined, dialyzed, and finally centrifuged to remove precipitates/impurities.

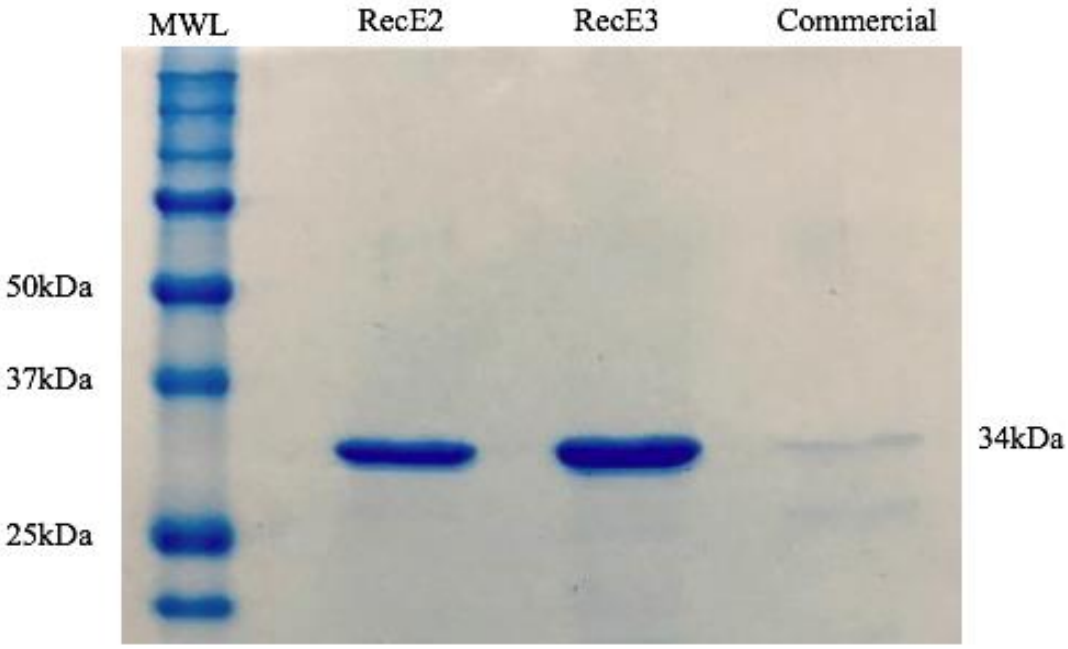


Figure 6: SDS-PAGE analysis of recombinant elastase post removal of precipitates: Lane 1 is the Molecular Weight Ladder, Lanes 2 and 3 contain the recombinant protein, and Lane 4 contains the commercially available *Pseudomonas* Elastase (34 kDa).

In Figure 6, a confirmation test was performed to assess and compare the size of our recombinant elastase to that of the commercially available one. The observed size (34 kDa) validates both recombinant elastase batches match that of the naturally produced and commercially available elastase. According to the SDS-PAGE analysis, the recombinants and commercial produce banding at 34 kDa. The extinction coefficient for RecE (ε, λ 280 nm) was 60,690 $M^{-1}cm^{-1}$ or 1.0937 $(mg/ml)^{-1}$ cm^{-1}.

Enzymatic Activity of the Recombinant Elastase

We employed two different biochemical methods to confirm the proteolytic as well as the elastolytic activity of the RecE product. Skim milk agar plates were designed with round filters saturated with four different concentrations of RecE as labeled in Figure 7. This test was used to identify the ability of RecE to proteolytically cleave casein contained in the skim milk plates. As observed in Fig 7, RecE contains high proteolytic activity.

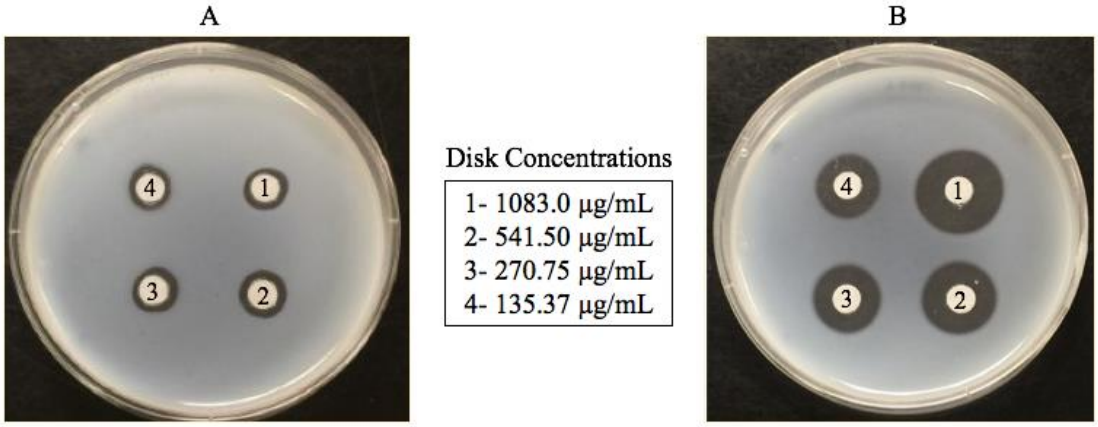


Figure 7: Proteolytic activity of the Recombinant Elastase in Skim milk agar diffusion plates: The plate displayed clearing zones around filter papers loaded with 10 µl of varying concentrations of RecE as indicated. Image A shows the ability of RecE to degrade casein at 5 minutes and after 24 hours (Image B).

The units of activity were then assessed using Elastin-Congo red as the substrate and subsequently calculated for Fig 8. The round filters (1-4) contained 1083.0, 541.50, 270.75, 135.37 µg/ml respectively. As presented in Fig 7A, RecE proteolytic activity was observed as hydrolysis areas, or transparent zones, within 5 minutes of the addition to the round filters. This was monitored every thirty minutes for a total of four hours and then imaged again at 24 hours post addition (Fig. 7B). The clearing zones were evidence of the ability of RecE to degrade the casein within the skim milk agar plates.

Once proteolytic activity was determined, the ability of the recombinant enzyme to degrade elastin was verified by Elastin-Congo Red (ERC) substrate assay. To test this, a standard curve was constructed and used to determine the Optical Density (OD) per mg of substrate solubilized in an hour at 37°C. With this knowledge, the amount of substrate solubilized by our recombinant elastase was calculated by adding known concentrations of substrate and RecE and then comparing the observed OD to the standard.

Elastolytic activity (in units) was determined by combining 50 µl of RecE and 10 mg of ERC substrate in 1 ml reactions. As a control and comparison, the commercially obtained elastase produced by *P. aeruginosa* was included in the protocol recommended by the manufacturer. The substrate solubilized for each reaction was determined by dividing the observed OD by the previously determined standard OD/mg substrate. From this value, the units of activity per mg of elastase (Units/mg elastase) was determined by dividing the mg substrate solubilized by the mg of elastase utilized. Table 1 compares the activity of the two recombinant elastases in comparison to the commercial. This experiment was performed in triplicate and the results presented as the means +/- standard deviation.

Elastin-Congo Red Assay

Sample	Optical Density (OD)	Substrate Solubilized (mg)	Units /mg Elastase	Specific Activity
Commercial	0.052	0.039	3.898 +/- 0.4	100.0
RecE2	0.265	0.198	*6.480 +/- 0.4	*166.2 +/- 9
RecE3	0.199	0.148	*6.844 +/- 0.1	*175.5 +/- 4

Table 1: Units of activity for RecE in comparison to commercial elastase: Both batches of RecE (RecE2 and RecE3) present increased activity in comparison to the commercial PE. Asterisks represent significant values from commercial.

Specific activity was determined by dividing the activity of the recombinant elastases by the activity of the commercial elastase. Specific activity is defined as the change in optical density (OD) at A_{485} per hour/mg of elastase written as a percentage in comparison to the commercial. As represented, we discovered RecE had a significant increase in activity in comparison to the naturally produced as evident by significant p values of student's t-test ($p<0.05$) for both batches.

In previous studies, elastolytic activity was determined at A_{495} rather than A_{485}. We utilized this same strategy to compare our product to previous research on cloning elastase. As Table 2 depicts, the specific activity of our RecE in comparison to our commercial is about a two-fold increase.

Elastin-Congo Red Assay

Sample	OD/mg Elastase	Specific Activity
Commercial	0.046	100.0
RecE2	0.089	*191.0 +/- 16
RecE3	0.094	*202.2 +/- 27

Table 2: Recombinant Elastase (RecE2 and RecE3) presents increased activity in comparison to the commercial *Pseudomonas* Elastase. Asterisks represent significant values from commercial.

This experiment was performed by using a constant known concentration of elastase (recombinant or commercial) and 10 mg of ERC substrate. The reactions were left on a shaker for 1 hour at 37°C before being placed in ice and subsequent absorbance read by spectrophotometer. The experiment was performed in triplicate and the results presented as an average of the three trials. Specific activity is defined as the change in optical density (OD) at 485 nm/hour/mg of elastase written as a percentage in comparison to the

commercial. Significant increases were determined using student t-test's comparing the specific activity of the commercial to the recombinants +/- standard deviation ($p<0.05$).

Activity was further characterized by demonstrating the ability of RecE to digest Elastin-Congo red substrate in a linear fashion. Reaction mixtures containing constant concentration of recombinant elastase and increasing values of substrate were combined. The OD was read at A_{485} after 1 hour at 37°C. Figure 8 depicts both recombinant batches having a linear relationship between the OD/mg elastase and the mg of substrate present in the reactions.

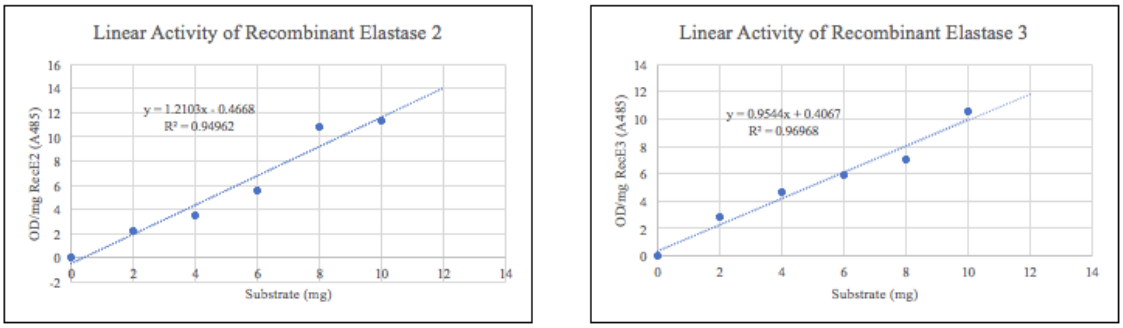


Figure 8: RecE demonstrates substrate linearity: A linear projection line with respective slopes and R^2 values indicate a linear relationship between the OD readout and Elastin-Congo red substrate added.

A trend line was fit to the data showing a linear forecast if substrate is increased. R^2 values were calculated and this determined our lines represent a predictive model for activity (RecE2, 0.95; RecE3, 0.97).

Virulence activity of RecE in vitro

The purpose of performing the following experiments was to demonstrate the ability of our RecE to function identically to the commercial elastase *in vitro*. Calu-3 monolayers were treated with various concentrations of Recombinant Elastase (1-8U/ml). These units of activity values reflect those of previous experiments in our lab. Figure 9

demonstrates the time course ability of a concentration at 2U/ml of Recombinant Elastase to make subtle morphological changes to Calu-3 cell anatomy in 2 hours.

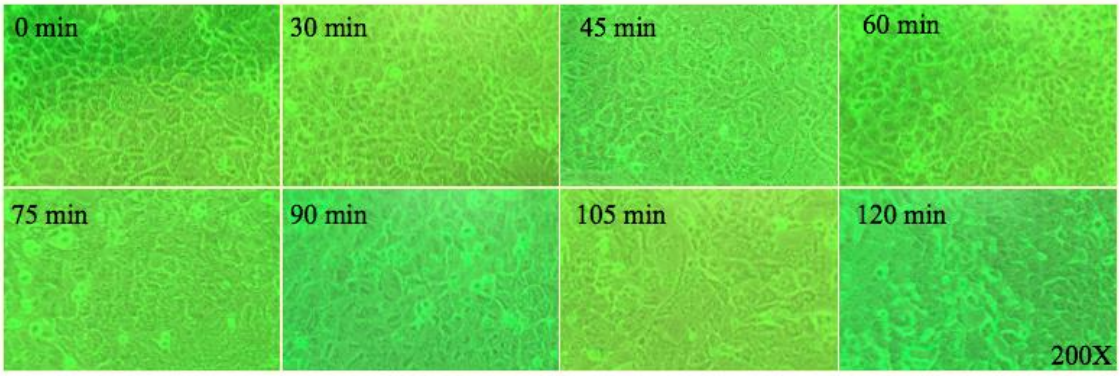


Figure 9: Time Course Analysis of the Effect of Recombinant Elastase on cell monolayers: Calu-3 cell monolayers were treated with 2 U/ml (a physiologic concentration) of RecE and images captured at 15 minute intervals for 2 hours (200x total magnification). Sublet morphology alterations were apparent after 60 minutes. Cell detachment was minimally observed at 120 minutes.

Monolayers were observed and images captured in 15 minute intervals for 2 hours. Figure 9 details that an *in vitro* examination of Recombinant Elastase displays similar physiological changes at low activity concentrations to the commercial Elastase. Figure 10 demonstrates the concentration effect of a RecE on the cell anatomy and monolayer. In comparison to 2U/ml, this higher concentration shows substantial morphological changes and cell detachment at 60 minutes and complete disruption by 2 hours.

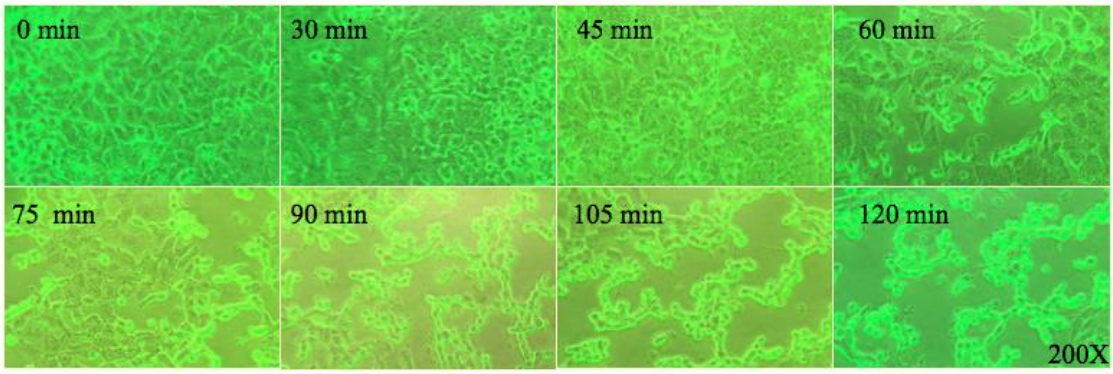


Figure 10: Dose Response Analysis of RecE Effect on cell monolayers: Calu-3 cell monolayers were treated with 8 U/ml of RecE and images captured at 15 minute intervals for 2 hours (200x total magnification). Morphological changes and cell detachment were apparent within 30 minutes.

Molecular Alterations in Tight Junction

We chose 1 or 2 U/ml of RecE activity for Immunofluorescent observations of tight junction protein, ZO-1, localization. ZO-1 is one of the proteins contained in the tight junction complex of cell monolayers. Therefore, normal expression of this protein is on the periphery of the cells to function as a barrier between the basal and apical surfaces.

Immunofluorescent (IF) analysis of ZO-1, a tight junction complex protein, localization was performed on Calu-3 cell monolayers in chamber slides. The cellular architecture was studied under immunofluorescent microscopy to look for changes after treatment with 1 or 2U/ml of Recombinant Elastase-2. Figure 12 displays the localization effect of treatment with Recombinant Elastase on ZO-1 (630X total magnification).

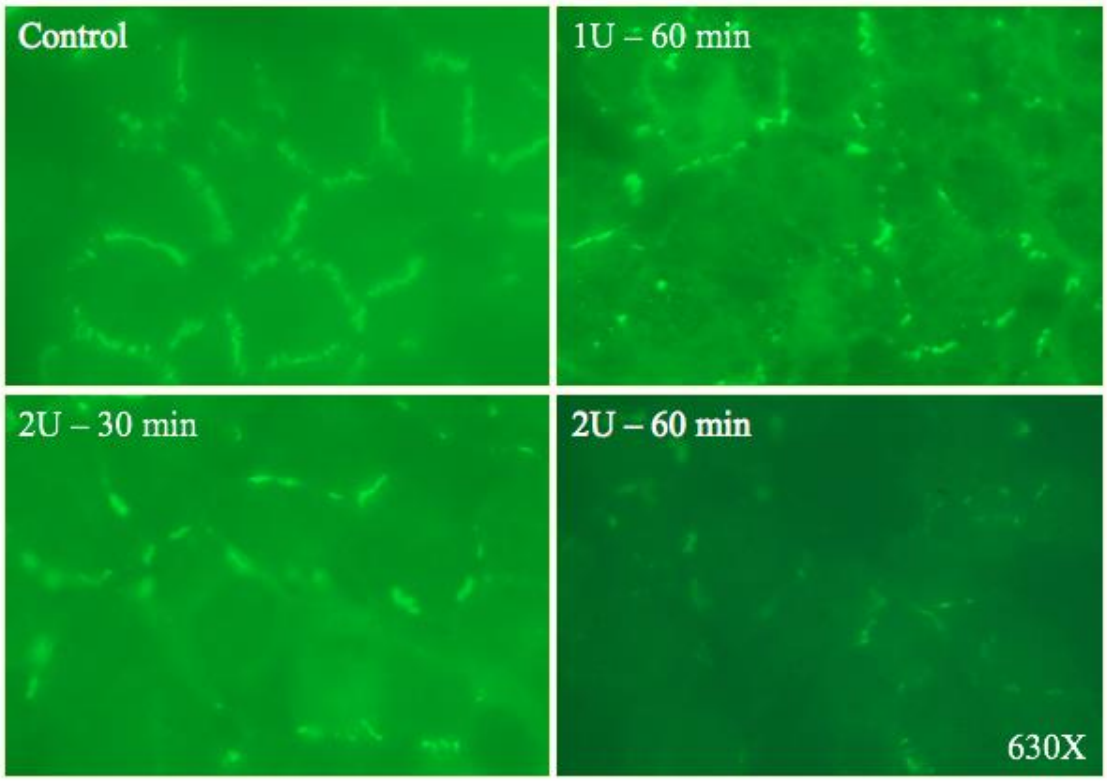


Figure 11: Immunofluorescence of Tight Junction Protein, ZO-1, localization after RecE treatment: Calu-3 cell monolayers were treated with RecE and images captured (630X total magnification). According to analysis, ZO-1 localization is time and dose dependent.

As presented by the IF results, the control displayed a uniformly distributed ZO-1 protein in cell peripheries. In treated cells, however, the continuous ring of ZO-1 was replaced by punctuate structures indicating degradation or relocation of the proteins. Therefore, ZO-1 organization is both a time-and dose-dependent effect of Recombinant Elastase treatment. These results complement the data reported in the lab's earlier research utilizing the commercial Elastase (107).

In conclusion, a Recombinant Elastase was cloned and expressed that properly reflects the size and enzymatic activity of the commercially available product from *P. aeruginosa*. The ability of RecE to cause anatomical changes *in vitro* via morphological and immunofluorescent analysis was characterized. Together, these results demonstrate

the ability to utilize the above protocol to produce and efficiently create a recombinant enzyme for further studying the pathophysiologic effects of *P. aeruginosa* elastase.

Chapter Four

Discussion

In this thesis, the goal to synthesize a Recombinant Elastase that is comparable to the commercially available elastase produced by *P.* aeruginosa was successfully accomplished. A recombinant lasB gene was cloned, inserted into pET28b plasmid, transformed electrocompetent recombinant expression *E. coli*, and purified a recombinant, active enzyme. Herein, this discussion describes the key points throughout the cloning process, improvements, and future research extensions.

Initially, it was planned to utilize two strains of *P. aeruginosa* during the cloning process. However, only the clinical isolate following amplification via PCR as the lasB gene of this strain had more amplification as assessed by the gel electrophoresis (Fig 1). This strain was isolated from patients with chronic infections of *P. aeruginosa* and likely contains an increased resistance to antibiotics. Furthermore, after sequencing the recombinant lasB gene sequence, the final product contained four base-pair changes in comparison to the listed sequence on GenBank. This comparison of the gene sequences suggests that the difference in nucleotide sequence may be attributed to strain diversity which can also account for increased virulence (122).

An IPTG lac operon promoter system was utilized for expression of the RecE protein. IPTG is a biological reagent that mimics allolactose in triggering the transcription of the lac operon. The first improvement in the cloning process involved the chosen plasmid, pET28b, which contains the lac operon along with nucleotides coding

for a 6x histidine tag. This improvement allowed for a Ni-NTA liquid chromatography system containing resin with a high affinity for histidine residues to successfully purify the RecE with high efficiency as detailed in Figs 5 and 6. Further, the induction process was enhanced, which improved the gene expression level by manipulating the temperature from the standard 37ºC to 30ºC to improve the low expression in the first few trials. In this case, the bacteria might be overwhelmed by production of the enzyme. Published research indicates the host strain does not prefer recombinant expression of proteases, therefore, potentially causing inclusion bodies to develop (123). Thus, to alleviate the stress mechanism caused by the overexpression of cloned genes in *E. coli*, an altered growth condition was performed during gene expression to improve the negative host response. Subsequently, modification in temperature increased the production of RecE and allowed for effective synthesis of a recombinant product.

Another challenge was the contaminating proteins that were observed at the high (55 kDa) and low (~17 kDa) banding presented in Fig 5. *Pseudomonas* produces the enzyme elastase as a pro-form protein of 55 kDa before being cleaved and subsequently activated upon release as a mature 33 kDa elastase, with loss of a 22 kDa fragment. The observed banding support the presence of pro-form elastase and fragments from its cleavage to produce the mature elastase. To improve production of mature elastase, lower temperature expression was performed and the protein was purified by Ni-affinity chromatography. Subsequent centrifugation to remove precipitates that formed after overnight storage of Ni-affinity purified protein produced RecE representing a clean, finished product, as judged by SDS-PAGE (Fig 6). These results suggest that the source

of the contamination stemmed from either degradation of the RecE, proenzyme, and potentially recombinant enzyme that was no longer enzymatically active.

The activity of the RecE was assessed to determine if RecE retained the published properties of the commercial enzyme. Using Skim milk agar diffusion plates and an Elastin-Congo red substrate assay the proteolytic and elastolytic activities of the recombinant enzyme was validated. The plates containing skim milk displayed clearing zones proving that the RecE can degrade casein as a proteolytic enzyme. From the Elastin-Congo red assay assessment, the RecE demonstrated an ability to degrade elastin. Astoundingly, RecE displayed an increased elastolytic ability in comparison to the commercial enzyme. This is important because enhanced activity can allow investigators to use less of the enzyme when studying its effects on lung tissue.

The elastolytic activity confirms the biochemical property of the RecE enzyme. Therefore, an assessment of whether RecE manipulated cell anatomy in a time and dose-dependent manner was examined. Microscopic evaluation of the cultured cells demonstrated that the RecE's alters the cell morphology in a dose and time dependent manner (Fig 9 and 10). The Azghani lab has shown that minute amounts of *Pseudomonas* Elastase are produced *in vivo* and this small amount minimally alters the lung epithelium (86, 98). As such, this subtle change in the host cell morphology by a physiologic concentration of RecE mimics previous findings. Therefore, morphological analysis helped determine the time and dose of RecE to properly emulate the natural observation in patients for the next examination using immunofluorescent studies.

Pseudomonas elastase has demonstrated an ability to alter the tight junction complex by manipulation of the ZO-1 protein (107). Through immunofluorescent

analysis using cell culture slides, the ability of RecE to manipulate the tight junction complex through ZO-1 reorganization or disruption was preliminarily verified (Fig 11). This validates previous studies from the Azghani lab that observed the same effect with the commercial elastase. In normal cell monolayers, the tight junctions are located uniformly on the periphery. However, with the addition of RecE, the ZO-1 protein is fragmented in its expression. Future studies should evaluate the manipulation of cell monolayers by RecE with Trans-epithelial Electrical Resistance (TER) analysis to confirm the immunofluorescent data presented in this project. *In vivo* studies will confirm the impact of RecE –induced alterations in tight junction complex in pathologic conditions including pulmonary edema.

In summary, the goals of this thesis were successfully achieved by completing the project on recombinant enzyme synthesis and extensively evaluating the activity of the RecE. The preliminary data reported here is only the beginning of using RecE to elucidate the pathophysiological effect on lung tissue. This product can effectively be utilized in the Azghani laboratory and potentially made into a commercial product for other labs to use in their respective research. The next step in this line of research will be examining the effect of RecE on paracellular permeability through Trans-epithelial Electrical Resistance (TER) and biochemical analysis, such as, western blot for ZO-1. Earlier research in the lab determined that treatment with elastase from *P. aeruginosa* transiently causes the activation of the Epidermal Growth Factor Receptor (EGFR) resulting in downstream physiologic effects such as inflammation and edema (99, 107). The exact mechanism for the activation of the EGFR receptor by PE is still unknown and therefore, RecE can be used to elucidate whether the activation is direct through

attachment or an indirect fashion through potential cleaving of extracellular pro-ligands. This will allow for alternative therapy options to be developed to either inhibit the activation of EGFR and/or potentially using monoclonal antibodies to neutralize *Pseudomonas* Elastase (99). The ultimate goal is to develop a method to better treat patients suffering from infections by this bacterium and hopefully this finding provides an opportunity for the Azghani laboratory and others to more efficiently study the pathogenesis of lung injury by *P. aeruginosa* elastase.

References

1. Driscoll JA, Brody SL, Kollef MH (2007). The Epidemiology, Pathogenesis and Treatment of *Pseudomonas aeruginosa* Infections. Drugs; 67:351- 368.
2. Van Delden C, Iglewski BH (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerg Infect Dis. 4(4):551- 60.
3. Jawetz E (1952). Infections with *Pseudomonas aeruginosa* treated with Polymyxin B. AMA Arch Intern Med. 89(1):90-98.
4. Chen S (2009). *Pseudomonas* Infection. Pseudomonas Infection: eMedicine Pediatrics: General Medicine. Web. 17 Feb 2017.
5. Allin AE (1941). Meningitis of the New-Born due to Pseudomonas Aeruginosa. Canadian Medical Association journal. 44(3):288-289.
6. Young LS (1984). Reviews of Infectious Diseases - *Pseudomonas aeruginosa*: Biology, Immunology, and Therapy: A Cefsulodin Symposium. Oxford University Press. 6(3):603-607.
7. Hunter CA, Ensign PR (1947). Epidemic of Diarrhea in a New-Born Nursery Caused by *Pseudomonas aeruginosa*. American Journal of Public Health and the Nations Health. 37:1166-1169.
8. Florman AL, Schifrin N (1950). Observations on a small outbreak of infantile diarrhea associated with Pseudomonas aeruginosa. The Journal of pediatrics. 36(6):758-766.

9. Davies J, Davies D (2010). Origins and evolution of antibiotic resistance. Mircrobiology and Molecular Biology Reviews. 74(3):417-433.
10. Rabin ER, Graber CD, Vogel E, Finkelstein RA, Tumbusch WA (1961). Fatal Pseudomonas Infection in Burned Patients — A Clinical, Bacteriologic and Anatomic Study. The New England Journal of Medicine. 265:1225-1231.
11. Sutter VL, Hurst V (1966). Sources of *Pseudomonas aeruginosa* infection in burns: study of wound and rectal cultures with phage typing. Ann Surg. 163(4):597-602.
12. Drake CH (1966). Evaluation of Culture Media for the Isolation and Enumeration of *Pseudomonas aeruginosa*. Health Lab Sci. 3:10.
13. Gould JC (1962). *Pseudomonas pyocyanea* Infections in Hospital. In Infections in Hosptials. Blackwell Scientific Publications. p119.
14. Kefalides NA, Arana JA, Bazan A, Velarde N, Rosenthal S (1964). Evaluation of Antibiotic Prophylaxis and Gamma-Globulin, Plasma, Albumin and Saline-Solution Therapy in Severe Burns. Ann Surg. 159:496.
15. Lowbury EJL, Fox J (1954). The epidemiology of infection with Pseudomonas pyocyanea in a burns unit. Journal of Hygiene. 52(3):403-416.
16. Maschler JA (1948). Case of Pyocyaneus Ring Abscess of the Cornea Treated with Streptomycin. The British journal of ophthalmology. 32(7):426–428.
17. Stanley MM (1947). Bacillus pyocyaneus infections: a review, report of cases and discussion of newer therapy including Streptomycin (concluded). The American journal of medicine. 2(4):347-367.

18. Thygeson P (1948). Acute central (hypopyon) ulcers of the cornea. California medicine. 69(1):18–21.
19. Weiss C (1943). Bacteriologic observations on infections of the eye. Archives of Ophthalmology. 30(1):110-137.
20. Thygeson P (1938). Treatment of Conjunctivitis. Archives of Ophthalmology. 19(4):586-608.
21. Fetzer AE, Werner AS, Hagstrom J (1947). Pathologic Features of Pseudomonal Pneumonia 1, 2. American Review of Respiratory Disease. 96(6):1121-1130.
22. Davies JC (2002). *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. Paediatr Respir Rev. 3(2):128-134.
23. Giamarellou H, Antoniadou A (2001). Antipseudomonal antibiotics. Med Clin North Am. 85(1):19-42.
24. Bendiak GN, Ratjen F (2009). The approach to *Pseudomonas aeruginosa* in cystic fibrosis. Respir Crit Care Med. 30(5):587-595.
25. Vinks AA, den Hollander JG, Overbeek SE, Jelliffe RW, Mouton JW (2003). Population Pharacokinetic Analysis of Nonlinear Behavior of Piperacillin during Intermittent or Continuous Infusion in Patients with Cystic Fibrosis. Antimicrob Agents Chemother. 47(2):541-547.
26. Alipour M, Suntres ZE, Halwani M, Azghani AO, Omri Abdelwahab (2009). Activity and Interactions of Liposomal Antibiotics in Presence of Polyanions and Sputum of Patients with Cystic Fibrosis. PLoS ONE 4(5): e5724. doi:10.1371/journal.pone.0005724.

27. Bergmans D, Bonten M (1999). Colonization and Infection with *Pseudomonas aeruginosa* in Intensive Care: Endogenous or Exogenous Origin. Yearbook of Intensive Care and Emergency Medicine. 19:131-140.
28. Gilboa-Garber N (1996). Towards Anti-*Pseudomonas aeruginosa* Adhesion Therapy. Advances in Experimental Medicine and Biology. 408:39-50.
29. Oliver A, Mulet, X, Lopez-Causape C, Juan C (2015). The increasing threat of *Pseudomonas aeruginosa* high-risk clones. Drug Resistance Updates. 21-22:41-59.
30. Erbguth FJ, Naumann M (1999). Historical Aspects of Botulinum Toxin. Justinius Kerner (1786-1862) and the "sausage poison." Journal of Neurology. 53:1850-1853.
31. Saraswathi P, Beuerman RW (2015). Corneal Biofilms: From Planktonic to Microcolony Formation in an Experimental keratitis Infection with *Pseudomonas aeruginosa*. 13(4):331-345.
32. Feldman M, Bryan R, Rajan S, Scheffler L, Brunnet S, Tang H, Prince A (1998). Role of Flagella in Pathogenesis of *Pseudomonas aeruginosa* Pulmonary Infection. Infection and Immunity. 66(1):43-51.
33. Azghani AO, Kondepudi AY, Johnson AR (1992). Interaction of *Pseudomonas aeruginosa* with human lung fibroblasts: role of bacterial elastase. Am J Respir Cell Mol Biol. 6(6):652-657.

34. Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jorgensen A, Molin S, Tolker-Nielsen T (2003). Biolfilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Mol Microbiol. 48(6):1511-1524
35. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995). Microbial biofilms. Annu Rev Microbiol. 49:711-745.
36. Costerton JW, Stewart PS, Greenberg EP (1991). Bacterial biofilms: a common cause of persistent infections. Science. 284:1318-1322.
37. Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S (2000). Development and dynamics of *Pseudomonas* sp. Biofilms. J Bacteriol. 182:6482-6489.
38. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol 184:1140-1154..
39. Pollack M (1984). The virulence of *Pseudomonas aeruginosa*. Review of Infectious Diseases. 6(3):617-626.
40. Brewer SC, Wunderink RG, Jones CB, Leeper-Chest KV (1996). Ventilator-associated pneumonia due to *Pseudomonas aeruginosa*. Chest. 109(4):1019-1029.
41. Martins MF, Abairos V (2002). Glycocalyx of lung epithelial cells. International Review of Cytology. 216:131-173.
42. Kautto L, Nguyen-Khuong T, Everest-Dass A, Leong A, Zhao Z, Willcox MDP, Packer NH, Peterson R (2016). Glycan involvement in the adhesion of *Pseudomonas aeruginosa* to tears. Experimental Eye Research. 145:278-288.

43. Stuer W, Jaeger KE, Winkler UK (1986). Purification of extracellular Lipase from *Pseudomonas aeruginosa*. 168(3):1070-1074.

44. Delden CV, Iglewski BH (1998). Cell-to-Cell Signaling and *Pseudomonas aeruginosa* Infections. Emerging Infectious Diseases. 4(4):551-560.

45. de Bentzmann S, Roger P, Dupuit F, Bajolet-Laudinat O, Fuchey C, Plotkowski MC, Puchelle E (1996). Asialo GM1 is a receptor for Pseudomonas aeruginosa adherence to regenerating respiratory epithelial cells. Infect Immun. 64(5):1582–1588.

46. Bergen GA, Shelhamer JH (1996). Pulmonary infiltrates in the cancer patient. New approaches to an old problem. Infect Dis Clin North Am. 10(2):297–325.

47. Lyczak JB, Cannon CL, Pier GB (2000). Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes and Infection. 2(9):1051-1060.

48. Nicas T, Iglewski B (1985). The Contribution of Exoproducts to Virulence of *Pseudomonas aeruginosa.* Canadian Journal of Microbiology. 31(4):387-392.

49. Kobayashi H, Kobayashi O, Kawai S (2009). Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. Journal of Infection and Chemotherapy. 15(3):125-142.

50. Rolsma S, Frank DW, Barbieri JT (2015). *Pseudomonas aeruginosa* toxins. The Comprehensive Sourcebook of Bacterial Protein Toxins. 1(4):133-160.

51. Rigoni M, Montecucco C (2015). Bacterial Protein Toxins as Tools in Cell Biology and Physiology. 2:794-798.

52. Holloway BW (1955). Genetic Recombination in *Pseudomonas aeruginosa.* Journal of General Microbiology. 13(3):572-581.

53. Ryan KJ, George C (2010). *Sherris Medical Microbiology* (5th ed.). New York: McGraw Hill Medical. 283(3):409-410.

54. Moayeri M, Welch RA (1998). Bacterial Exotoxins. Methods in Microbiology. 27:287-300.

55. Balfanz J, Rautenberg P, Ullmann U (1996). Molecular Mechanisms of Action of Bacterial Exotoxins. Zbl. Bakt. 284:170-206.

56. Liu PV (1974). Extracellular Toxins of *Pseudomonas aeruginosa.* The Journal of Infectious Diseases. 131(2):192.

57. Scott BB, Barclay GR, Smith DGE, McLoughlin F, Poxton IR (1990). IgG Antibodies to Gram-negative endotoxin in human sera. Lipopolysaccharide (LPS) cross-reactivity due to antibodies to LPS. Serodiagnosis and Immunotherapy in Infectious Disease. 4(1):25-38.

58. Morihara K (1963). *Pseudomonas aeruginosa* proteinase: I. Purification and general properties. Biochemica et Biophysica Acta. 73(1):113-124.

59. Inoue H, Nakagawa T, Morihara K (1963). *Pseudomonas aeruginosa* Proteinase: II. Molecular Weight and Molecular Dimension. Biochemica Et Biophysica Acta. 73(1):125-131.

60. Patzer J, Nielsen H, Kharazmi A (1989). *Pseudomonas aeruginosa* exotoxin A primes human monocyte oxidative burst response *in vitro*. Microbial Pathogenesis. 6(4):287-295.

61. Wretlind B, Bjorklind A, Pavlovskis OR (1987). Role of exotoxin A and elastase in the pathogenicity of *Pseudomonas aeruginosa* strain PAO experimental mouse burn infection. Microbial Pathogenesis. 2(6):397-404.

62. Michalska M, Wolf P (2015). *Pseudomonas* Exotoxin A: optimized by evolution for effective killing. Front Microbiol. 6:963.

63. Barker AP, Vasil AI, Filloux A, Ball G, Wilderman PJ, Vasil ML (2004). A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. Mol Microbiol. 53(4):1089-1098.

64. Ohman DE, Cryz SJ, Iglewski BH (1980). Isolation and characterization of Pseudomonas aeruginosa PAO mutant that produces altered elastase. Journal of Bacteriology. 142(3):836-842.

65. Liu PV (1966). The Roles of Various Fractions of *Pseudomonas aeruginosa* in its Pathogenesis. The Journal of Infectious Disease. 130:94-99.

66. Lynn TC (1974). Purification and Characterization of *Pseudomonas aeruginosa* Exotoxin. Infection and Immunity. 9(1):113-118.

67. Bever RA, Iglewski BH (1988). Molecular Characterization and Nucleotide Sequence of *Pseudomonas aeruginosa* Elastase Structural Gene. Journal of Bacteriology. 170(9):4309-4314.

68. Morihara K, Tsuzuki H, Oka T, Inoue H, Ebata M (1965). *Pseudomonas aeruginosa* Elastase: Isolation, Crystallization, and Preliminary Characterization. The Journal of Biological Chemistry. 240(8):3295-3304.

69. Morihara K (1964). Production of Elastase and Proteinase by *Pseudomonas aeruginosa*. Journal of Bacteriology. 88:747-757.

70. Kawaharajo K, Homma JY, Aoyama Y, Okada K, Morihara K (1975). Effects of protease and elastase from *Pseudomonas aeruginosa* on skin. Japanese Journal of Experimental Medicine. 45(2):79-88.
71. Kawaharajo K, Homma JY, Aoyama Y, Morihara K (1975). *In vivo* studies on protease and elastase from *Pseudomonas aeruginosa*. Japanese Journal of Experimental Medicine. 45(2):89-100.
72. Morihara K, Tsuzuki H (1977). Production of protease and elastase by *Pseudomonas aeruginosa* strains isolated from patients. Infection and Immunity. 15(3):679-685.
73. Kessler E, Spierer A (1984). Inhibition by phosphoramidon on *Pseudomonas aeruginosa* elastase injected intracorneally in rabbit eyes. Current Eye Research. 3(8):1075-1078.
74. Morihara K, Tsuzuki H (1978). Phosphoramidon as an inhibitor of elastase from *Pseudomonas aeruginosa.* Japanese Journal of Experimental Medicine. 48(1):81-84.
75. Yamamoto S, Fukishima J, Atsumi Y, Takeuchi H, Kawamoto S, Okuda K, Morihara K (1988). Cloning and characterization of elastase structural gene from *Pseudomonas aeruginosa* IFO 3455.
76. Morihara K, Fukushima J, Okuda K (1989). Primary Structure of *Pseudomonas aeruginosa*: a comparison with Thermolysin. Antibiotic Chemotherapy. 42:36-41.

77. Fukushima J, Yamamoto S, Morihara K, Atsumi Y, Takeuchi H, Kawamoto S, Okuda K (1989). Structural gene and complete amino acid sequence of *Pseudomonas aeruginosa* IFO3455 elastase. Journal of Bacteriology. 171(3):1698-1704.

78. Thayer MM, Flaherty KM, McKay DB (1991). Three-Dimensional Structure of the Elastase of *Pseudomonas aeruginosa* at 1.5- Å Resolution. J of Biol Chem. 266(5):2864-2871.

79. Schad PA, Bever RA, Nicas TI, Leduc F, Hanne LF, Iglewski BH (1987). Cloning and characterization of elastase genes from *Pseudomonas aeruginosa*. Journal of Bacteriology. 169(6):2691-2696.

80. Schad PA, Iglewski BH (1988). Nucleotide sequence and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* elastase structural gene. Journal of Bacteriology. 170(6):2784-2789.

81. Gambello MJ, Iglewski BH (1991). Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. Journal of Bacteriology. 173(9):3000-3009.

82. Peters JE, Galloway DR (1990). Purificatoin and characterization of an active fragment of the LasA protein from *Pseudomonas aeruginosa*: enhancement of elastase activity. Journal of Bacteriology. 172(5):2236-2240.

83. Toder DS, Gambello MJ, Iglewski BH (1991). *Pseudomonas aeruginosa* LasA: a second elastase under the transcriptional control of lasR. Molecular Microbiology. 5(8):2003-2010.

84. Rodriquez-Boulan D, Nelson WJ (1989). Morphogenesis of the polarized epithelial cell phenotype. Science. 254:718-725.

85. Simons K, Fuller SD (1985). Cell surface polarity in epithelia. Annual Review in Cell Biology. 1:243-288.

86. Azghani AO, Connelly JC, Peterson BT, Gray LD, Collins ML, Johnson AR (1990). Effects of *Pseudomonas aeruginosa* Elastase on Alveolar Epithelium Permeability in Guinea Pigs. Infection and Immunity. 58(2):433-438.

87. Homma JY, Abe C, Tanamoto K, Hirao Y, Morihara K, Tsuzuki H, Yanagawa R, Honda E, Aoi Y, Fujimoto Y, Goryo M, Imazeki N, Noda H, Goda A, Takeuchi S, Ishihara T (1978). Effectiveness of immunization with single and multi-component vaccines prepared from a common antigen (OEP), protease and elastase toxoids of *Pseudomonas aeruginosa* on protection against hemorrhagic pneumonia in mink due to *P. aeruginosa.* Japanese Journal of Experimental Medicine. 48:111-133.

88. Hartsock A, Nelson WJ (2008). Adherens and Tight Junctions: Structure, Function and Connections to the Actin Cytoskeleton. Biochim Biophys Acta. 1778(3):660-669.

89. Farquhar MG, Palade GE (1963). Junctional complexes in various epithelia. Journal of Cellular Biology. 17:375-412.

90. Gumbiner B (1987). The structure, biochemistry, and assembly of epithelial tight junctions. American Journal of Physiology. 253:749-758.

91. Madara JL (1989). Loosening tight junctions Lessons from the intestine. Journal of Clinical Investigation. 83:1089-1094.

92. Molitoris BA, Nelson WJ (1990). Alterations in the establishment and maintenance of epithelial cell polarity as a basis for disease process. Journal of Clinical Investigation. 85:3-9.

93. Welsh MJ, Shasby DM, Husted RM (1985). Oxidants increase paracellular permeability in a cultured epithelial cell line. Journal of Clinical Investigation. 76:1155-1168.

94. Hecht G, Pothoulakis C, LaMont JT, Madara J (1988). *Clostridium difficile* toxin A perturbs cytoskeletal structure and tight junction permeability of cultured intestinal epithelial monolayers. Journal of Clinical Investigation. 82:1516-1524.

95. Phipps RJ, Torrealba PJ, Lauredo IT, Denas SM, Sielezak MW, Ahmed A, Araham WM, Wanner A (1987). Bacterial pneumonia stimulates macromolecule secretion and ion and water fluxes in sheep trachea. Journal of Applied Physiology. 62:2388-2397.

96. Azghani AO, Gray LD, Johnson AR (1993). A Bacterial Protease Perturbs the Paracellular Barrier Function of Transporting Epithelial Monolayers in Culture. Infection and Immunity. 61(6):2681-2186.

97. Azghani AO, Miller EJ, Peterson BT (2000). Virulence factors from *Pseudomonas aeruginosa* increase lung epithelial permeability. Lung. 178(5):261-269.

98. Azghani AO, Bedinghaus T, Klein R (2000). Detection of elastase form *Pseudomonas aeruginosa* in sputum and its potential role in epithelial cell permeability. Lung. 178(3):181-189.

99. Azghani AO (1996). *Pseudomonas aeruginosa* and epithelial permeability: role of virulence factors elastase and exotoxin A. American Journal of Respiratory Cell and Molecular Biology. 15(1):132-140.

100. Blackwood LL, Stone RM, Iglewski BH, Pennington JE (1983). Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. Infection and Immunity. 39(1):198-201.

101. Azghani AO, Baker JW, Shetty S, Miller EJ, Bhat GJ (2002). *Pseudomonas aeruginosa* stimulates ERK signaling pathway and enhances IL-8 production by alveolar epithelial cells in culture. Inflammation Research. 51:506-510.

102. Kida Y, Higashimoto Y, Inoue H, Shimizu T, Kuwano K (2008). A novel secreated protease from *Pseudomonas aeruginosa* activates NFkB through protease-activated receptors. Cell Microbiology. 10:1491-1504.

103. Leduc D, Beaufort N, de Bentzmann S, Roussell JC, Namane A, Chignard M, Pidard D (2007). The *Pseudomonas aeruginosa* LasB metalloproteinase regulates the human urokinase-type plasminogen activator receptor through domain-specific endoproteolysis. Infection and Immunity. 75:3848-3858.

104. Helfrish I, Schmitz A, Zigrio P, Michels C, Haase I, Bivic AI, Leitges M, Niessen CM (2006). Role of APKC isoforms and their binding partners PAR3 and PAR6 in epidermal barrier formation. Journal of Invest Dermatol. 127:782-791.

105. Chen ML, Pothoulakis C, LaMont JT (2002). Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to *Clostridium difficile* toxin A. Journal of Biological Chemistry. 277:4247-4254.

106. Andreeva AY, Krause E, Muller EC, Blasig IE, Utepbergenov DI (2001). Protein kinase C regulates the phosphorylation and cellular localization of occludin. Journal of Biological Chemistry. 276:38480-38486.

107. Clark CA, Thomas LK, Azghani AO (2011). Inhibition of Protein Kinase C Attenuates *Pseudomonas aeruginosa* Elastase-Induced Epithelial Barrier Disruption. American Journal of Respiratory Cell and Molecular Biology. 45(6):1263-1271.

108. Klapper L, Kirschbaum MH, Sela M, Yarden Y (2000). Biochemical and clinical implications of the erbB/HER signaling network of growth factor receptors. Adv Cancer Res;77: 25–79.

109. Takeyama K, Jung B, Shim JJ, Burgel PR, Dao-Pick, Ueki IF, Protin U, Kroschel P, Nadel JA (2001). Activation of epidermal growth factor receptors is responsible for mucin synthesis induced by cigarette smoke. Am J Physiol Lung Cell Mol Physiol;280: L165–72.

110. Takeyama K, Dabbagh K, Shim JJ, Dao-Pick T, Ueki IF, Nadel JA (2000). Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. J Immunol. 164:1546–52.

111. Voldborg BR, Damstrup L, Spang-Thomsen M, Poulsen HS (1997). Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. Ann. Oncol. 8(12):1197-1206.

112. Clauditz TS, Gontarewicz A, Lebok P, Tsourlakis MC, Grob TJ, Munscher A, Sauter G, Bokemeyer C, Knecht R, Wilczak W (2012). Epidermal growth factor receptor (EGFR) in salivary gland carcinomas: potentials as therapeutic target. Oral Oncol. 48(10):991-996.
113. Rojas AM, Fuentes G, Rausell A, Valencia A (2012). The Ras superfamily: evolutionary tree and role of conserved amino acids. J. Cell Biol. 196:189–201.
114. Madaule P, Axel R (1985). A novel ras-related gene family. Cell. 41, 31–40.
115. Hall A (1998). Rho GTPases and the actin cytoskeleton. Science. 279:509–514.
116. Pierce KL, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ (2001). Epidermal Growth Factor (EGF) Receptor-dependent ERK Activation by G Protein-coupled Receptors. The Journal of Biological Chemistry. 276(25):23155-23160.
117. Azghani AO, Neal K, Idell S, Amaro R, Baker JW, Omri A, Pendurthi UR (2014). Mechanism of fibroblast inflammatory responses to *Pseudomonas aeruginosa* elastase. Microbiology. 160:547-555.
118. Roudabush FL, Pierce KL, Maudsley S, Khan KD, Luttrell LM (2000). Transactivation of the EGF receptor mediates IGF-1-stimulated Shc phosphorylation and ERK1/2 activation in COS-7 cells. J Biol Chem 275:22583–22589.
119. Dong J, Opresko LK, Dempsey PJ (1999). Metalloprotease-mediated ligand release regulates autocrine signaling through the epidermal growth factor receptor. Proc Natl Acad Sci USA. 96:6235–40.

120. Tirabassi R, Bio B (2017). Foundation of Molecular Cloning – Past, present, and future. New England BioLabs. Internet Source. Retrieved 03.08.2017.

121. Xijin L, Wentao X, Kunlun H, Xiaohong M, Zhihon L, Zhemin L, Jingxin G, YunBo L (2009). Cloning, expression and characterization of recombinant elastase from *Pseudomonas aeruginosa* in *Picha pastoris*. Protein Expr and Purif. 63:69-74.

122. Raftari M, Ghafourian S, Sadeghifar N, Abu Bakar F (2013). Cloning and overexpression of extracellular elastase from *Pseudomonas aeruginosa*. Eur J of Inflamm. 11(1):55-60.

123. Kane JF, Hartley DL (1988). Formation of recombinant protein inclusion bodies in *Escherichia coli*. Trends in Biotechnology. 6(5):95-101.